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DANILO GUSTAVO PULITA ALANIS

ÁCIDO HIALURÔNICO AUMENTA A DEPOSIÇÃO DE MATRIZ ÓSSEA ASSOCIADO AO MAIOR NÚMERO DE CÉLULAS BMPR-IB POSITIVAS.

CURITIBA
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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos à obtenção do título de Mestre em Odontologia – Área de concentração: Periodontia.

Orientadora:Prof^{a}.Dr^{a}. Vula Papalexiou.

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ÁCIDO HIALURÔNICO AUMENTA A DEPOSIÇÃO DE MATRIZ ÓSSEA ASSOCIADO AO MAIOR NÚMERO DE CÉLULAS POSITIVAS PARA O RECEPTOR 1B DA PROTEINA OSTEOMORFOGENÉTICA.

RESUMO

A recuperação de tecido ósseo perdido, com baixa morbidade e alta taxa de regeneração tecidual, tem sido fundamental para reabilitação de alguns pacientes. Muitos biomateriais tem sido testados, isoladamente ou em associação, como o ácido hialurônico (AH). O objetivo deste estudo foi analisar a expressão de BMPR-IB, por meio de análise imunoistoquímica, em defeitos criados artificialmente em tíbia de coelho, com e sem a presença de AH gel. Para tanto, 45 coelhos albinos machos receberam duas perfurações na tíbia, medindo 4,1 mm de diâmetro e 2,0 mm de profundidade. Um defeito foi preenchido por coágulo (grupo controle) e outro por AH (grupo experimental). As peças cirúrgicas foram removidas após a morte dos animais com 20, 30 e 40 dias e foram fixadas, desmineralizadas e incluídas em parafina para análise imunoistoquímica. Foi quantificada a percentagem de matriz óssea neoformada e contado o número de células BMPR-1B+. Nos grupos controles, o número de células BMPR-IB+ aumentou ($p<0.05$), enquanto nos grupos experimentais houve diminuição do número destas células ($p<0.05$) com o tempo de tratamento. No entanto, no grupo controle de 40 dias, o número de células BMPR-IB+ foi menor que no grupo experimental de 20 dias. A utilização do AH promoveu aumento da deposição de matriz óssea comparada ao coágulo ($p<0,05$) e esta ocorreu de forma mais rápida e densa, resultando em maior formação de tecido ósseo.

Palavras-chave: Ácido hialurônico, reparo ósseo, proliferação celular, osteoblasto, imunoistoquímica.

INTRODUÇÃO

Biomateriais com propriedades osteocondutora e osteoindutora tem sido estudados como substitutos ósseos para restabelecer o processo de reparo, contribuindo para um suficiente aumento do volume ósseo e permitindo a reabilitação de eventuais, perdas ósseas por entidades patológicas distintas, traumas, ou mesmo, ausência dos dentes. As técnicas cirúrgicas empregadas para recuperação óssea geralmente utilizam a enxertia para alcançar a regeneração, o que mantém as características teciduais, possibilitando a reabilitação.

A regeneração óssea requer a presença de células progenitoras com a capacidade de diferenciação e proliferação em tecido com fenótipo apropriado para restaurar o dano sofrido [1]. Quando isto não ocorre, um tecido inapropriado é formado no processo cicatricial, formando áreas fibrosas. Buscando promover maior e mais rápido reparo, muitos biomateriais tem sido testados, isoladamente ou em associação [2]. Em estudos recentes [3,4,5], o ácido hialurônico tem se mostrado um material eficiente não só pela facilidade de utilização em gel, mas também pelos resultados apresentados.

O ácido hialurônico (AH) é um polissacarídeo longo e simples, de alto peso molecular, formado por repetidos dissacarídeos de estrutura [Ácido D-Glucorônico (1-B-3) N-acetil-glicosamina (1-B-4)] [6]. É encontrado na matriz extracelular e sintetizado na membrana plasmática celular. Tem carga eletronegativa, o que o torna agregador de proteínas também relacionadas com neoformação óssea, tais como CD44, RHAMM e LYVE-1 [7]. Degradada por

hialuronidases, esta macromolécula é fragmentada em moléculas de menor peso molecular, que tem demonstrado potencial de ação diferenciado no processo de reparo [8]. O AH de baixo peso molecular parece estimular angiogênese, enquanto o de alto peso molecular parece estar relacionado a maior formação óssea [4,9]. Também chamado hialuronana ou hialuronato de sódio, o AH tem implicação direta na interação de célula a célula, adesão de célula a matriz, mobilidade celular e organização da matriz extracelular. Além do papel importante na migração, diferenciação e adesão celular, promove a morfogênese de tecidos mesenquimais, inclusive no recrutamento, proliferação e diferenciação de osteoblastos, também estimula a mineralização de tecido ósseo [10]. Zou et al.[10] estudaram a estimulação de células de medula óssea suína com diferentes concentrações de AH, mostrando a aceleração da proliferação e o aumento da diferenciação celular.

As Proteinas Morfogenéticas Ósseas (BMPs) são uma família de proteínas que induzem a formação de osso e cartilagem in vivo [11]. Kawano et al.[3] observaram a melhora da ação de BMP-2 na indução de diferenciação de osteoblastos na presença do AH.

As funções biológicas das BMPs são mediadas por via de transdução de sinal por meio de três receptores de BMP: BMPR-IA, BMPR-IB e BMPR-II [12]. O BMPR-IB é o principal receptor para desenvolvimento da condensação condroblástica em ossos longos, como também no processo de ossificação craniofacial e endocondral. Segundo Chen et al. [13], a falta e/ou perda da expressão desta proteína inibe o processo osteogênico, contribuindo para a formação de células adiposas.

Assim, o objetivo deste estudo foi comparar, por meio de análise imunoistoquímica, a expressão de BMPR-IB e analisar histomorfometricamente a percentagem de reparo ósseo em defeitos criados artificialmente em tíbias de coelhos, com e sem a presença de ácido hialurônico gel.

MATERIAIS E MÉTODOS

O protocolo experimental do presente estudo foi aprovado pelo Comitê de Ética no Uso de Animais da Pontifícia Universidade Católica do Paraná (172/07).

Foram utilizados 45 coelhos albinos machos da raça Nova Zelândia, com peso entre 2 a 2,5 kg. Estes foram sedados por meio da administração via endovenosa de cloridrato de ketamina 50 mg (6-10 mg/kg) e xilazina 2 g (0,1 mL/kg). Para anestesia local foi utilizada lidocaína 2% com adrenalina 1:100.000, com objetivo de promover vasoconstrição e controle da dor. Uma incisão de 6 cm foi realizada na região médio-proximal de uma das tíbias, utilizando lâmina de bisturi 15. Após o descolamento músculo-periosteal dos tecidos, dois defeitos ósseos agudos foram confeccionados, utilizando trefinas com 4,1 mm de diâmetro e 2 mm de profundidade, totalizando duas cavidades por animal sendo mantida distância de 4 mm entre os defeitos.

Os defeitos ósseos foram divididos em dois grupos: no grupo controle (C), as cavidades foram preenchidas com coágulo; no grupo experimental (E), as cavidades foram preenchidas com gel de ácido hialurônico (Suplasyn®, solução injetável 10 mg / 1 mL em embalagem com 1 seringa preenchida contendo 20 mg / 2 mL). A substância ativa de Suplasyn® é uma fração de ácido hialurônico com um comprimento de cadeia molecular definido de 500 - 1.000 KDa, que lhe confere um alto peso molecular. Produzido por meio da fermentação de bactérias, não contém nenhum componente de origem animal, não promovendo reações anti-genicas. Cada animal apresentou um defeito ósseo do grupo controle e um defeito ósseo do grupo experimental. A sutura foi

realizada com fio de seda 3.0, e retirada após 7 dias. Foi realizada antibioticoterapia com 40.000 UI/kg de penicilina G benzatina por uma semana. Os animais foram mortos com 20, 30 e 40 dias [7], utilizando sobredosagem dos sedativos. Desta maneira, o grupo controle foi subdividido em C20, C30 e C40,e o grupo experimental em E20, E30 e E40, de acordo com os tempos de tratamento.

