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SELANTE HETERÓLOGO DE FIBRINA COMO ARCABOUÇO PARA CÉLULAS TRONCO MESENQUIMAIS ASSOCIADOS AO FOSFATO DE CÁLCIO E APLICADOS EM DEFEITO CRÍTICO DE FÊMURES DE RATOS

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestre em Doenças Tropicais.

Orientador: Prof. Dr. Benedito Barraviera
Coorientador: Prof. Dr Rui Seabra Ferreira Júnior
Coorientadora: Dra. Elenize Jamas Pereira

Botucatu (SP)
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RESUMO

O reparo do tecido ósseo é um desafio antigo da ciência mundial. A partir das décadas de 70-80, os biomateriais passaram a ser explorados e hoje desempenham importante papel na engenharia tecidual. Estes, quando aplicados na enxertia óssea, devem ser bioativos, biocompatíveis e reabsorvíveis. A integração entre os diversos materiais de origem sintética e os arcabouços biológicos possui amplo espectro de uso. Dentre os arcabouços destacam-se os compostos à base de fibrina por suas propriedades hemostáticas, estabilidade e morfologia favorável à proliferação celular. A presente revisão abordou a estrutura e constituição do tecido ósseo e suas limitações no processo de reparo, a relevância da engenharia tecidual aplicada à esta finalidade e os biomateriais utilizados para otimizar tal processo, com ênfase especial aos materiais compostos por fosfatos de cálcio, células tronco mesenquimais e sua aplicação junto ao arcabouço biológico selante de fibrina, com destaque para o selante heterólogo de fibrina, agora denominado biopolímero de fibrina, um produto único e em fase de testes em experimentação animal e estudos clínicos. Propõe-se, ao final desta, uma nova abordagem para a regeneração de defeitos ósseos de ratos baseada na associação entre o selante heterólogo de fibrina, o fosfato de cálcio bifásico e células tronco mesenquimais.

Palavras-chave: Regeneração óssea, biomateriais, scaffolds, biopolímero de fibrina, fosfato de cálcio, células tronco mesenquimais.

ABSTRACT

Bone tissue repair is an ancient challenge of world science. From the 70s and 80s, biomaterials began to be explored and nowadays play an important role in tissue engineering. These, when applied in bone grafting, have to be bioactive, biocompatible and resorbable. The integration between the various materials of synthetic origin and biological scaffolds has a wide spectrum of use. Among these scaffolds, fibrin-based compounds are distinguished by their hemostatic properties, stability and morphology favorable to cell proliferation. The present review addressed the structure and constitution of bone tissue and its limitations in the repair process, the relevance of tissue engineering approaches applied to this purpose and the biomaterials used to optimize this process, with special emphasis on materials composed of calcium phosphates, mesenchymal stem cells and its application with the biological sealant fibrin scaffold, highlighting the heterologous fibrin sealant, now named as fibrin biopolymer, a unique product testing in animal experimentation and clinical studies. At the end of this, a new approach is proposed for the regeneration of rat bone defects based on the association between heterologous fibrin sealant, biphasic calcium phosphate and mesenchymal stem cells.

Key-words: Bone regeneration, biomaterials, scaffolds, fibrin biopolymer, calcium phospate, mesenchymal stem cells.

LISTA DE ILUSTRAÇÕES

Figure 1. Presentation of the fibrin biopolymer. 47

Figure 2. Presentation of the biphasic calcium phosphate and aspect of the granules..... 48

Figure 3. Femoral 5 milimeter critical sized defect site..... 51

Figure 4. Flow citometry analysis of mesenchymal stem cells characterized by surface markers..... 53

Figure 5. Stable clot formed by fibrin biopolymer containing mesenchymal stem cells and biphasic calcium phosphate 54

Figure 6. Implantation of the composite in the bone defect site..... 54

Figure 7. Mean of Hounsfiled Unit (UH) value at the defect site of all groups 30 and 60 days after surgical procedure. Observation by micro-CT scan..... 55

Figure 8. Representative images on coronal plane of the right femur of rats subbmited to bone defect 30 and 60 days after surgical procedure by micro-computed tomography scan. 57

Figure 9. Representative images of the region of interest, macroscopic and scanning electron microscopy view, 30 days after surgical procedure. 59

Figure 10. Representative images of the region of interest, macroscopic and scanning electron microscopy view, 60 days after surgical procedure 60

Figure 11. Representative images of histological sections stained by Hematoxilin-Eosin on the region of interest 30 days after surgical procedure. 62

Figure 12. Representative images of histological sections stained by Hematoxilin-Eosin on the region of interest 60 days after surgical procedure. 63

Figure 13. Relative quantification of the defect site of all groups 30 and 60 days after procedure observed on histological sections.. 64

Figure 14. Relative quantification of progression of bone formation of treated groups 30 and 60 days after procedure observed on histological sections.. 64

Figure 15. Bone formation inside biphasic calcium phosphate granules in receptor site..... 65

Figure 16. Progressive resorption of calcium phosphate granules in receptor site. a) 30 and b) 60 days after implantation... 65

LISTA DE TABELAS

Table 1. Qualitative parameters for bone growth and consolidation. Observation by micro-CT scan 56

LISTA DE ABREVIATURAS E SIGLAS

- **Anvisa:** Agência Nacional de Vigilância Sanitária
- **BPF:** Biopolímero de fibrina
- **FBP**: Fibrin biopolymer
- **BCP**: Biphasic calcium phosphate
- **MSC**: Mesenchymal stem cells
- **Micro-CT**: Micro-computed tomography
- **SEM:** Scanning electron microscopy
- **HE staining**: Hematoxilin and Eosin staining

SUMÁRIO

CAPÍTULO I – REVISÃO DE LITERATURA

REVISÃO DE LITERATURA 19
O tecido ósseo 19
Estrutura e constituição 19
O processo de regeneração óssea e suas limitações 19
Engenharia tecidual aplicada a regeneração óssea 21
Biomateriais como substitutos ósseos: os fosfatos de cálcio 22
Células tronco mesenquimais: potencial e aplicações 24
Arcabouços biológicos (*scaffolds*): os selantes de fibrin e biopolímero de fibrina 26

OBJETIVOS 30
Objetivo geral 30
Objetivos específicos 30

REFERÊNCIAS 31

CAPÍTULO II – SUBMITED MANUSCRIPT "Fibrin biopolymer as scaffold to treat bone critical defects"

Abstract 44

INTRODUCTION 45

EXPERIMENTAL PROCEDURES 47
Animals 47
Fibrin Biopolymer 47
Biphasic calcium phosphate biomaterial 48

Mesenchymal stem cells 48
Isolation and expansion 48
Characterization 49
Association of fibrin biopolymer with mesenchymal stem cells and calcium phosphate 49
In vivo experimental procedures 50
Experimental design 50
Surgical procedure of critical sized femur defect 50
Euthanasia 51
Micro-computed tomography analysis 51
Scanning eletron microscopy (SEM) analysis 52
Histological analysis 52

RESULTS 53
Mesenchymal stem cell characterization 53
Surgery performing and euthanasia 54
Micro-CT analysis 55
Macroscopic aspect and SEM analysis 58
Hystological analysis 61

DISCUSSION 66

CONCLUSIONS 71

REFERENCES 72

APPENDIX I- Surface cell markers used on mesenchymal stem cells characterization by flow cytometry 83

APPENDIX II- Specifications of tomography Shimadzu® SCT-7800TC – helical single channel and obtained scans 84

APPENDIX III- Equipment and software used on histological analysis ... 85

APPENDIX IV- Qualitative analysis of images obtained by micro-computed tomography Equipment and software used on histological analysis 86

APPENDIX V- Weibel pattern applyied on stereological analysis (example) 87

APPENDIX VI- Numerical results of stereological analysis 88

CONCLUSÃO 92

APÊNDICE 93
Anexo I: Aprovação pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu 94

Anexo II: Instrução Normativa para a elaboração de teses e dissertações do programa de Doenças Tropicais da Faculdade de Medicina de Botucatu (FMB) 96

Capítulo I

1 REVISÃO DE LITERATURA

1.1 O TECIDO ÓSSEO

1.1.2 Estrutura e constituição

O tecido ósseo é um tipo especializado de tecido conjuntivo responsável por funções corporais tais como suporte, locomoção, proteção de tecidos moles e armazenamento e liberação de íons cálcio e fosfato. Este tecido é formado por células integradas a uma matriz óssea e constituído por osteoblastos, osteócitos e osteoclastos. Os osteócitos situam-se no interior da matriz e conectam-se por esta através de canalículos sendo responsáveis pela manutenção da matriz; os osteoblastos são produtores da parte orgânica da matriz, como colágeno, proteoglicanos e glicoproteínas sendo importante no processo de mineralização desta estrutura; e, por fim, os osteoclastos são células intrínsecas à reabsorção do tecido participando do processo de remodelação óssea. Já a porção acelular é representada pela matriz óssea constituída essencialmente por material extracelular calcificado. Os íons mais abundantes são o fosfato e o cálcio. Sua porção orgânica é constituída majoritariamente por fibras colágenas. Além disso, o tecido ósseo é recoberto pelo endósteo e pelo periósteo, superfícies internas e externas formadas por tecido conjuntivo e células osteogênicas. Os ossos longos são formados a partir da ossificação endocondral, que é caracterizada por ter seu início sobre uma porção de tecido cartilaginoso (cartilagem hialina) (1,2).

1.1.2 O processo de regeneração óssea e suas limitações

A regeneração óssea é um processo complexo e bem ordenado, que envolve fenômenos de osteoindução e osteocondução, iniciado quando há dano ao osso, e resultando no reestabelecimento da estrutura e na função do esqueleto (3).

Na fratura óssea ocorre inicialmente uma hemorragia, seguida por destruição de matriz e morte das células. A reparação se inicia com a re-

moção destes restos pelos macrófagos, enquanto o periósteo e o endósteo próximos à área lesionada iniciam um intenso processo de proliferação, formando um tecido rico em células osteoprogenitoras que penetra entre as extremidades ósseas rompidas. No tecido conjuntivo das extremidades fraturadas, surge o tecido ósseo primário ou imaturo através da ossificação endocondral de pequenos fragmentos de cartilagem formados e também por ossificação intramembranosa. Portanto, são encontrados simultaneamente no local da reparação áreas de cartilagem ossificada intramembranosa e ossificação endocondral. Após algum tempo, este conjunto evolui para um calo que envolve as extremidades da região fraturada e é constituído pelo tecido ósseo imaturo que une provisoriamente estas extremidades (1,3).

Durante toda a vida, o tecido ósseo é constantemente renovado em um ciclo de remoção do osso antigo pelos osteoclastos e sua substituição por tecido recém-formado por osteoblastos (4,5). Em condições naturais, o local da lesão repara-se sem a formação de tecido cicatricial, e as condições pré-injúria são restabelecidas, muitas vezes não sendo possível diferenciar o tecido lesionado do pré-existente adjacente (3,6).

Entretanto, diversos fatores podem prejudicar o processo de reparo, como casos em que ocorre não junção ou junção tardia da fratura. Esta falha pode ser causada por diversos fatores, tais como defeitos ósseos muito extensos, onde a necessidade de novo tecido excede a capacidade de auto cura, sendo estes causados por trauma, infecção, tumores, anomalias ósseas e malformações congênitas. Outro fator de risco é a associação de uma segunda doença limitante ao processo de reparo tecidual, como a osteoporose, doenças microvasculares, presença de infecções, tabagismo ou diabetes *mellitus* (3,7,8).

Estima-se que existam mais de 150 doenças e síndromes relacionadas a problemas esqueléticos e articulares (9). São registrados no mundo cerca de 15 milhões de fraturas, as quais exigem 2,2 milhões de

procedimentos de enxertia óssea anualmente (10,11). Para a regeneração deste tecido, muitas vezes é necessário o uso de enxertos ou implantes, que atuam como suporte e interagem como arcabouço, estimulando o processo de restauração tecidual (12).

Considerando a incidência de doenças relacionadas ao tecido ósseo, o número de procedimentos cirúrgicos e as limitações de alguns casos, o estudo de estratégias para a otimização da regeneração óssea é um assunto de grande interesse.

1.2 ENGENHARIA TECIDUAL APLICADA A REGENERAÇÃO ÓSSEA

O termo engenharia tecidual refere-se à aplicação de métodos da engenharia para a compreensão das relações entre a estrutura e a função dos tecidos normais e doentes e o desenvolvimento de substitutos biológicos para restaurar, manter e otimizar a função tecidual (8,13).

A engenharia tecidual aplicada à medicina regenerativa dos ossos tem como objetivo restaurar a forma e a função deste, utilizando-se de combinações entre células, moléculas sinalizadoras e materiais biocompatíveis, preferencialmente associados a um arcabouço (14). Objetiva-se, assim, a combinação e aplicação de materiais que estimulem o processo de reparo e sejam gradativamente reabsorvidos e substituídos por um tecido biológico recém-formado (8).

Um candidato promissor para a regeneração óssea deve ser bioativo, ter propriedades osteogênicas, osteocondutoras e osteoindutoras (14). Entende-se por osteogênese o processo biológico onde células osteoprogenitoras diferenciam-se em osteoblastos os quais, posteriormente, mineralizam-se e dão origem a um novo tecido ósseo. Osteocondução, por sua vez, refere-se à capacidade de uma superfície ser favorável ao crescimento ósseo adequado, normalmente através de poros, canais ou tubos. Por fim, osteoindução é o processo de recrutamento de células osteoprogeni-

toras para o local da lesão e subsequente diferenciação destas em osteoblastos (14,15). Além disso, o candidato dever ser biocompatível, ou seja, possuir a habilidade de integrar-se com as moléculas e células presentes no tecido lesionado sem acometer as áreas adjacentes (8,16).

1.2.1 Biomateriais como substitutos ósseos: os fosfatos de cálcio

O uso de biomateriais de diversas origens para dar suporte às células ósseas e, simultaneamente, estimular o crescimento deste tecido é um campo de interesse de longa data, sendo alvo de diversas pesquisas a fim de se investigar e otimizar a ação destes materiais *in vivo* (17).

Os biomateriais são em geral de origem sintética, natural ou natural modificada, destinados a interagir com o sistema biológico receptor e exercer diversas funções tais como tratar, reparar, reconstruir, aumentar ou substituir órgãos ou funções do corpo humano (18,19).

Um biomaterial deve ser biocompatível com o tecido onde será implantado, bioativo, biodegradável, não apresentar toxicidade e estimular uma resposta adequada dos tecidos vivos (8,20,21).

Preferencialmente, um enxerto ósseo deve unir propriedades de osteoindução, ou seja, capacidade de promover a diferenciação de células osteoprogenitoras em osteoblastos; osteocondução, o suporte ao crescimento ósseo e à proliferação tecidual, e osteointegração, a habilidade em integrar-se ao tecido ósseo adjacente (8,22).

Os enxertos ósseos de origem autógena, ou seja, retirados do próprio paciente, são considerados o padrão ouro na enxertia, pois apresentam propriedades osteogênicas, osteocondutoras, osteoindutoras e menor chance de rejeição (23). Entretanto, sua aplicação apresenta inconvenientes como a localização e o tamanho do defeito e a quantidade limitada de tecido doador. Além disso, há a necessidade de uma segunda intervenção cirúrgica para retirada do enxerto, com aumento do tempo de recuperação e custo, e aumento na morbidade do sítio doador (24,25).

Dessa forma, enxertos ósseos sintéticos são alternativas a estes enxertos biológicos devido à disponibilidade ilimitada (26). Os compostos a base de fosfatos de cálcio representam a principal classe de biomateriais empregados na reposição e na regeneração do tecido ósseo. Tais compostos foram introduzidos há mais de duas décadas, e representaram um grande avanço no campo das biocerâmicas, resultando em benefícios para diversas aplicações clínicas (27-29). Tais materiais podem ser empregados no tratamento de fraturas de diversas etiologias, defeitos e deformidades maxilofaciais, reparação e reconstrução óssea, fixação de implantes e liberação de medicamentos (30-33).

Materiais derivados de fosfatos de cálcio apresentam como principais características: biocompatibilidade com a fase mineral de ossos, dentes e tecidos calcificados devido à sua semelhança química com a parte inorgânica do osso humano; bioatividade, ou seja, capacidade de induzir uma resposta biológica similar àquela gerada durante a remodelação óssea; ausência de toxicidade, sendo metabolizados naturalmente e sem causar alterações nos níveis destes íons na urina, plasma ou órgãos; não são imunogênicos e são excelentes materiais osteocondutores, além de aumentar consideravelmente a resistência mecânica no local do defeito (34,35).

Dentre tais vantagens, destaca-se a osteocondução, processo descrito anteriormente, onde o recrutamento e estímulo de células pluripotentes e indiferenciadas para a forma de células formadoras de ossos, como pré osteoblastos, osteoblastos e, finalmente, osteócitos acontece na presença dos fosfatos de cálcio (36).

Estes materiais possuem diversas apresentações, como grânulos, blocos ou discos, e sistemas injetáveis, ampliando seu campo de aplicação de acordo com as necessidades do local da lesão, como estabilização, resistência mecânica, permeabilidade ou osteointegração. Em todos estes sistemas, a presença de poros é necessária para a difusão de fato-

res de crescimento e migração, proliferação e adesão de células osteo-progenitoras (37-41).

Dentre os materiais compostos por fosfatos de cálcio destacam-se a hidroxiapatita (HA), o βtri fosfato de cálcio (TCP) e composições que reúnem os dois materiais, chamadas de fosfatos de cálcio bifásicos (BCP) (42).

Destes, os compostos bifásicos possuem ampla utilização, pois apresentam a vantagem adicional do controle do nível de degradação, ajustando-se a proporção entre os dois componentes, a qual normalmente é representada por 60% de HA e 40% de TCP (21,43-45). Tais materiais combinam a baixa solubilidade e estabilidade da hidroxiapatita com a fase mais solúvel do tri fosfato de cálcio e a diferença no tamanho dos poros dos dois componentes, a qual influencia na solubilidade e difusão de bio-moléculas, reabsorção da área lesionada e diferenciação de células em osteoblastos, enquanto é gradativamente reabsorvido pelos osteoclastos (46,47). Além disso, estudos demonstraram que a combinação HA/TCP apresenta maior osteocondutividade do que estes separadamente (45,46).

Biomateriais desenvolvidos a partir de fosfatos de cálcio não são osteoindutivos, porém, tem sido demonstrado que a tal propriedade pode ser induzida combinando estes com fatores de crescimento, proteínas bi-oativas ou células osteogênicas ou tronco indiferenciadas (21,48-50).

A associação de materiais a base de fosfato de cálcio e células tronco mesenquimais apresenta bom potencial osteogênico, visto que tal biomaterial possui superfície apropriada para a adesão e atividade celular, atuando como estímulo para a diferenciação em osteoblastos devido à sua semelhança com a fração mineral do osso (33,50-54).

1.2.2 Células tronco mesenquimais: potencial e aplicações

O uso de células tronco é promissor na engenharia tecidual devido a habilidade de proliferação e diferenciação destas nas três linhagens

germinativas. O principal objetivo da aplicação de células tronco no local da lesão é que estas iniciem o remodelamento e crescimento do novo tecido, quando houver uma falha parcial ou completa no mecanismo de reparação (55-57).

Diversos estudos têm demonstrado que o uso de células tronco, notadamente as mesenquimais, podem ser aplicadas a diversas terapias de regeneração óssea como câncer, osteonecrose, osteoastroses, fraturas, doenças periodontais e fusões espinhais (58,59).

Tais células são multipotentes e, portanto, dotadas de grande versatilidade. Estas podem se diferenciar em linhagens osteogênicas, mioblásticas, adipogênicas, condrogênicas, endoteliais e neurogênicas, originando diversos tecidos, além de possibilitar um transplante alogênico sem a necessidade de imunossupressão, podendo inclusive ser isoladas de uma fonte autóloga, sem prejuízos ao doador (60-63).

Células tronco mesenquimais são isoladas a partir de diversas fontes tais como periósteo, cérebro, fígado, medula óssea, tecido adiposo e músculo esquelético. As isoladas da medula óssea representam uma importante fonte de células tronco (57). Células provenientes de tal sítio doador possuem grande potencial de diferenciação em linhagem osteogênica e, ampla capacidade de expansão *in vitro* (62). A partir de um pequeno volume, tais células podem ser isoladas, cultivadas e manter sua funcionalidade após expansão ou criopreservação, devido a sua elevada capacidade proliferativa (64,65).

Estudos demonstraram que tais células têm a habilidade de migrar para sítios de tecido injuriado e possuem propensão à secreção de fatores sinalizadores importantes para o reparo ósseo (57,62,66). Dessa forma, a capacidade única das células mesenquimais em promover a cicatrização óssea, combinado com sua facilidade de isolamento, fornecem uma terapia mais efetiva, mais barata, com menos morbidade para os pacientes que sofrem com limitações na regeneração deste tecido (62,67).