Após a morte dos animais e remoção dos espécimes, as peças cirúrgicas foram fixadas em solução de formol a 10% durante 24 horas e desmineralizadas em solução de EDTA a 4,13% por 120 dias. Foi realizada a inclusão em parafina.

Processamento imunoistoquímico

Para a imunomarcação, cortes seriados medindo 3 µm de espessura foram desparafinados em xilol em estufa a 60ºC, e hidratados em cadeia descendente de alcoóis (absoluto, 95%, 80%, respectivamente). Após a hidratação, os espécimes foram submetidos à recuperação antigênica em solução de tripsina a 1% (pH 7,2) por 45 minutos a 37°C em estufa.

As lâminas contendo os cortes histológicos seguiram para lavagem e, posteriormente, foram imersas em água oxigenada a 3% por 30 minutos para extinguir a atividade da peroxidase endógena. Após lavagem em água destilada, os espécimes foram imersos em solução tampão salina (PBS, pH 7,4) para manter constantes as propriedades químicas da reação. Posteriormente, os cortes foram incubados com o anticorpo primário anti BMPR-IB (concentração 200 µg/mL, Santa Cruz Byotechnology, Santa Cruz, EUA), com fator de diluição de 1:100 em PBS, por 18 horas (*overnight*). Para

detectar o anticorpo primário, foi utilizado kit universal streptavidina biotina peroxidase (Diagnostic BioSystems, Pleasanton, EUA), conforme instruções do fabricante. A reação imune foi revelada com solução de tetracloreto de diaminobenzidina (Sigma, St Louis, EUA) por 2 minutos, produzindo um precipitado marrom no local do antígeno. Os espécimes foram contra-corados com hematoxilina de Harris por 3 minutos. Para controle negativo, utilizou-se o isotipo policlonal IgG de coelho (2 mg/mL), por 10 minutos de incubação, em temperatura ambiente como anticorpo primário.

Análise das imagens

Imagens de todos os espécimes foram capturadas por uma câmera digital (Samsung, Coréia do Sul), acoplada a um microscópio de luz (Zeiss) com aumento original de 200×. Cada imagem digital foi salva com resolução de 600 dpi, produzindo uma imagem virtual de 117 × 80 cm. Como não foi possível capturar todo o defeito em uma única imagem com a magnificação utilizada, um quadro digital composto por todo o defeito, foi então, construído combinando quatro imagens menores, baseadas em estruturas de referência histológica, principalmente de tecido ósseo depositado e vasos sanguíneos [14].

Análise histomorfométrica

Todas as medidas histomorfométricas foram realizadas utilizando o software Image Tool 2.00 (Universidade do Texas, EUA).

Os perímetros referentes à área da matriz óssea depositada e a quantidade de tecido conjuntivo remanescente foram traçados manualmente e

as respectivas áreas foram mensuradas. As células positivas para BMPR-IB foram marcadas e contadas. Uma imagem com escala de 200 μm foi usada para calibrar todas as mensurações realizadas. As lâminas foram então analisadas para cada um dos parâmetros acima e os dados foram expressos em percentagem de osso neoformado e número de células que exibiram positividade para o anticorpo BMPR-IB. Uma média de três medições, com intervalos de 1 semana, para cada parâmetro foi calculada para cada espécime.

Análise estatística

Os valores obtidos para cada variável foram registrados e tabulados em planilhas no software estatístico SPSS versão 15,0 para Windows (SPSS Inc, Chicago, IL, EUA). Para normalidade das variáveis foi utilizado o teste de Kolmogorov-Smirnov e para homogeneidade de variâncias foi utilizado o teste de Levene. Para os grupos que apresentaram distribuição normal, foi utilizado o teste de Anova a 01 critério. Quando Anova a 01 critério acusou diferenças entre as médias dos grupos, utilizou-se o teste de Games-Howell para as variáveis que não apresentaram homogeneidade de variâncias entre os grupos. Todos os testes estatísticos foram realizados com nível de significância de 5% ($p<0,05$).

RESULTADOS

A amostra foi composta por 6 grupos, divididos em três grupos controles (C20, C30 e C40), e três grupos experimentais (E20, E30 e E40), de acordo com o tempo de tratamento dos animais.

Houve distribuição normal dos dados para as variáveis estudadas nos diferentes grupos ($p > 0,05$). Não houve homogeneidade de variâncias na amostra avaliada ($p < 0,05$).

Houve diferença entre grupos para as variáveis percentagem de osso neoformado e número de osteoblástos ($p < 0,001$) (Tabelas 1 e 2). As Tabelas 1 e 2 mostram os valores médios, desvios-padrão e valores de p das variáveis percentagem de osso neoformado e número de células nos grupos controles e experimentais, respectivamente.

Tabela 1: Valores médios, desvios-padrão e valores de p das variáveis percentagem de osso neoformado (%) e número de células nos grupos controles (C20, C30 e C40).

	C20 X (DP)	C30 X (DP)	C40 X (DP)	Valor p
Percentagem de osso	14,14 (0,81)	15,45 (0,96)	17,27 (0,88)	0,000
BMPR-1B+	452,80 (55,76)	657,93 (58,82)	1174,20 (67,90)	0,000

Para a variável percentagem de osso neoformado, houve diferença significante entre os grupos C20 e C30 (p=0,004), C30 e C40 (p=0,00012), e C20 e C40 (p=0,000).

Para a variável número de células, houve diferença significante entre os grupos C20 e C30 (p=0,000), C30 e C40 (p=0,000), e C20 e C40 (p=0,000).

Tabela 2: Valores médios, desvios-padrão e valores de p das variáveis percentagem de osso neoformado (%) e número de células nos grupos experimentais (E20, E30 e E40).

	E20 X (DP)	E30 X (DP)	E40 X (DP)	Valor p
Percentagem de osso	17,91 (2,87)	19,80 (1,45)	23,15 (1,82)	0,000
BMPR-1B+	1477,67 (89,01)	1232,13 (80,38)	579,73 (47,12)	0,000

Para a variável percentagem de osso neoformado, houve diferença significante entre os grupos E30 e E40 (p=0,00009), E20 e E40 (p=0,00005).

Para a variável número de células, houve diferença significante entre os grupos E20 e E30 (p=0,000), E30 e E40 (p=0,000), e E20 e E40 (p=0,000).

Os gráficos 1 e 2 ilustram a distribuição da média dos valores das variáveis percentagem de neoformação óssea e número de células nos diferentes grupos estudados, respectivamente.

Gráfico 1: Distribuição da média dos valores da variável percentagem de osso nos diferentes grupos estudados.

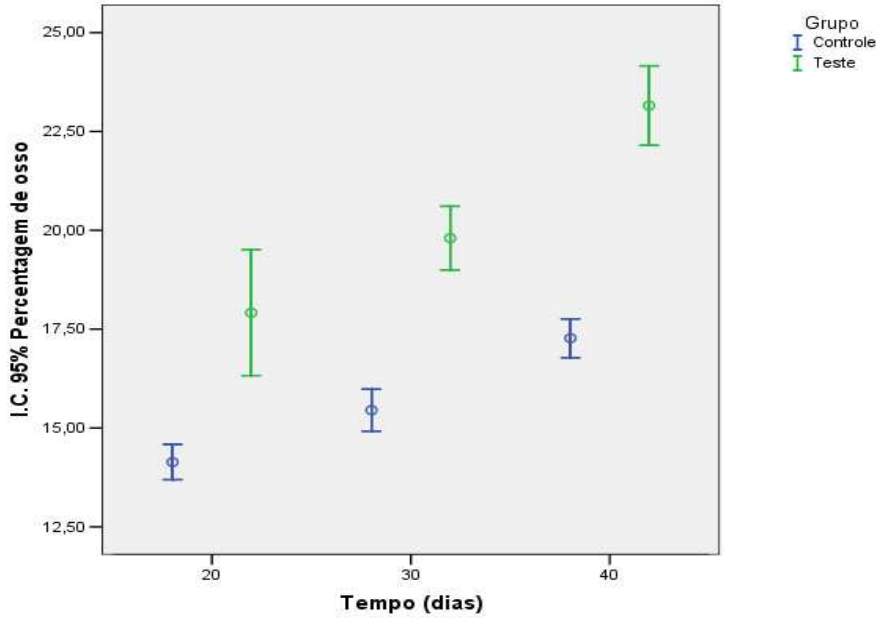
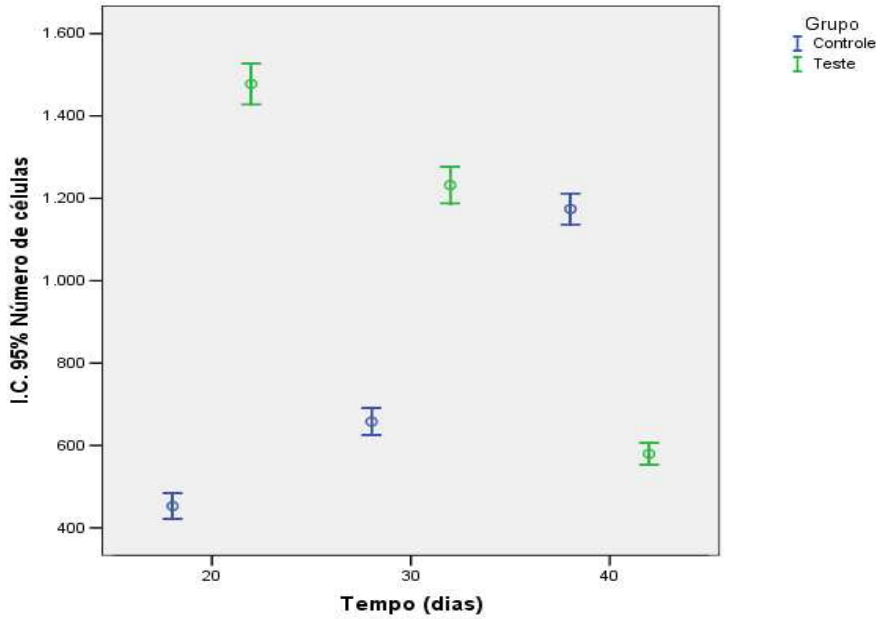


Gráfico 2: Distribuição da média dos valores da variável número de células nos diferentes grupos estudados.



Os grupos controle e experimental foram comparados entre si de acordo com os diferentes tempos de tratamento. Para a variável percentagem de osso

neoformado, houve diferença significante entre os grupos nos seguintes tempos: 20 (p=0,001), 30 (p=0,000) e 40 dias (p=0,000). Para a variável número de células, houve diferença significante entre os grupos controle e experimental nos tempos: 20 (p=0,000), 30 (p=0,000) e 40 dias (p=0,000).

RESULTADOS HISTOLÓGICOS

Grupo controle

Os aspectos microscópicos observados no grupo controle foram similares em todos os períodos analisados. Aos vinte dias de pós-operatório, pouca quantidade de tecido ósseo neoformado foi observado e estava concentrado apenas nas margens do defeito, em contigüidade ao tecido ósseo remanescente. Embora a quantidade de matriz óssea depositada tenha aumentado nos períodos de 30 e 40 dias, o padrão de distribuição foi similar aos 20 dias, isto é, seguiu um padrão de crescimento centrípeto por todo defeito circular, mantendo um arcabouço ósseo cortical, enquanto no centro do defeito cirúrgico, um alto contingente de células adiposas foram encontradas.

A figura 1 e 2 ilustram fotomicrografias dos defeitos ósseos em tíbias de coelhos preenchidos por coágulo (grupo controle) e AH (grupo experimental), nos períodos de 20, 30 e 40 dias e em maior aumento a presença de osteoblastos no processo de reparo.

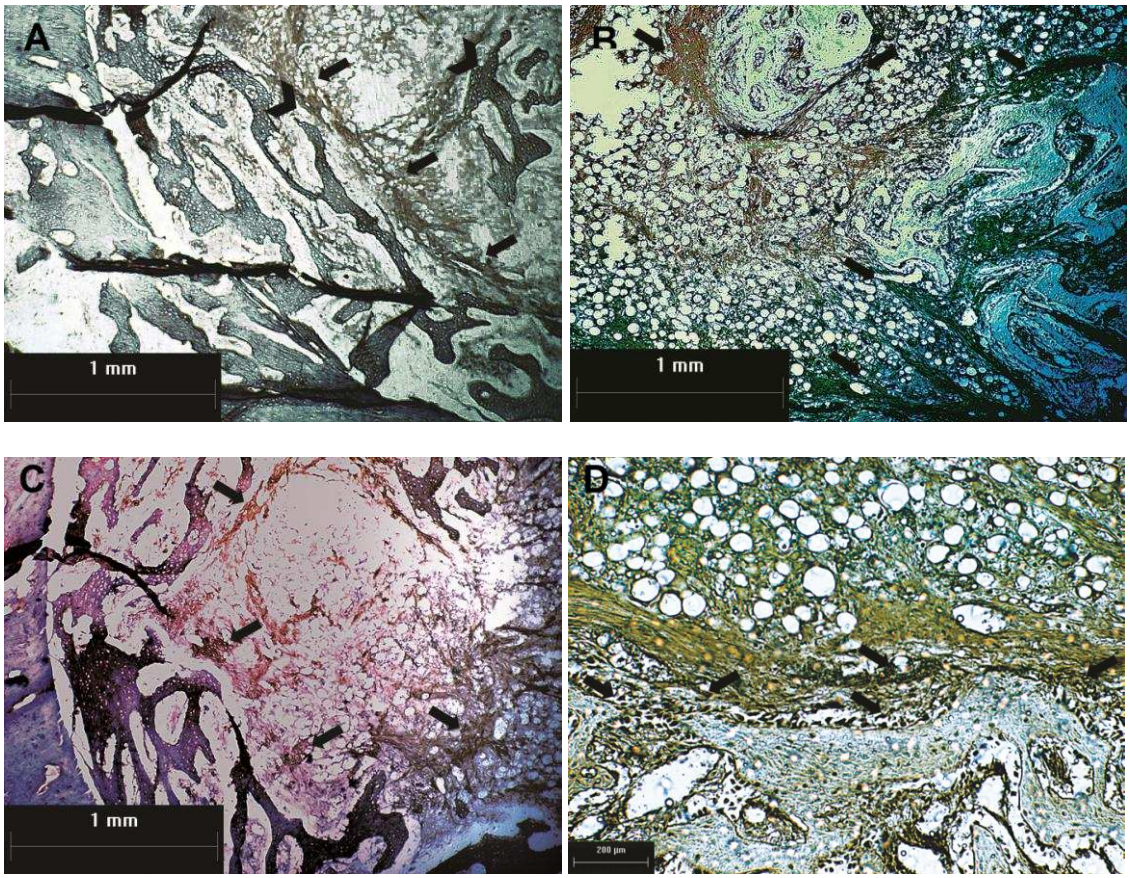


Figura 1 - Micrografia referente a imunomarcação da proteína BMPR-IB nos grupos controles analisados. (A) revela o aspecto do padrão de distribuição da proteína BMPR-IB (➡) circundando e delineando área adjacente do remanescente ósseo (❱) em 20 dias de pós-operatório. Esse padrão de distribuição é similar nos períodos de 30 (B) e 40 dias (C), em maior aumento podemos os osteoblastos marcados (➡) no grupo C 20 (D). Nesses períodos é possível também observar intensa deposição de medula adiposa e finas trabéculas de osso neoformado em áreas coincidentes a proteína BMPR-IB.