Embora alguns estudos demonstrem que tais células podem ser aplicadas sozinhas na reparação do tecido ósseo (68, 69), outros afirmam que células isoladas não possuem grande potencial em formar um tecido novo, tornando necessário um ambiente que inclua a presença de um material para oferecer suporte estrutural, a fim de que ocorra o crescimento adequado (70-73).

Abordagens de engenharia óssea tecidual usando biomateriais naturais ou sintéticos e células adultas ou indiferenciadas obtiveram um grande número de sucessos em modelos animais (8,74,75). Além disso, o comportamento celular é fortemente influenciado por sinais biológicos e bioquímicos da matriz extracelular. Dessa forma, o uso de arcabouços como *delivery systems* para fatores de crescimento, peptídeos de adesão e citocinas têm recebido grande atenção da comunidade científica (76,77).

1.2.3 Arcabouços biológicos (*scaffolds*): os selantes de fibrina e bipolímero de fibrina

Os arcabouços biológicos têm como função criar um ambiente biomimético, ou seja, oferecer as condições ideais e semelhantes às dos tecidos originais para que células ou tecidos se estabeleçam e proliferem satisfatoriamente (78,79).

Arcabouços porosos tridimensionais proporcionam o ambiente adequado para a regeneração de tecidos e órgãos. Estes agem como modelo para a formação óssea e são implantados juntamente com células, biomateriais ou fatores de crescimento. Fornecem ainda estabilidade estrutural e ambiental para a regeneração celular e mimetizam o tecido em funcionalidade (76,80-82).

A fibrina é um dos principais constituintes do processo de cicatrização e tem como principal função o reestabelecimento da homeostase, além de servir como arcabouço para a migração, adesão e diferenciação celular (83-85).

Os selantes de fibrina são materiais biológicos dotados de propriedades adesivas e hemostáticas, são capazes de promover adesão e a estabilidade dos tecidos, controlam sangramentos em áreas cirúrgicas e aceleram o processo de cicatrização tecidual. Possuem amplo uso como arcabouços (*scaffolds*) e em sistemas para entrega de fármacos (como *drug delivery*), possibilitando a aplicação precisa de sistemas compostos por outros biomateriais, células, enzimas ou fatores de crescimento (87-89).

Estes materiais são compostos basicamente por trombina e fibrinogênio. A trombina converte o fibrinogênio em fibrina formando uma rede estável e mimetizando o passo final da cascata de coagulação. Tradicionalmente, a trombina utilizada é obtida por meio de sangue humano ou bovino, e o fibrinogênio a partir de sangue humano (88,89). Os selantes de fibrina foram desaprovados para uso pela FDA (Food and Drug Administration's) dos EUA em 1998 (90,91). Esta agência regulatória se preocupava com a possibilidade de transmissão de doenças infecciosas, em especial as virais, por intermédio do sangue humano (90-94).

Tendo em vista tal problemática, a ideia de produzir um novo selante foi proposta em 1989 por um grupo de pesquisadores do Centro de Estudos de Venenos e Animais Peçonhentos da UNESP (CEVAP). O principal objetivo do projeto foi produzir um selante de fibrina que não utilizasse produtos derivados do sangue humano. Para tanto, foram utilizados a giroxina, uma serinoprotease trombina-símile isolada do veneno de *Crotalus durissus terrificus*, e o crioprecipitado extraído do sangue de grandes animais. Este último substituiria o fibrinogênio humano. Este selante foi patenteado, utilizado em experimentação animal em diversos modelos e aprovado pela ANVISA (Agência Nacional de Vigilância Sanitária) para ensaios clínicos fase I/II no tratamento de úlceras venosas crônicas em seres humanos. Este se mostrou um produto seguro e deverá ser no futuro avaliado a eficácia tratando-se pacientes com úlceras venosas crônicas

num ensaio clínico fase III. (95-99). Estudos de nosso grupo têm validado as múltiplas funções deste bioproduto, as quais abrangem não somente suas propriedades selantes, mas também a hemostasia, superfície ideal para adesão celular e estabilidade dos tecidos e veículo para aplicação precisa de biomateriais, células, enzimas ou fatores de crescimento; desas forma, a designação de "biopolímero de fibrina" é mais adequada para este biomaterial inovador.

No início do século XXI diversos autores (100-102) analisaram o efeito do selante de fibrina comercial sobre o comportamento das células tronco mesenquimais. Estas mostraram-se viáveis, com aumento da sobrevivência e da estabilidade sem afetar a capacidade migratória (70,100-102). Gasparotto et al. (103) pela primeira vez mostraram que especificamente o novo biopolímero de fibrina produzido pelo CEVAP tem excelente interação com tais células derivadas da medula óssea de ratos, formando uma estrutura tridimensional que possibilita a adesão, diferenciação e proliferação das mesmas.

O uso dos selantes de fibrina na regeneração óssea apresentaram bons resultados, pois os materiais a base de fibrina iniciam o processo de hemostasia no local aplicado e agem como uma matriz bioativa (104,105). Além disso, a manipulação cirúrgica de biomateriais como os fosfatos de cálcio associados aos selantes de fibrina melhora e amplia seu campo de aplicação em cirurgias ósseas (48), pois tal associação forma um material moldável, de estabilidade imediata e com propriedades que favorecem a osteogênese no sítio de implantação, além de produzir um composto que pode ser acomodado no defeito sem deixar espaços vazios (26,48,70,106).

Assim, a estrutura formada por este arcabouço biomaterial, juntamente com as células tronco e os biomoduladores formam um molde ideal, constituído de sítios para a adesão e crescimento celular, poros que permitem a difusão de nutrientes, resistência capaz de suportar as forças

exercidas sobre o local de implantação, e taxa de degradação semelhante às da neoformação óssea (13,107-110).

Cunha et al (111) obtiveram resultados satisfatórios associando o biopolímero fibrina e a hidroxiapatita sintética no tratamento de defeitos craniais em ratos. Neste caso o selante apresentou propriedades biofuncionais e osteocondutoras. Recentemente Orsi e colaboradores (112) validaram a associação entre tal bioproduto à base de fibrina e células tronco mesenquimais tratando defeitos críticos experimentais no fêmur de ratos osteoporóticos.

Dessa forma, a associação entre biomateriais, células tronco e aplicação junto a um arcabouço a base de fibrina abre um novo campo para as aplicações dos enxertos ósseos, visto que as propriedades químicas, físicas e biológicas somam-se para o preparo de substitutos ósseos (113-115).

Portanto, considerando-se que o biopolímero de fibrina é um arcabouço biológico tridimensional adequado para as células tronco mesenquimais, a associação com outros biomateriais com propriedades osteocondutoras a base de fosfato de cálcio, poder-se-á encontrar alternativas terapêuticas mais eficientes e viáveis para o tratamento de reparos ósseos.

2 OBJETIVOS

2.1 Objetivo geral

O objetivo do presente estudo foi avaliar a influência do material osteocondutor fosfato de cálcio bifásico, associado ou não com células tronco mesenquimais (CTMs), e aplicados com o arcabouço biológico tridimensional e biodegradável denominado biopolímero de fibrina no processo de reparo ósseo de ratos submetidos a defeito crítico do fêmur.

2.2 Objetivos específicos

- Padronizar a técnica de produção do defeito crítico em fêmures de ratos;
- Verificar a ação do biopolímero de fibrina como arcabouço biológico no reparo ósseo;
- Verificar o efeito do fosfato de cálcio aplicado sobre o reparo ósseo;
- Verificar a ação do biopolímero de fibrina em associação ao material osteocondutor;
- Coletar e cultivar *in vitro* as CTMs a partir da medula óssea de ratos;
- Caracterizar as CTMs obtidas a partir da medula óssea de ratos por meio da citometria de fluxo;
- Verificar o efeito da aplicação das CTMS em associação ou não com o fosfato de cálcio e biopolímero fibrina;
- Avaliar a área da lesão no fêmur dos ratos tratados, através de imagens de tomografia 30 e 60 dias após o procedimento cirúrgico;
- Avaliar a cobertura óssea através de microscopia eletrônica de varredura 30 e 60 dias após o procedimento cirúrgico;
- Avaliar o reparo ósseo por meio da análise histológica aos 30 e 60 dias após o procedimento cirúrgico.

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Capítulo II

CHAPTER II: MANUSCRIPT SUBMITTED TO THE JOURNAL "MATERIALS RESEARCH: IBERO-AMERICAN JOURNAL OF MATERIALS"

HETEROLOGOUS FIBRIN SEALANT AS SCAFFOLD TO MESENCHYMAL STEM CELLS ASSOCIATED TO CALCIUM PHOSPHATE AND APPLIED IN CRITICAL DEFECTS ON FEMUR OF RATS

Running title: Fibrin biopolymer as scaffold to treat bone critical defects

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The bone tissue repair remains a challenge on tissue engineering. Currently, new synthetic materials are being applied and often integrated to live cells and biological scaffolds. Among these last, the unique fibrin biopolymers (FBP) produced by CEVAP has hemostastic, sealant, adhesive and scaffold properties. It was evaluated the regenerative potential of a FBP, biphasic calcium phosphate (BCP) and mesenchymal stem cells (MSCs) association applied on critical sized defects in femurs of rats. It was showed that FBP is a suitable scaffold for bone defects due to its stable clot allowing cell adhesion and proliferation. This association was bioactive, biocompatible, with satisfactory integration between materials applied and formed bone. Progressive deposition of bone matrix on defect site was observed on all experimental groups 30 and 60 days after the procedure, more significantly on group treated with FBP and MSCs. According to the literature and these results, late assessment will be necessary for a definitive conclusion on these associations.

Key words: bone regeneration, fibrin sealant, fibrin biopolymer, calcium phospate, mesenchymal stem cells.

1. Introduction

The bone regeneration starts right after its damage, and involves osteoinduction and osteoconduction phenomena that results in the reestablishment of the structure and function [1]. During the life and under normal conditions this tissue is constantly renewed [2,3]. However, several factors may impair the repair process, such as very extensive defects, trauma, infection, tumors, bone abnormalities and congenital malformations. When non-unions occur its often required the use of grafts or implants that act as support and stimulators to the process of tissue restoration. Thus, tissue engineering approaches aims to develop regenerative therapies for bone tissue in order to restore its shape and function combining cells, signaling molecules and biocompatible materials, preferably associated with a bioactive scaffold [4].

Currently, autogenous bone grafts are still considered the gold standard in bone grafting due to its osteogenic properties[1]. However, its application presents drawbacks such as the limited amount of donor bone tissue and the need for a second surgical to remove the graft, causing an increase in recovery time. In this scenario, synthetic bone grafts present an important alternative due to their unlimited availability[5]. Calcium phosphates compounds are the main class of biomaterials used in the replacement and in the regeneration of bone tissue and present as main characteristics biocompatibility, bioactivity and absence of toxicity and immunogenicity; in addition, these materials are excellent osteoconductors and increases mechanical resistance at the defect site [6,7]. Among these materials are hydroxyapatite (HA), calcium β-phosphate (TCP) and the combination among them, known as biphasic calcium phosphates (BCP) [8]. This last compound is widely used due to its additional advantage of controlling the level of degradation by adjusting the proportion between the two components [7].

The association between calcium phosphate materials and mesenchymal stem cells presents good osteogenic potential, since these stem cells have an appropriate surface for cellular adhesion and activity, acting as a stimulus for the differentiation in osteoblasts [9-13]. In addition, the unique ability of mesenchymal cells to promote bone healing, combined with their ease of isolation, provides a more appropriate therapy for patients who suffer from limitations in the regeneration of this tissue [14,15]. However, for satisfactory cell action, adequate anchoring at the application site

is imperative, which may be provided by a biological scaffold. Three-dimensional scaffolds provide suitable environment for tissue regeneration, acting as a framework for bone formation and are easily implanted with cells, biomaterials or growth factors, providing structural and environmental stability for cell and tissue regeneration[16,17-19].

Fibrin sealants are natural fibrin biopolymers endowed with adhesive, sealant, and hemostatic properties and suitable for cell adhesion and tissue stability. They are widely used as scaffolds and in drug delivery systems, enabling the precise application of biomaterials, cells, enzymes or growth factors[20-22]. Traditionally commercial fibrin sealants are produced from thrombin obtained from human or bovine blood, and fibrinogen extracted from human blood[22,23]. This biomaterial, under exceptional conditions, can transmit infectious diseases by human blood [23,24]. In view of this, researchers from The Center for the Study of Venoms and Venomous Animals of UNESP (CEVAP-Brazil) proposed the production of a new fibrin biopolymer (FBP), previously named of heterologous fibrin sealant that did not use products derived from human blood. That way, a thrombin-like serine protease enzyme isolated from *Crotalus durissus terrificus* venom, and a cryoprecipitate rich in fibrinogen extracted from *Bubalus bubalis* were used [25]. This new product was patented, tested on several animal experimentation and approved by Anvisa for phase I / II clinical trials in the treatment of patients with chronic venous ulcers, where has proved to be a safe product [25-45].

It was showed by our research team that the fibrin biopolymer produced by CEVAP has an excellent interaction with the mesenchymal stem cells derived from the bone marrow of rats[30], forming a three-dimensional structure that enables cell adhesion, differentiation and proliferation, properties also observed on commercial polymers[46,47]. In addition, satisfactory results was obtained by associating FBP with synthetic hydroxyapatite in the treatment of cranial defects in rats[43]. Recently, we also validated the association between FBP and mesenchymal stem cells in femoral defects of osteoporotic rats[45].

Therefore, the objective of the present study was to evaluate for the first time the potential of repair of calcium phosphate biomaterial applied together FBP associated or not with mesenchymal stem cells in rats submitted to a femoral critical sized defect.

2. Experimental Procedures

The present study was approved by the Animal Ethics Committee of Botucatu Medical School, São Paulo State University (protocol 1164/2016).

2.1 Animals

Eighty male Wistar rats (*Rattus norvegicus*) of 350±20 kilograms average weight was randomly divided into five experimental groups, and ten rats of twenty-one days age was designed to bone marrow harvest aiming mesenchymal stem cell expansion. The rats were provided by rodent house from Laboratory of Experimental Medicine, from UNESP Botucatu. All animals were kept at 21 ± 2 °C under a 12 h light/dark cycle, with *ad libitum* access to food and water.

2.2 Fibrin Biopolymer

The Fibrin Biopolymer (FBP) (Fig. 1) was supplied by The Center for the Study of Venoms and Venomous Animals (CEVAP) at São Paulo State University, Brazil. The components and formula of the applied product are contained in its patents (registry number: BR1020140114327 and BR1020140114360). The components were mixed upon use on the following: 10 µL of Fraction 1 (gyroxin), 25 µL of Fraction 2 (fibinogen cryoprecipitate) containing and 15 µL of Fraction 3 (diluent) were pipetted and homogenized, resulting in a final volume of 50 µL of sealant and keeping standard concentration.

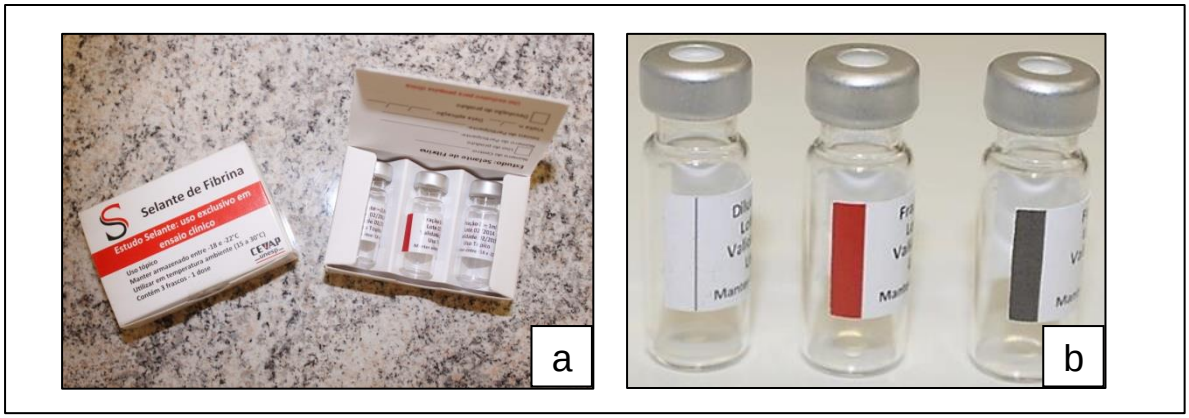

Figure 1. **a** and **b**) Presentation of the fibrin biopolymer.

2.3 Biphasic calcium phosphate biomaterial

The biphasic calcium phosphate (BCP) (Graftys® BCP – Anvisa n. 80517190002) (Fig. 2) was kindly supplied by LAS Brasil importer. The synthetic material was presented in granules varying between 0,5 and 1 millimeter and consists of 60% of hydroxiapatite and 40% of β- tricalcium phosphate. Its porosity varied between ≤ 10 µm and 100 µm. Fifteen milligrams of previously aliquoted and storaged material were implanted in the site of interest.

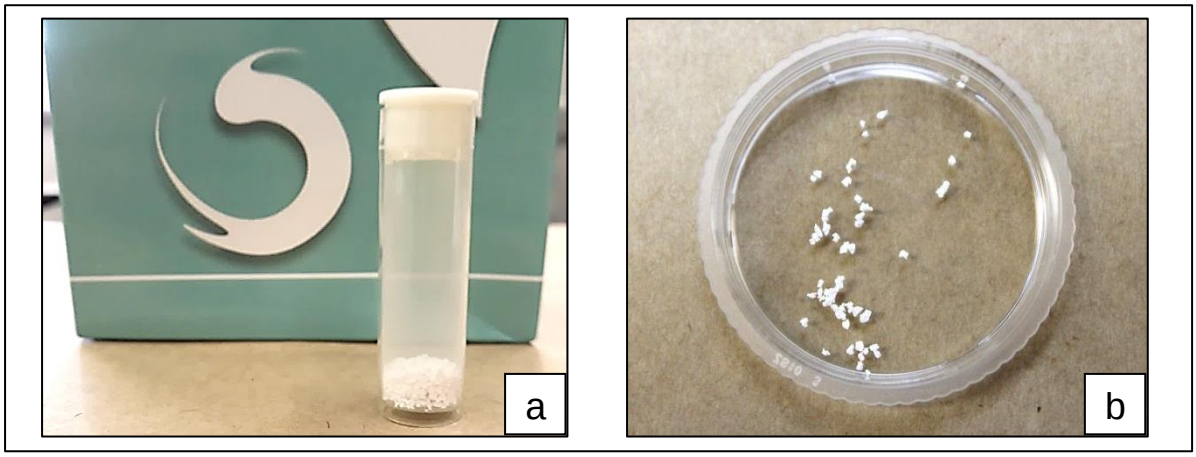

Figure 2. **a**) Presentation of the biphasic calcium phosphate **b)** aspect of the granules.

2.4 Mesenchymal stem cells

2.4.1 Isolation and expansion

Aiming the harvesting of the cells from bone marrow, euthanasia of rats was performed with Isofluorane overdose (MAC > 5%) and cervical dislocation after the unconsciousness of the animals was confirmed. Bone marrow cells were obtained by centrifugation of the femurs and tibias, which were vertically positioned in conical tubes containing 1000 µL of DMEM® (Dulbecco's Modified Eagle Medium) high glucose, and centrifuged at 2.500 rpm for 4 minutes. The bones were then discarded and the precipitate was resuspended. The obtained solution was transferred to 25cm^2 culture flasks containing 4mL of DMEM® (supplemented with 20% Fetal Bovine Serum, 100µg/mL Penicillin/ Streptomycin and 3µg/mL Amphotericin B), making a total volume of 5 mL. The flasks were kept in an incubator on 5% CO_2 atmosphere and at 37.5°C. Replacements to the culture medium were performed every 3 to 5 days, depending on the growth of cells and adherence on the bottle surface, both

monitored by light microscopy. At the moment cells reached about 90% confluence, the cell passage was performed. For this process, the culture medium in the flask was discarded, 2mL of PBS was added for washing and discarded thereafter; after washing, 2mL of trypsin was added and the flask was maintained in the incubator oven for 5 minutes. With the cells in suspension, 2 mL of supplemented DMEM® was added. The solution was then transferred to 15mL conical tubes and centrifuged at 1700 rpm for 10 minutes. After centrifugation, the supernatant was discarded and the pellet containing the cells was resuspended in 1000 µl of DMEM®. The solution was then transferred to flasks of the appropriate size.

2.4.2 Characterization

Cultivated mesenchymal stem cells (MSCs) on fourth passage was characterized by flow cytometry (FACS Calibur®; BD Pharmingen) using the positive surface markers CD90, CD44 and ICAM-1, and negative expression markers CD45, CD11b, MHC class I and RT1-aw2 49 . Specifications of each marker are presented in appendix 1. The preparation consisted on an adjustment in cell volume to $2x10^5$ cells per tube/marker. Antibodies were added according to specific dilution also described in appendix 1. The samples were then incubated at room temperature for 1 hour and washed with PBS [45].