Grupo Experimental

Em nenhum dos períodos analisados e em nenhum espécime tratado com AH o defeito cirúrgico fechou completamente, mesmo em 40 dias de pós-operatório. Aos 20 dias de pós-operatório, foi identificada intensa quantidade de células BMPR-IB positivas aglomeradas, que se estendiam ao interior do defeito cirúrgico num padrão plexiforme. Áreas de deposição mineral óssea eram identificadas em associação com o conteúdo celular positivo ao fenótipo BMPR-IB. Esse padrão foi semelhante nos 30 e 40 dias de pós-cirúrgico.

Um fato digno de nota é que, diferentemente do grupo controle, áreas de tecido adiposo foram encontradas focalmente nos grupos experimentais, apenas no centro do defeito.

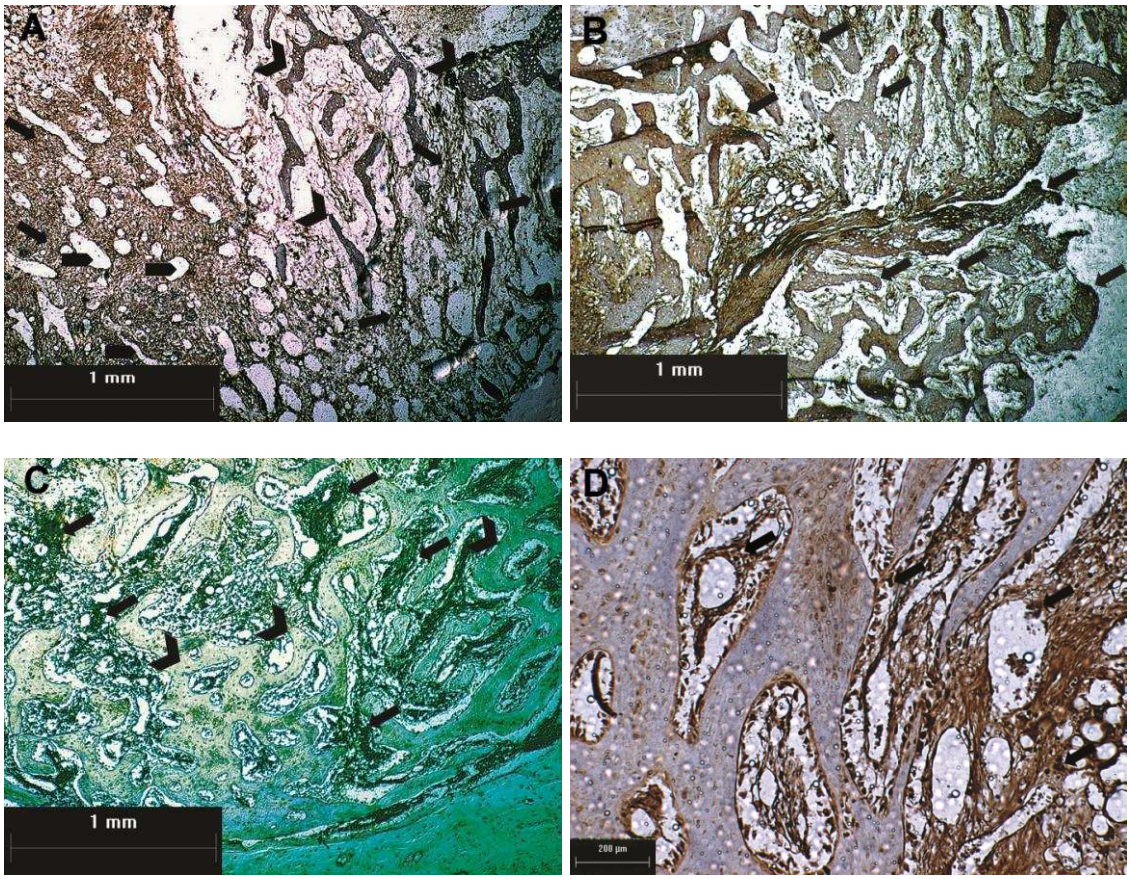


Figura 2 - Micrografias A a C revelam o padrão de imunomarcação da BMPR-IB nos grupos tratados com AH. Em A verifica-se robusta deposição dessa proteína disposta em padrão plexiforme(➡), incitando a disposição arquitetural de novas trabéculas ósseas (❱). É possível observar também numerosos espaços vasculares (➡) circundados por área celular BMPR-IB+. Padrão que segue também aos 30 (B) e 40 (E) dia pós operatório, em maior aumento podemos os osteoblastos marcados (➡) no grupo E 20 (D). Nesses períodos verifica-se escassez de células adiposas, e intensas e robustas trabéculas ósseas neoformadas, delimitando o espaço medular.

DISCUSSÃO

No presente estudo, duas perfurações de 4,1 mm de diâmetro e 2,0 mm de profundidade foram realizadas em tíbias de coelhos, caracterizando um defeito ósseo não crítico. Um defeito foi preenchido por coágulo e o outro por AH para verificar o potencial de reparo ósseo deste biomaterial. Para interpretação dos resultados, as peças cirúrgicas foram analisadas histologicamente por meio da quantificação percentual da matriz óssea neoformada e da contagem do número de células BMPR-IB positivas.

Os nossos resultados demonstraram que a utilização do AH como material de preenchimento de defeitos ósseos em tíbias de coelhos promoveu aumento estatisticamente significante da matriz óssea depositada quando comparado ao grupo controle. Este resultado corrobora achados prévios [3,10,15], que enfaticamente descrevem que o AH pode aumentar o índice de massa óssea em leito de reconstrução óssea. Nesse sentido, Zou et al.[10] mostraram em seus espécimes uma aceleração proliferativa e diferenciação osteoblástica em defeitos submetidos a diferentes concentrações de AH. De forma similar, Kawano et al.[3] evidenciaram melhora na ação da proteína BMP-2 sob condução do AH na diferenciação de osteoblasto. Por outro lado, alguns estudos não demonstraram eficiência do AH no reparo ósseo [16,17]. Isto pode estar relacionado ao peso molecular, ao grau de viscosidade e à concentração do AH empregado em cada estudo [18,19,20]. Além disso, o aumento significativo da matriz óssea depositada no presente estudo coincidiu com o aumento e distribuição precoce (grupos de 20 dias) de células que exibiram o imunofenótipo BMPR-IB.

No presente estudo, o reparo ósseo observado nos grupos experimentais e controles tiveram características diferentes quanto ao número de células positivas para BMPR-IB. Nos grupos controles houve aumento do número destas células. Apesar deste aumento, o número de células com 40 dias de pós-operatório (grupo C40) ainda era menor do que o número de células no grupo experimental de 20 dias (grupo E20). Nos grupos experimentais houve diminuição do número de células BMPR-IB positivas. No entanto, o maior número destas células foi observado em 20 dias (grupo E20). Isto pode ter ocorrido pelo fato das proteínas BMPR-IB estarem presentes em osteoblastos em fase inicial de diferenciação [12]. Na fase tardia, outros marcadores, tais como, a osteocalcina, estão relacionados com a diferenciação osteoblástica [12].