2.5 Association of fibrin biopolymer with mesenchymal stem cells and calcium phosphate

At the bone defect site and depending on the group, 50 µL of FBP, 15 milligrams of biphasic calcium phosphate, and 10 µL of complete culture medium containing an average of 3×105 cells previously characterized was added. In the group where only the biphasic calcium phosphate was implanted, it was made directly in the place, adding and compacting the granules with the help of a spatula. For the association of BCP and FBP, the fractions 2 and 3 of the second were homogenized in a cell culture plate, the calcium phosphate was added and, finnaly, Fraction 1 was added; the clot formed was then transferred to the defect site. For the association of the two materials with the mesenchymal stem cells, after counting of these, transfer to 2 ml microtubes and centrifugation, the cells were resuspended in 2ml microtubes, with volume of 10 µL of complete medium, and then added to a microtube containing

the calcium-based biomaterial and the fractions of the FBP as previously described. The formed clot was then transferred to the lesion site. In the control group, no material was received.

2.6 In vivo experimental procedures

2.6.1 Experimental design

A five-millimeter critical sized defect was performed on right femur of each rat, as described afterwards, and divided into 5 experimental groups, namely:

- Group 1: Critical defect without material filling (control group)
- Group 2: Critical defect filled with BCP
- Group 3: Critical defect filled with FBP+BCP
- Group 4: Critical defect filled with FBP+ MSCs
- Group 5: Critical defect filled with FBP+BCP+MSC

2.6.2 Surgical procedure of critical sized femur defect

All the animal experiments were performed according to the Brazilian College for Animal Experimentation (COBEA) guidelines 49.

Animals were anesthetized with Isoflurane at concentration (MAC) of 3% for induction and 1.5% for maintenance, no need for mechanical ventilation. After confirmation of anesthetic induction, the animals were placed in lateral decubitus position and trichotomy was performed on the anterior region of the right thigh. A medial longitudinal incision was made on the skin of the anterior distal third of the region. The skin was laterally removed, and then the femoral quadriceps muscle was separated exposing the periosteum, which was sectioned transversely and disjointed from the bone surface. After exposure of the bone tissue, a critical defect of 5 mm diameter and 2 mm width was performed using a drill bit coupled to a dental micromotor (BELTEC ® model LB100) at about 3000 rpm, thus promoting a bone defect in the proximal third of the right femur diaphysis (Fig. 3). During the procedure of the defect, the lesion site was constantly irrigated with physiological solution in order to avoid overheating on the region. According to the group, the mixture of components was added to the promoted bone defect. After stabilization of the graft, the suture was performed. On the postoperative period, the animals received food and water *ad libitum* and were not immobilized at any time. The rats received postoperative analgesia

consisting on 5 mg/kg of tramadol hydrochloride, 5 mg/kg of ketoprofen (Ketofen® 10%) and 8 mg/kg enrofloxacin hydrochloride (Chemitril® 2.5%) subcutaneously in an interval of twelve hours during three days[51].

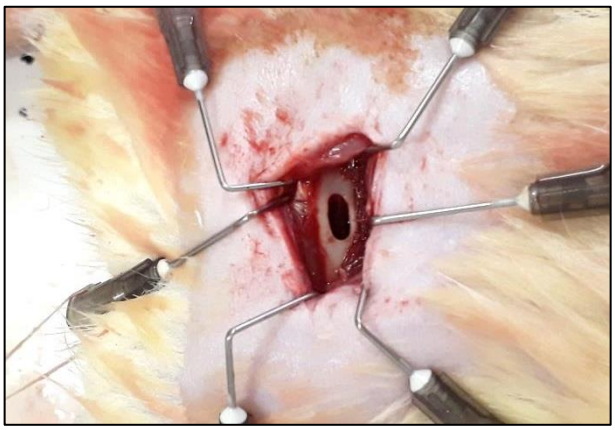

Figure 3. Femoral 5 milimeter critical sized defect site.

2.6.3 Euthanasia

Animals were euthanized by Isoflurane overdose (MAC > 5%) and cervical dislocation after the unconsciousness of the animals was confirmed. The procedure occurred in two periods for observation and analysis of samples: 30 and 60 days after surgery. On these periods and before euthanasia, micro-computed tomography scan of the region of interest were performed. The collected bones were then forwarded for histological and for scanning electron microscopy analysis. The macroscopic aspect of the femurs and surrounding area at the moment of euthanasia was also considered.

2.7 Micro-computed tomography analysis

At 30 and 60 days after the surgical procedure, the animals were submitted to micro-computed tomography (micro-CT) scan in order to evaluate the repair process on the lesion site. The procedure was carried by a helical single channel tomograph (Shimadzu® SCT-7800TC), additional specifications of the equipment is contained in appendix 2. To the scan, animals were previously anesthetized with ketamine and xylazine hydrochloride at the dose of 0.10 mL / 100 grams of body mass and positioned in dorsal decubitus. The images obtained were studied in the cross-sectional, longitudinal and coronal sections, and the tomographic-graphical appearance of the

implants were evaluated considering adjacent opacification, Hounsfield Units values, bone proliferation, consolidation and remodeling processes on the region of interest [52].

2.8 Scanning eletron microscopy (SEM) analysis

After collecting of the femurs, fixation with Karnovsky's solution for 36 hours was initially performed. After fixation, samples were washed in phosphate buffer and immersed in 0.5% osmium tetroxide solution for 60 minutes and posteriorly dryed to the critical point, positioned over stubbs and coated with gold (sputtering process). The samples were analyzed using ta Jeol® JSM 5800LV microscope on 10 kV.

2.9 Histological analysis

The femurs were collected and fixed in 10% formaldehyde solution for 24 hours. Subsequently, the material was subjected to decalcification in 30% formic acid solution during 15 days. After decalcification, the bones were reduced to the region of interest, fixed in 70% alcohol for 12 hours, dehydrated in an increasing series of ethanol, diaphanized in xylol and, finally, embedded in paraffin. Semi-serial cross sections of 5µm of the bone tissue were obtained, which were stained with Hematoxylin and Eosin (HE). The slides obtained were visualized and photographed with help of equipments described on appendix 3 on magnification of 4x, 10x and 20x. Stereological analysis was performed based on images obtained by 10x magnify according to methodology described for Weible et al [53] in order to quantify formed bone, biomaterial, bone marrow and cellularized connective tissue on lesion area.

3. Results

3.1 Mesenchymal stem cell characterization

The mesenchymal stem cells used in the present study characterized by flow cytometry before its application showed the expressions represented on histograms contained on Figure 4. The cells presented positive expression for markers CD90, CD44 and ICAM1, and negative for CD45, CD11b RT1-aw2 and MHC Class II.

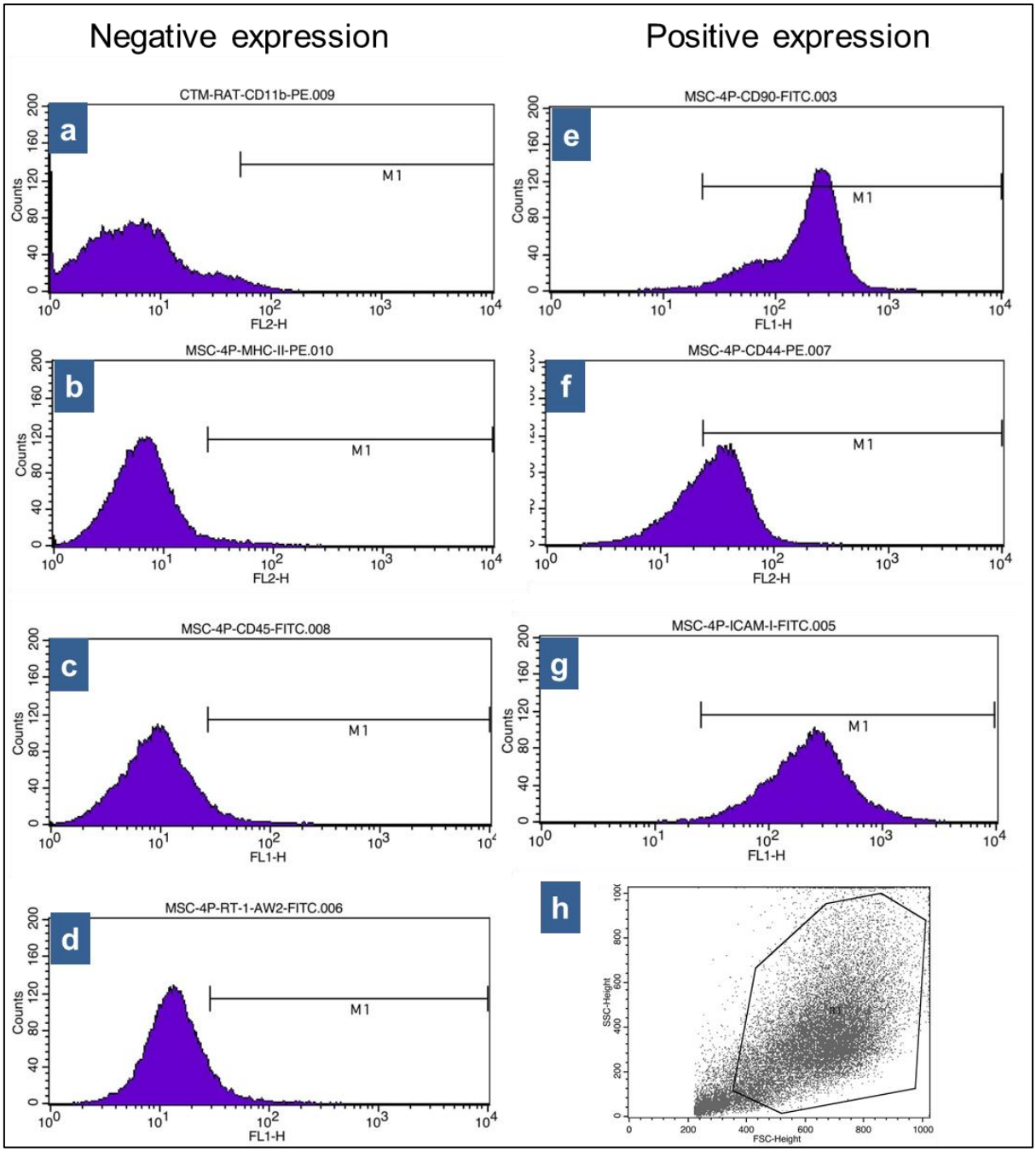

Figure 4. Flow citometry analysis of mesenchymal stem cells characterized by surface markers. M1 (related to x axis) represents the limit required to positive expression be considered. **a)** CD11B, **b)** MHC Class II, **c)** CD45, **d)** RT1-Aw2, **e)** CD90, **f)** CD44, **g)** ICAM1 **h)** gate of analysed cell population.