Outra justificativa para a diminuição do número de células BMPR-IB+ no grupo experimental seria a diminuição da concentração de AH, pela ação de hialuronidase, principal agente na quebra do AH em moléculas menores [7,8].

A concentração e peso molecular ideais para a maior estimulação da formação óssea ainda necessita de mais estudos. Porém, as evidências de aumento celular precoce e taxas superiores de osso nos grupos experimentais quando comparadas aos controles, mostram que o AH apresenta um comportamento favorável a neoformação óssea. Corroborando esta hipótese, Sasaki, Watanabe [9] mostraram que a presença de AH de alto peso molecular acelera a neoformação óssea por induzir a diferenciação de células tronco mesenquimais.

No presente estudo, foi observado componente vascular bem formado, circundado pelas células BMPR-IB+. West et al. [21] demonstraram que

produtos de degradação do AH induzem a angiogenese. O AH de baixo peso molecular, associado a matriz óssea desmineralizada, inserido em defeitos ósseos de tíbias de ratos, estimulou neovascularização e neoformação óssea [4]. Embora os vasos sanguíneos não tenham sido avaliados no presente estudo, podemos inferir que a ação angiogênica do AH também contribua para a aceleração da deposição da matriz óssea nos defeitos tratados por AH.

Apesar de, neste estudo, em nenhum espécime ter ocorrido a completa reparação do defeito ósseo (em 40 dias), os grupos tratados com AH mostraram aumento na neoformação óssea, fato que leva a interpretação de que o AH demonstra papel de osteoindução[19,22]. Além disso, foi identificada mudança no padrão de ossificação, ocorrendo de forma mais rápida e efetiva, sugerindo que o AH parece ser também um acelerador da ossificação endocondral [9,23]. Isto foi revelado pela presença de escassa área endocondral formada e intensa área de neoformação óssea propriamente dita. Estes achados coincidiram com a distribuição de células BMPR-IB+, organizadas em padrão plexiforme.

CONCLUSÃO

Com base na metodologia empregada neste estudo, pode-se concluir que a presença do ácido hialurônico aumenta a expressão de células BMPR-IB+ e acelera a deposição de matriz óssea.

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2. ARTIGO EM INGLÊS

HYALURONIC ACID INCREASES BONE MATRIX DEPOSITION ASSOCIATED WITH THE LARGEST NUMBER OF BMPR-IB POSITIVE CELLS.

ABSTRACT

The recovery of lost bone tissue with low morbidity and high rate of tissue regeneration has been indispensable in the rehabilitation of some patients. Many biomaterials have been tested, alone or in combination, such as hyaluronic acid (HA). The aim of this study was to analyze the expression of BMPR-IB in artificially created defects in rabbit tibia, with and without the presence of HA gel, by means of immunohistochemical analysis. For this purpose, two defects (4.1 mm diameter, 2.0 mm deep) were performed in the tibia of 45 male albino rabbits. A defect was filled with blood clot (control group) and another with HA (experimental group). The specimens were removed after the death of the animals with 20, 30 and 40 days, and were fixed, demineralized and embedded in paraffin for immunohistochemical analysis. The percentage of neoformed bone matrix was quantified and the number of BMPR-IB+ cells was counted. In control groups, the number of BMPR-IB+ cells increased ($p<0.05$), while in the experimental groups the number of these cells decreased ($p <0.05$) with the time of treatment. However, in C40, the number of BMPR-IB+ cells was still lower than in group E20. The use of HA promoted increased deposition of bone matrix compared to clot ($p <0.05$) and this occurred faster and denser, resulting in increased bone formation.

Keywords: Hyaluronic acid, bone repair, cell proliferation, osteoblast, immunohistochemistry.

INTRODUCTION

Biomaterials with osteoconductive and osteoinductive properties have been studied recently as alternative bone substitutes to restore the repair process, contributing to a sufficient increase in bone volume and allowing for the rehabilitation of bone loss by different pathologies, trauma, or even absence of teeth. Surgical procedures for bone recovering generally use grafting as the main alternative to achieve regeneration, which keeps the tissue characteristics, allowing for the rehabilitation.

The bone tissue regeneration requires the presence of progenitor cells with the capacity for differentiation and proliferation in tissue with phenotype appropriate to restore the damage [1]. When this does not occur, inappropriate tissue is formed in the healing process, forming fibrous areas. Aiming to promote greater and faster repair, many biomaterials have been tested, alone or in combination [2]. In recent studies [3,4,5], hyaluronic acid has been shown to be a efficient material due to the ease of use in gel, and due to the results presented.

Hyaluronic acid (HA) is a long polysaccharide, yet simple, with high molecular weight, consisting of repeated disaccharide with the following structure [D-glucoronic acid (1-B-3) N-acetyl-glucosamine (1-B-4)] [6]. It is found in the extracellular matrix and synthesized in the cell plasma membrane. HA is electronegative, which allows it to make aggregation of some proteins also related to bone formation, such as CD44, RHAMM and LYVE-1 [7]. Degraded by hyaluronidases, this macromolecule is fragmented into molecules of lower molecular weight, which have demonstrated distinguished potential of action in

the repair process [8]. The HA of low molecular weight appears to stimulate angiogenesis, while the HA of high molecular weight seems to be related to increased bone formation [4,9]. Also called hyaluronan or sodium hialorunate, HA is directly implicated in the cell-to-cell interaction, adhesion between cell to matrix, cell motility, and organization of the extracellular matrix. Besides the role in migration, differentiation and cell adhesion, HA promotes the morphogenesis of mesenchymal tissues, including the recruitment, proliferation and differentiation of osteoblasts [10]. It also stimulates the mineralization of bone tissue. Zou et al. [10] studied the stimulation of bone marrow cells in pigs with different concentrations of HA, showing the acceleration of proliferation and the increase of cell differentiation.

Kawano et al. [3] observed an improvement in the action of BMP-2 in the induction of osteoblast differentiation in the presence of HA. The Bone Morphogenetic Proteins (BMPs) are a family of proteins that induce the formation of bone and cartilage in vivo [11].

The biological functions of BMPs are mediated by signal transduction via three BMP receptors, BMPR-IA, BMPR-IB and BMPR-II [12]. The BMPR-IB is the major receptor for the development of chondroblastic condensation in long bones, but also in the process of endochondral and craniofacial ossification. According to Chen et al. [13], the absence and/or loss of expression of this protein inhibits the osteogenic process, contributing to the formation of fat cells.

Thus, the purpose of this study was to compare, by means of immunohistochemical analysis, the expression of BMPR-IB and analyze the percentage of histomorphometric bone repair in defects defects in rabbit tibiae, with and without the presence of hyaluronic acid gel.

MATERIALS E METHODS

The experimental protocol of this study was approved by the Ethics Committee on Animal Use of the Pontifical Catholic University of Parana (172/07).

Forty-five male albino New Zealand rabbits, weighing between 2 and 2.5 kg were used in this study. They were sedated by intravenous administration of ketamine hydrochloride 50 mg (6-10 mg/kg) and xylazine 2 g (0.1 mL/kg). For local anesthesia, it was used 2% lidocaine with 1:100,000 epinephrine, aiming to promote vasoconstriction and pain control. A 6 cm incision was made in mid-proximal region of the tibia, using a scalpel blade 15. After removal of the muscle-periosteal tissues, two sharp defects were made using trephine with 4.1 mm diameter and 2 mm deep, totaling two defects per animal. A distance of 4 mm between the defects was maintained.

Bone defects were divided into two groups: in the control group (C), the defects were filled with blood clot; in the experimental group (E), the defects were filled with hyaluronic acid gel (Suplasyn®, injectable solution 10 mg/1 mL packaged with a prefilled syringe containing 20 mg/2 mL). The active ingredient of Suplasyn® is a specific fraction of HA with a defined molecular chain length, produced by fermentation of bacteria. Suplasyn® does not contain any components of animal origin, which gives a high molecular weight from 500 to 1000 KDa. Each animal had a bone defect in the control group and a bone defect in the experimental group. The suture was performed with 3.0 silk thread, and removed after 7 days. Antibiotic therapy was performed with 40,000 IU/kg of benzathine penicillin G for a week. The animals were killed with 20, 30 and

40 days [7], using an overdose of sedatives. Thus, control groups were subdivided into C20, C30 and C40, and the experimental groups at E20, E30 and E40, according to the time of treatment.

After the death of animals and removal of specimens, the specimens were fixed in formalin 10% for 24 hours and demineralized in 4.13% EDTA solution for 120 days. Thus, they were embedded in paraffin.

Immunohistochemical processing

For immunostaining, serial sections measuring 3 mm thick were dewaxed in xylene in an oven at 60 °C, and hydrated in descending chain alcohols (absolute, 95%, 80%, respectively). After hydration, the specimens were subjected to antigen retrieval in a solution of 1% trypsin (pH 7.2) for 45 minutes at 37 °C in an oven.

The slides containing histological sections were washed and then were immersed in 3% hydrogen peroxide for 30 minutes to eliminate endogenous peroxidase activity. After washing with distilled water, specimens were immersed in buffered saline (PBS, pH 7.4) to maintain constant the chemical reaction. Subsequently, the sections were incubated with primary antibody anti BMPR-IB (concentration 200 mg/mL, Santa Cruz Byotechnology, Santa Cruz, USA) with a dilution factor of 1:100 in PBS, during 18 hours (overnight). To detect the primary antibody it was used streptavidin biotin universal kit (Diagnostic BioSystems, Pleasanton, USA) according to manufacturer's directions. The immune reaction was revealed with a solution of diaminobenzidine tetrachloride (Sigma, St Louis, USA) for 2 minutes, producing a brown precipitate at the site of antigen. The specimens were counterstained

with Harris hematoxylin for 3 minutes. For negative control, it was used the polyclonal rabbit IgG isotype (2 mg/mL) for 10 min incubation at room temperature as primary antibody.

Image analysis

Images of all specimens (immunohistochemistry) were captured by a digital camera (Samsung, South Korea), coupled with a light microscope (Zeiss) with 200 × original magnification. Each digital image was saved with 600 dpi resolution, producing a virtual image of 117 × 80 cm. Because it was not possible to capture all the defect in a single image with the magnification used, a digital picture made up of any defect, was then constructed by combining four smaller images, based on histological structures of reference, such as deposited bone tissue and blood vessels [14].

Histomorphometric analysis

All histomorphometric measurements were performed using the software Image Tool 2.00 (University of Texas, USA).

The perimeters regarding the area of deposited bone matrix and the amount of remaining fibrous tissue were manually traced and their areas were measured. The positive cells for BMPR-IB were marked and counted. An image scale of 200 μm was used to calibrate all measurements. The slides were then analyzed for each of the above parameters and the data were expressed as a percentage of new bone formation and number of positive cells for BMPR-IB antibody. An average of three measurements with 1-week interval for each parameter was calculated for each sample.

Statistical analysis

The values obtained for each variable were recorded and tabulated into spreadsheets in SPSS version 15.0 for Windows (SPSS Inc, Chicago, IL, USA). The normality of variables and homogeneity of variance were analyzed by the Kolmogorov-Smirnov and Levene tests, respectively. For groups that showed normal distribution, Anova test at one criterion was used. When the Anova test showed differences between groups, it was used the Games-Howell test for the variables that showed no homogeneity of variances between groups. All statistical tests were performed with a significance level of 5% ($p < 0.05$).

RESULTS

The sample consisted of six groups that were divided into three control groups (C20, C30 and C40) and three experimental groups (E20, E30 and E40), according to the time of treatment of animals.

There was normal distribution of data for the variables in the different groups ($p>0.05$). There was no homogeneity of variance in the sample ($p<0.05$).

There were differences between groups for the variables percentage of bone formation and number of osteoblasts ($p<0.001$) (Tables 1 and 2). Tables 1 and 2 show the mean values, standard deviations and p values of the variables percentage of new bone formation and cell number in control and experimental groups, respectively.

Table 1: Mean values, standard deviations and p values of the variables percentage of new bone formation (%) and cell number in control groups (C20, C30 and C40).

	C20 X (SD)	C30 X (SD)	C40 X (SD)	P value
Bone percentage	14.14 (0.81)	15.45 (0.96)	17.27 (0.88)	0.000
Cell number	452.80 (55.76)	657.93 (58.82)	1174.20 (67.90)	0.000

For the variable percentage of bone formation, there was significant difference between groups C20 and C30 ($p = 0.004$), C30 and C40 ($p = 0.00012$), and C20 and C40 ($p = 0.000$).

For the variable number of cells, there was significant difference between groups C20 and C30 (p = 0.000), C30 and C40 (p = 0.000), and C20 and C40 (p = 0.000).

Table 2: Mean values, standard deviations and p values of the variables percentage of new bone formation (%) and number of cells in the experimental groups (E20, E30 and E40).

	E20 X (SD)	E30 X (SD)	E40 X (SD)	P value
Bone percentage	17.91 (2.87)	19.80 (1.45)	23.15 (1.82)	0.000
Cell number	1477.67 (89.01)	1232.13 (80.38)	579.73 (47.12)	0.000

For the variable percentage of bone formation, there was significant difference between groups E30 and E40 (p = 0.00009), E20 and E40 (p = 0.00005).

For the variable number of cells, there was significant difference between groups E20 and E30 (p = 0.000), E30 and E40 (p = 0.000), and E20 and E40 (p = 0.000).

Figures 1 and 2 illustrate the distribution of mean values of the variables bone percentage and number of cells in different groups, respectively.

Figure 1: Distribution of average values of the percentage of bone in different groups.