3.2 *Surgery performing and euthanasia*

At the time of surgery, it was verified the application of the materials in the group with the fibrin biopolymer was facilitated by this since the clot formed, containing the calcium phosphate biomaterial and stem cells (Fig. 5), was much easier to be handled, molded and accommodated at the injury site (Fig. 6).

After the surgery, the incision site showed no signs of inflammation and animals presented normal locomotion. At the time of euthanasia, no inflammatory infiltrates or rejection at the graft site were observed (Fig. 8).

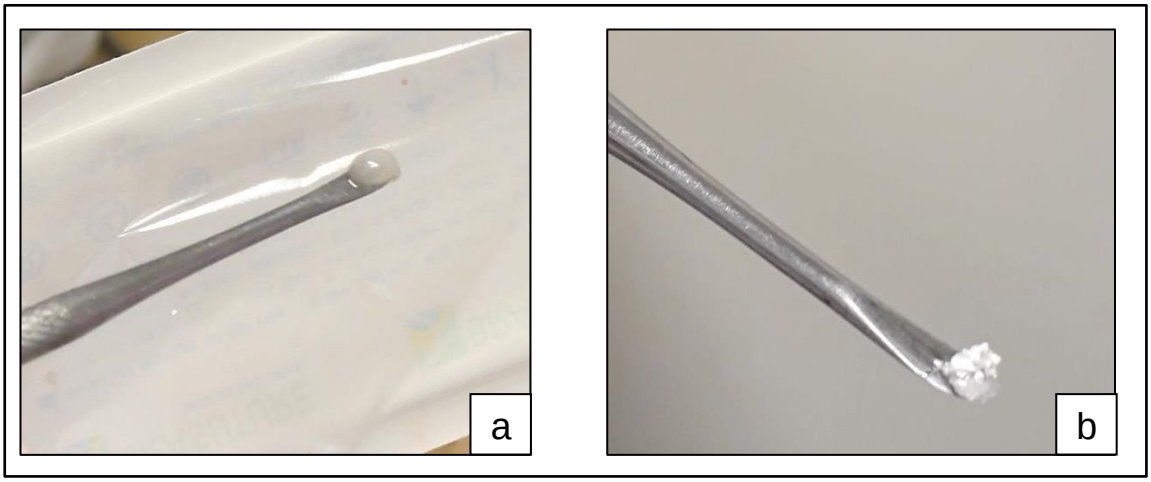

Figure 5. Stable clot formed by fibrin biopolymer containing **a)** mesenchymal stem cells and **b)** biphasic calcium phosphate.

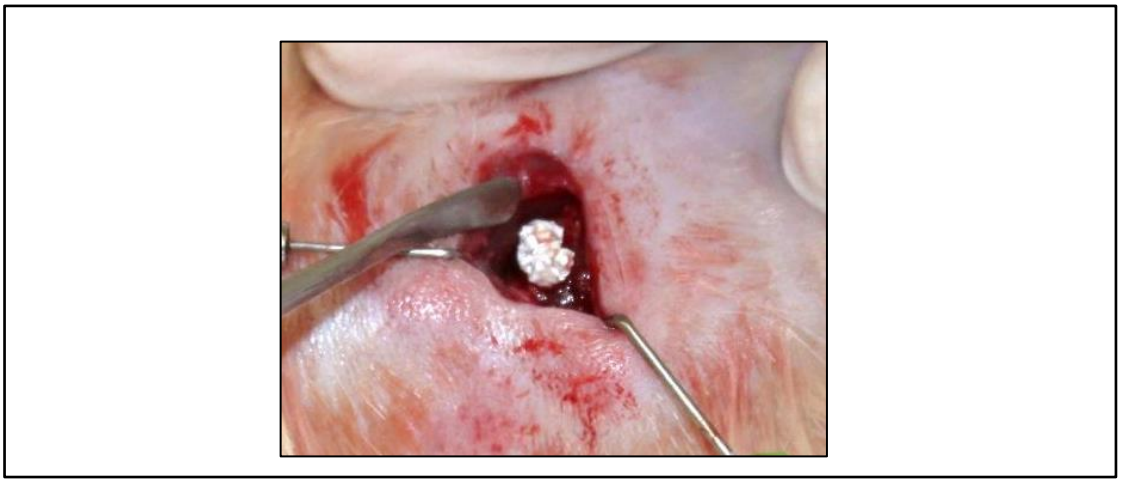

Figure 6. Implantation of the composite in the bone defect site.

3.3 Micro-CT Analysis

Through the images obtained by tomography (Fig. 8), lesion site was visible in all groups and periods. The quantitative parameter evaluated was the UH value (Hounsfield units) (Fig.7), which is related to the density of the tissue in formation, and in the normal cortical bone tissue has a value of 1000 UH or more [52]. At 30 days, the mean value per group was less than 1000 only in the control group. At 60 days, UH value was higher than 1000 in all groups, but control still presented the lowest value among all groups.

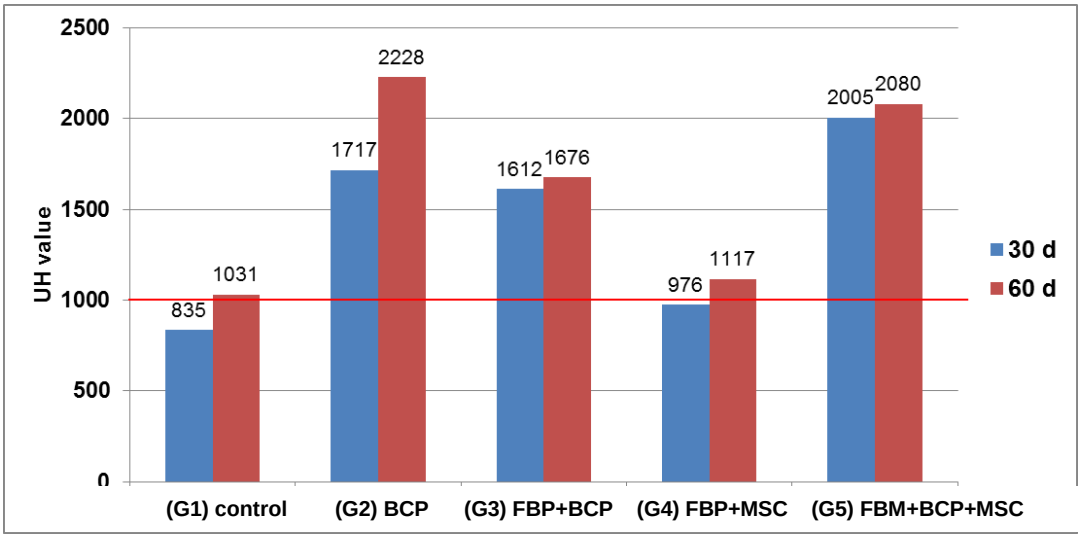

Figure 7. Mean of Hounsfiled Unit (UH) value at the defect site of all groups 30 and 60 days after surgical procedure. Observation by micro-CT scan. Red line indicates minimum value expected for regular cortical bone.

Based on main parameters to evaluate bone growth and consolidation, the qualitative analysis considered the following parameters: bone proliferation, remodeling and consolidation, and opacification of the region of interest in relation to the adjacent bone. According to the qualitative parameters considered, the proliferation, which indicates bone activity at the lesion site, was observed in all groups in the final period of observation, indicating that the total consolidation had not yet occurred. In addition, remodeling in the region was also active at 60 days, but the control group showed cortical misalignment and separation between the edges of the lesion (Table 1). Opacification, a parameter that indicates the similarity of the radiopacity area of the lesion with adjacent intact tissue, was greater in the groups where biphasic calcium phosphate was applied. Detailed information about the parameters evaluated is contained in appendix 4.

Table 1. Qualitative parameters for bone growth and consolidation. Observation by micro-CT scan. **(-):** absent **(-+)** small significance. **(+)** medium. **(++)** significative.

	G1		G2		G3		G4		G5	
	30 d	60 d	30 d	60 d	30 d	60 d	30 d	60 d	30 d	60 d
Proliferation	-	-+	+	+	+	+	+	++	+	+
Remodeling	-+	-+	+	+	+	+	++	++	+	++
Opacification	-+	-+	++	++	++	++	+	+	++	++

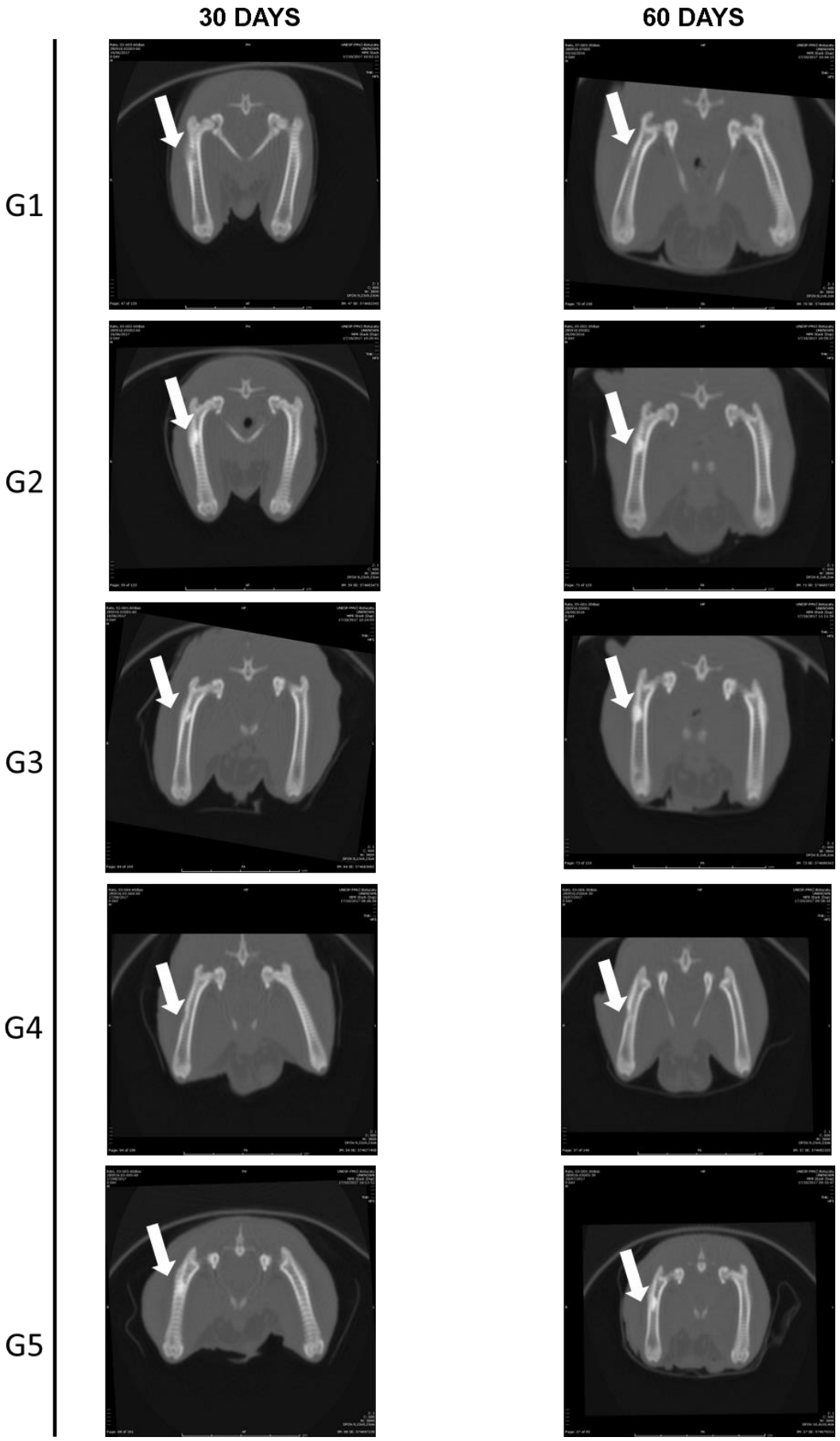

Figure 8. Representative images on coronal plane of the right femur of rats subbmited to bone defect (white arrows) 30 and 60 days after surgical procedure by micro-CT scan.

3.*4 Macroscopic aspect and SEM analysis*

Figures 9 and 10 shows general aspect of the lesion region at 30 and 60 days after the surgical procedure, at the moment of the collection of the material after euthanasia and by Scanning Electron Microscopy (SEM). Macroscopically and at 30 days, it is possible to distinguish the edges of the lesion in all experimental groups, with a significant approximation of the edges of the lesion in group 4. Through microscopic images, groups 1 and 4 (both without biphasic calcium phosphate material) show uniform coverage at the lesion site, while the groups treated with calcium phosphate allows visualization of the characteristic morphology of the granules. At 60 days, it is perceptive the approximation of the edges of the lesion in all the groups, with integration of the lesion edges to the applied association, however without apparent resorption in the central region in the groups that received the biomaterial. The images obtained through the scan corroborate the approach of the edges of the lesion and it is not possible to visualize the lesions. The control group and the fibrin biopolymer and cells association (group 4) present total superficial coverage in the lesion area. Over again, calcium phosphate remained without great resorption of this in the central region of the lesion.

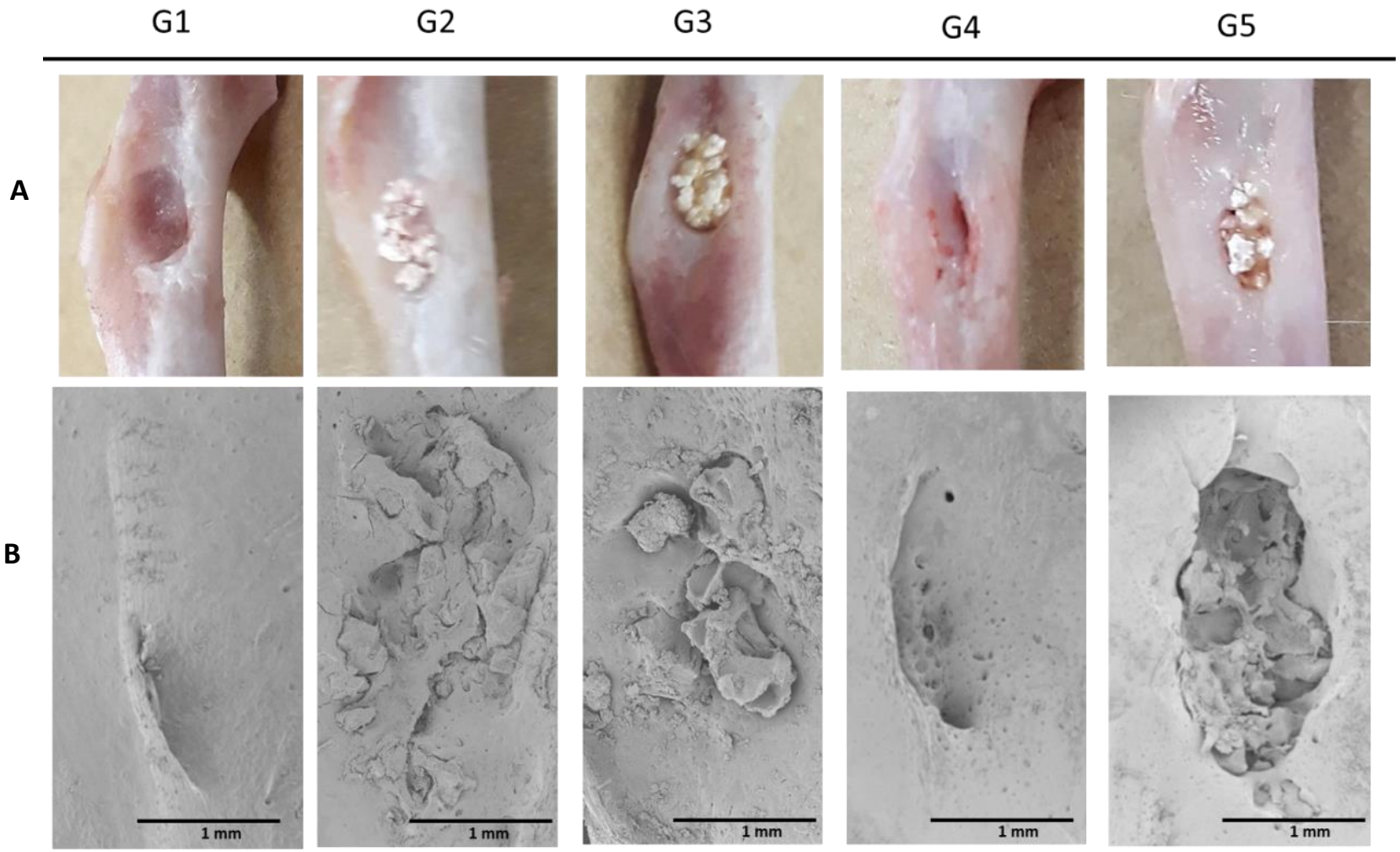

Figure 9. Representative images of the region of interest, **(a)** macroscopic and **(b)** by scanning electron microscopy view, **30 days** after surgical procedure.

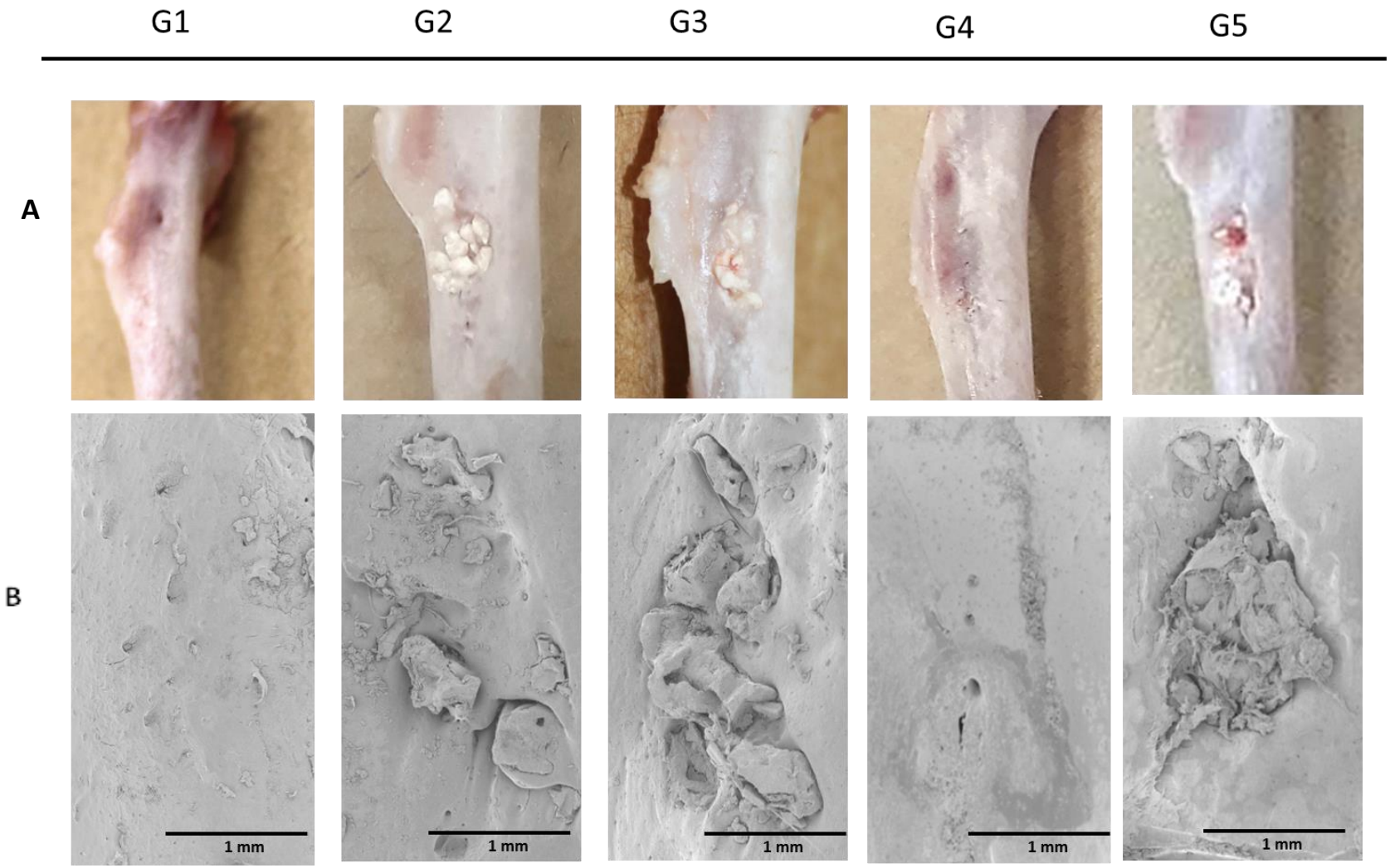

Figure 10. Representative images of the region of interest, **(a)** macroscopic and **(b)** by scanning electron microscopy view, **60 days** after surgical procedure.