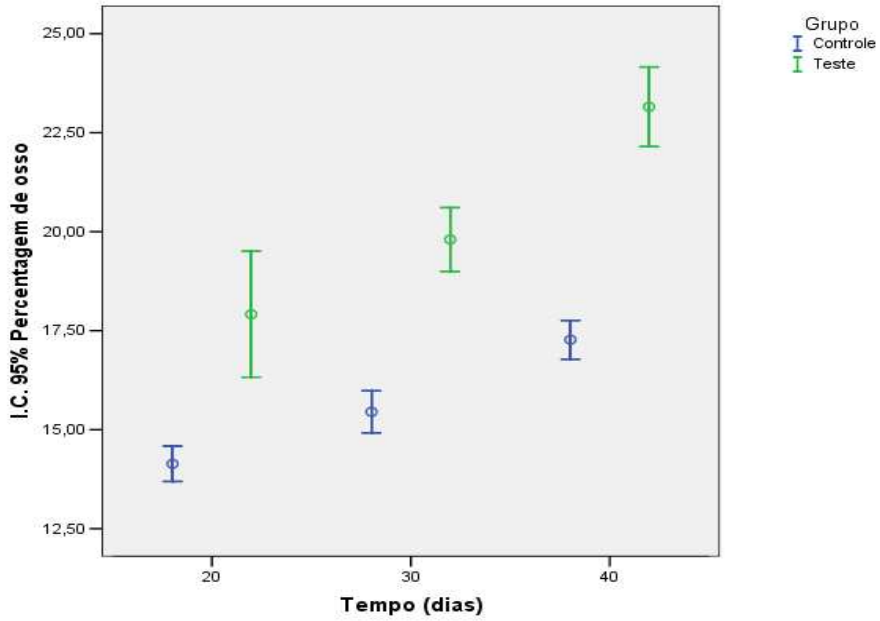
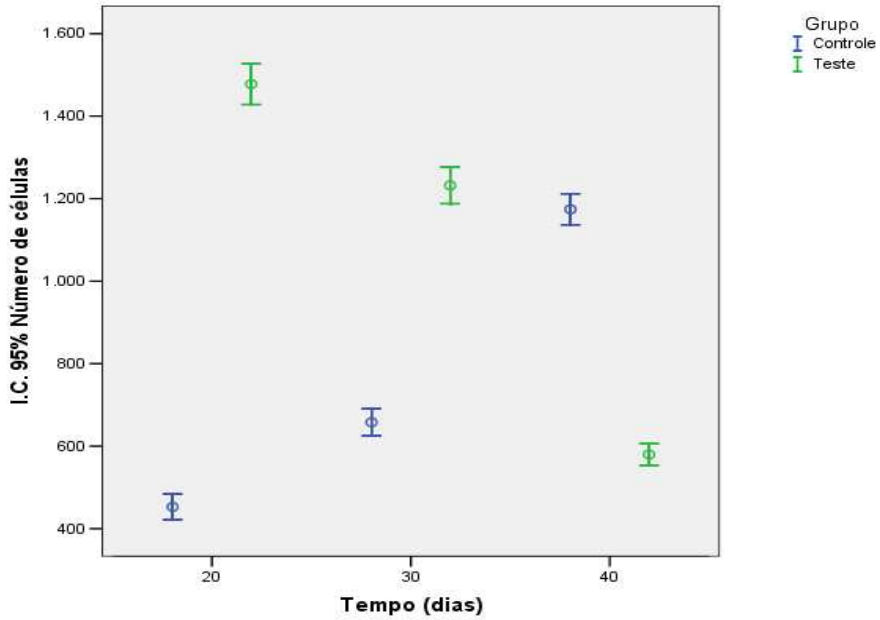


Figure 2: Distribution of average values of the variable number of cells in different groups.



The control and experimental groups were compared according to the different treatment times. For the variable percentage of new bone formation, there was significant difference between groups in the following periods of time:

20 (p = 0.001), 30 (p = 0.000) and 40 days (p = 0.000). For the variable number of cells, there was significant difference between control and experimental groups at times: 20 (p = 0.000), 30 (p = 0.000) and 40 days (p = 0.000).

HISTOLOGICAL RESULTS

Control Group

The microscopic features observed in the control group were similar in all studied periods. At 20 days after surgery, a small amount of newly formed bone tissue was observed and it was focused only on the margins of the defect, in proximity to the remaining bone tissue. Although the amount of deposited bone matrix has increased in periods of 30 and 40 days, the distribution pattern was similar at 20 days, i.e. it followed a centripetal pattern of growth across circular defect, maintaining a framework cortical bone, while in the center of the surgical defect, a high number of fat cells were found.

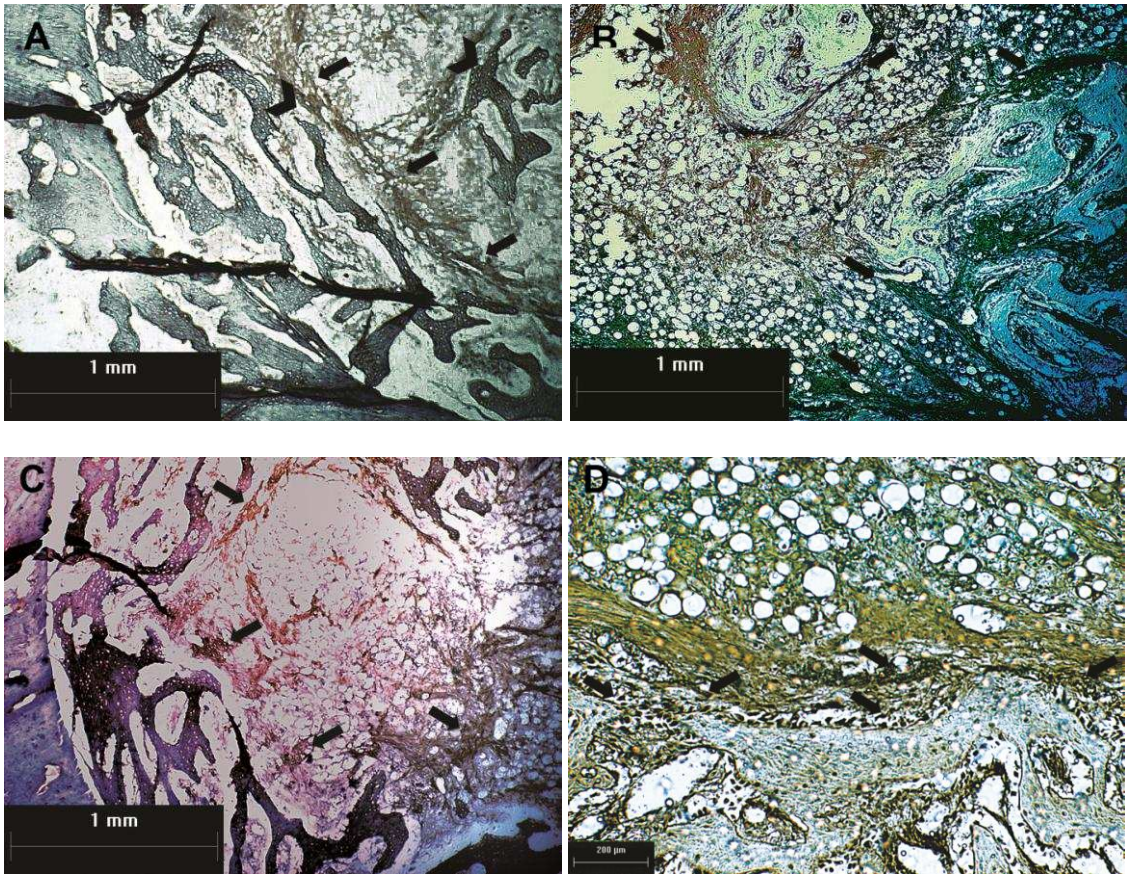


Figure 3 - Micrographs regarding immunostaining of BMPR-IB in analysed groups. (A) shows the appearance of the distribution pattern of BMPR-IB protein (arrow), surrounding and delineating adjacent area of the bone remaining at 20 days postoperatively. This distribution pattern is similar for periods of 30 (B) and 40 days postoperatively (C), in a largest magnification, osteoblasts are seen in group E 20 (D). During these periods it is also possible to observe intense deposition of marrow fat (arrowhead) and thin trabeculae of newly formed bone in the overlapping areas of BMPR-IB protein.

Experimental Group

In no period and in no specimen treated with HA the surgical defect has closed completely, even at 40 days postoperatively. At 20 days after surgery, it was identified intense amount of BMPR-IB positive cells clustered, which spread to the interior of the surgical defect in a plexiform pattern. Areas of bone mineral deposition were identified in association with the positive cell phenotype BMPR-IB. This pattern was similar in 30 and 40 days after surgery.

It was worth noting that, unlike the control group, areas of adipose tissue were found focally in the experimental groups, only in the center of the defect.

Figure 3 shows photomicrographs of bone defects in rabbit tibiae filled with blood clot (control group) and HA (experimental group), in periods of 20, 30 and 40 days.

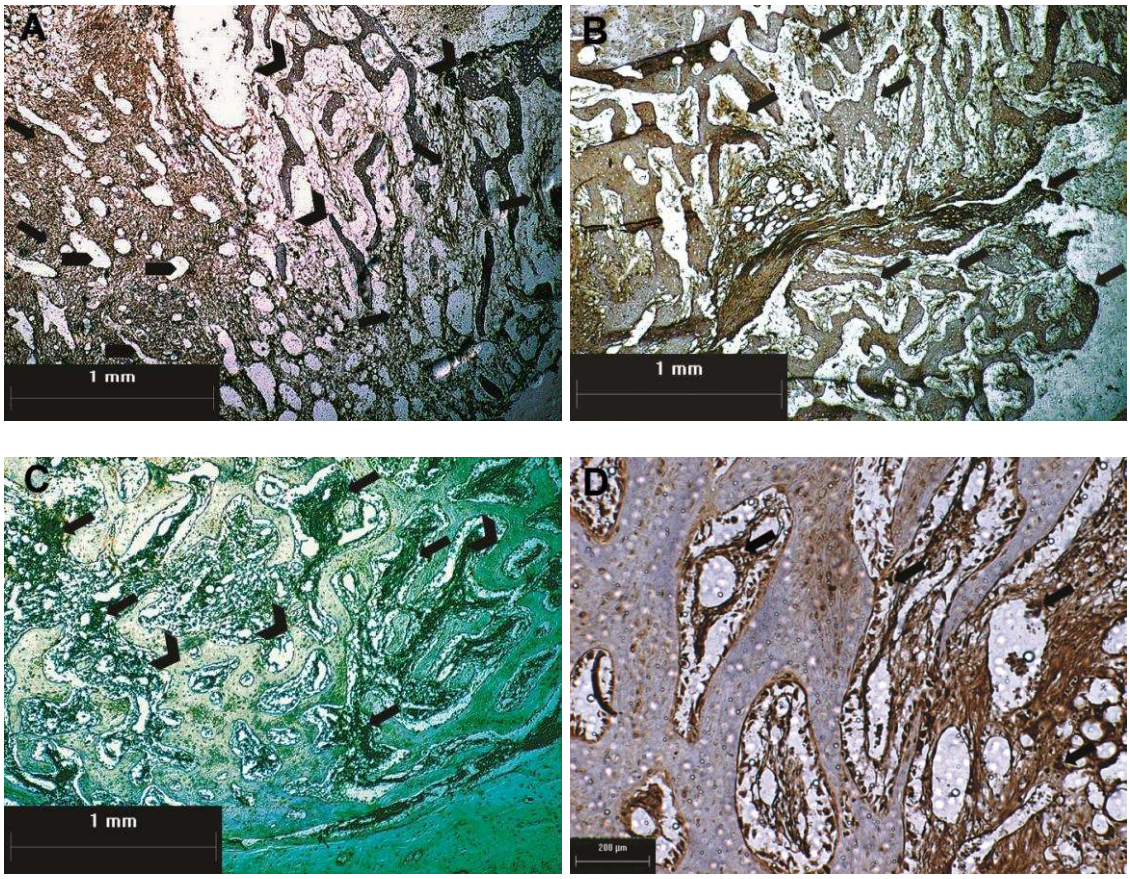


Figure 4 - Micrographs A-C show the pattern of immunohistochemical labeling of BMPR-IB in the groups treated with HA. In A there is strong deposition of this protein arranged in plexiform pattern, prompting the architectural layout of new bone trabeculae. It is also possible to observe numerous vascular spaces (arrow notched) surrounded by cell area BMPR-IB +. This pattern also follows in 30 (B) and 40 (C) postoperative days, in a largest magnification, osteoblasts are seen in group E 20 (D). During these periods there is scarcity of fat cells, and intense and robust neoformed bone trabeculae, delimiting the medullary space.

DISCUSSION

In this study, two defects of 4.1 mm in diameter and 2.0 mm in depth were performed in rabbit tibiae, characterizing a non-critical bone defect. A defect was filled with blood clot and the other by HA in order to investigate the potential of bone repair of this biomaterial. To interpret the results, the specimens were analyzed histologically by quantifying the percentage of newly formed bone matrix and counting the number of positive cells for BMPR-IB.

Our results demonstrate that the use of HA as a filling material for bone defects in rabbit tibiae caused a significant increase of deposited bone matrix when compared with controls. This result confirms previous findings [3,10,15], which emphatically describe that HA can increase bone mass in the bed of bone reconstruction. In this sense, Zou et al. [10] showed an acceleration in osteoblast differentiation and proliferation in defects subjected to different concentrations of HA. Similarly, Kawano et al. [3] showed improvement in the action of BMP-2 protein in the HA in driving differentiation of osteoblast. Conversely, some studies have not demonstrated efficiency of HA in bone repair [16,17]. This may be related to molecular weight, degree of viscosity and concentration of HA used in each study [18,19,20]. Furthermore, the significant increase of deposited bone matrix in the present study coincided with the increase and early distribution (groups of 20 days) of cells that exhibited the immunophenotype BMPR-IB.

In the present study, bone repair observed in experimental and control groups showed different characteristics regarding the number of positive cells for BMPR-IB. In control groups there was an increase in the number of these

cells. Despite this increase, the number of cells with 40 days postoperatively (group C40) was still lower than the number of cells in the experimental group treated for 20 days (group E20). In the experimental groups there was a decrease in the number of BMPR-IB+ cells. However, the greatest number of these cells was observed at 20 days (group E20). This can be explained by the fact that the cells marked by BMPR-IB are in early stages of osteoblast differentiation [12]. In the late stage, other markers, such as osteocalcin, are related to osteoblast differentiation [12].

Another explanation for the decreased number of BMPR-IB+ cells in the experimental group would be a decrease in the concentration of HA by the action of hyaluronidase, the main agent in the breakdown of HA into smaller molecules [7,8].

The ideal concentration and molecular weight for the greater stimulation of bone formation still needs further studies. However, the evidences of earlier cell growth and higher rates of bone in experimental groups compared with controls show that HA has a behavior conducive to bone formation. Confirming this hypothesis, Sasaki, Watanabe [9] showed that the presence of high molecular weight HA accelerates bone formation by inducing the differentiation of mesenchymal stem cells.

In this study, it was found well-formed vascular component, surrounded by BMPR-IB+ cells. West et al. [21] showed that degradation products of HA induce angiogenesis. The HA of low molecular weight, combined with demineralized bone matrix, inserted into bone defects of tibiae of rats, stimulated neovascularization and bone formation [4]. Although blood vessels were not counted in the present study, we can infer that the angiogenic action of

HA will also contribute to accelerating the deposition of bone matrix in the defects treated with HA.

Although a complete repair of bone defect has not occurred in any specimen of this study (in 40 days), HA-treated groups showed increased bone formation, which suggests that HA has an osteoconductive paper [19,22]. Furthermore, changes in the pattern of ossification were observed, occurring more quickly and effectively, suggesting that HA also appears to be an accelerator of endochondral ossification [9,23]. This was revealed by the presence of sparse endochondral formed area and intense area of bone neoformation. These findings coincided with the distribution of BMPR-IB+ cells, arranged in plexiform pattern.

CONCLUSION

Based on the methodology employed in this study, we can conclude that the presence of hyaluronic acid increases the expression of BMPR-IB+ cells and accelerates the deposition of bone matrix.

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