3.5 Histological analysis

Histological analysis was performed to evaluate the morphological characteristics of the tissue formation at the lesion site. Figures 11 and 12 present the images of the histological sections stained by Hematoxylin and Eosin of the defect region 30 and 60 days after implantation. It is possible to observe progressive deposition of bone matrix in all groups. Groups 2, 3 and 5 showed integration of the calcium phosphate biomaterial to the adjacent newly formed matrix, with a high presence of resorption lacunae, these in a smaller number in group 2. In group 4, the bone formation was very exuberant, linear and organized, with few bone marrow infiltrates. In groups 2 and 5 and at 30 days, a higher presence of highly cellular connective tissue was observed. At 60 days, the deposition of bone matrix was similar in groups 2, 3, and 5, and more evident in group 4, which received the association of fibrin biopolymer and mesenchymal stem cells. Therefore, the bone formation of the treated groups, quantified by stereological analysis (Figs.13 and 14), can be represented by: FBP + MSC> FBP + BCP + MSC> FBP + BCP> BCP.

In the control group, the bone matrix still presented a disorganized pattern and permeated by bone marrow. Although there was bone formation in the control group, it did not evolved significantly from 30 to 60 days.

Despite the high bone formation around the calcium phosphate granules, no noteworthy cell proliferation was observed inside the biomaterial granules until 60 days after implantation (Fig. 15). Due to the presence of resorption gaps and the difference in its appearance at 30 and 60 days (Fig. 16), it was observed that the reabsorption of these materials is occurring. In turn, the fibrin biopolymer could not be visualized after 30 days of implantation. In the groups where phosphate and polymer was applied, with or without addition of stem cells, there was at 30 days of observation abundant presence of highly cellularized connective tissue.

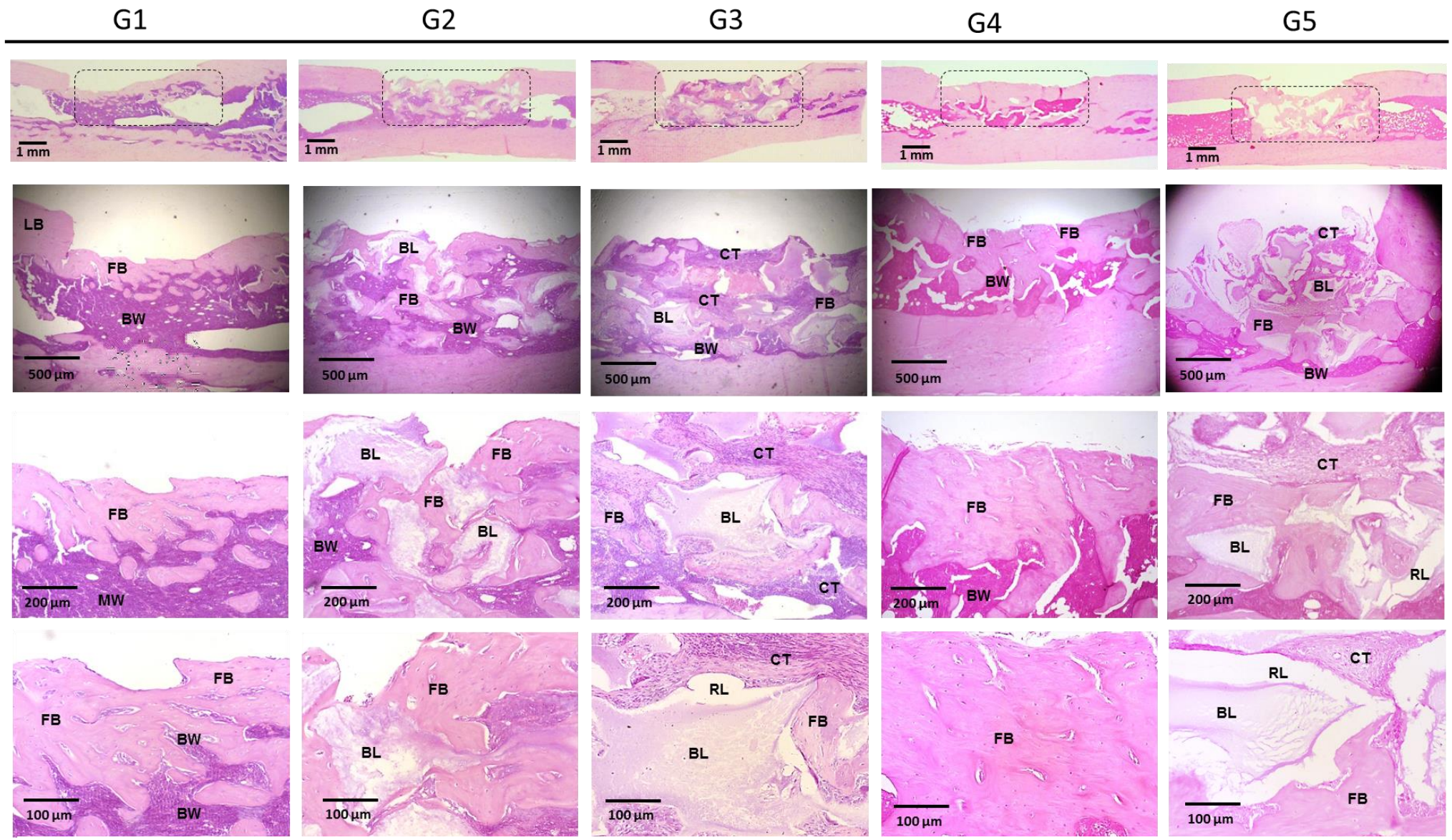

Figure 11. Representative images of histological sections stained by Hematoxilin-Eosin on the region of interest **30 days** after surgical procedure. **LB**: adjacent lamellar bone. **FB**: newly formed bone. **BL**: calcium phosphate biomaterial. **RL**: resorption lacunae **BW**: bone marrow **CT**: cellular connective tissue.

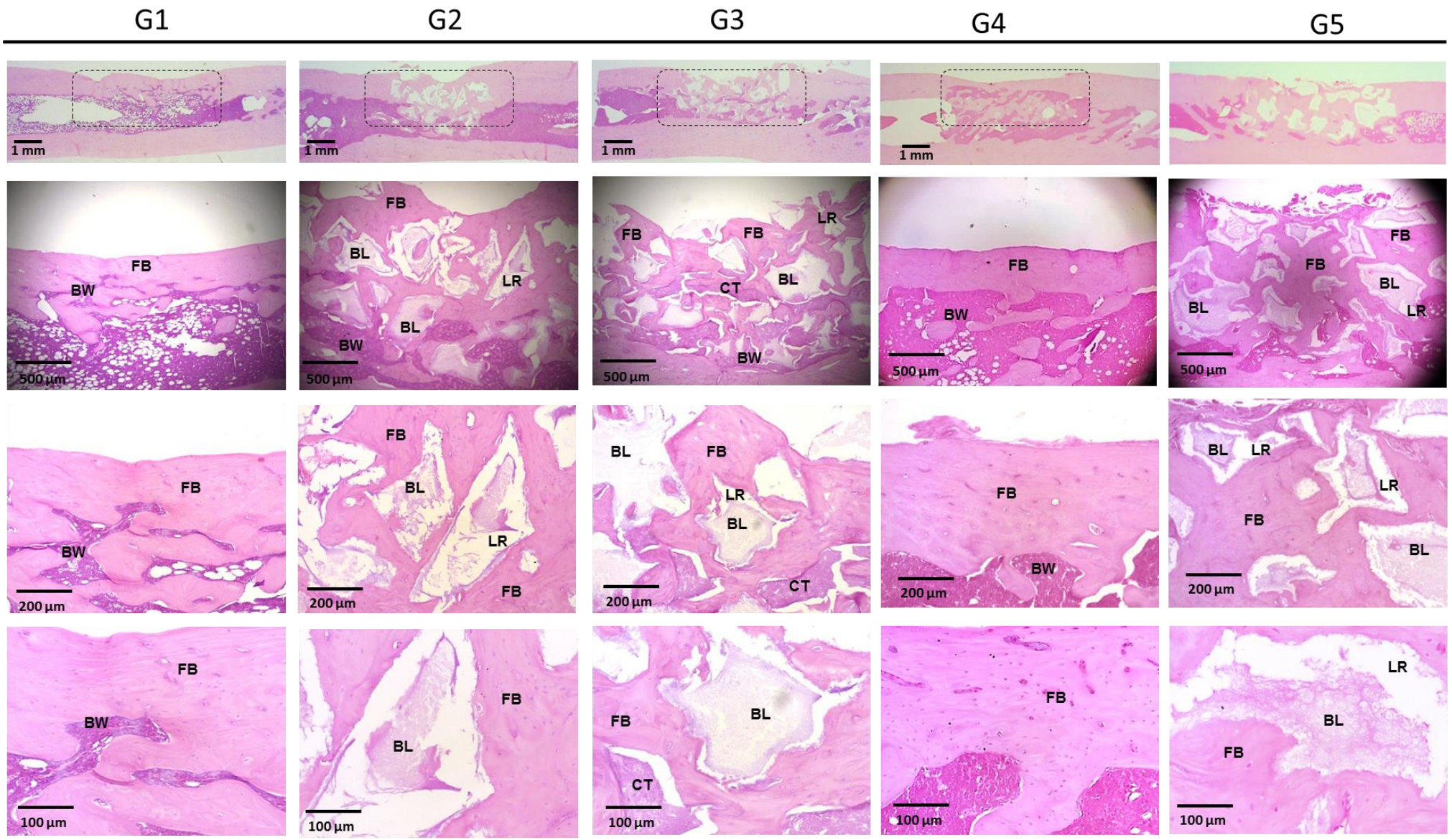

Figure 12. Representative images of histological sections stained by Hematoxilin-Eosin on the region of interest **60 days** after surgical procedure. **LB**: adjacent lamellar bone. **FB**: newly formed bone. **BL**: calcium phosphate biomaterial. **RL**: resorption lacunae **BW**: bone marrow **CT**: cellular connective tissue.

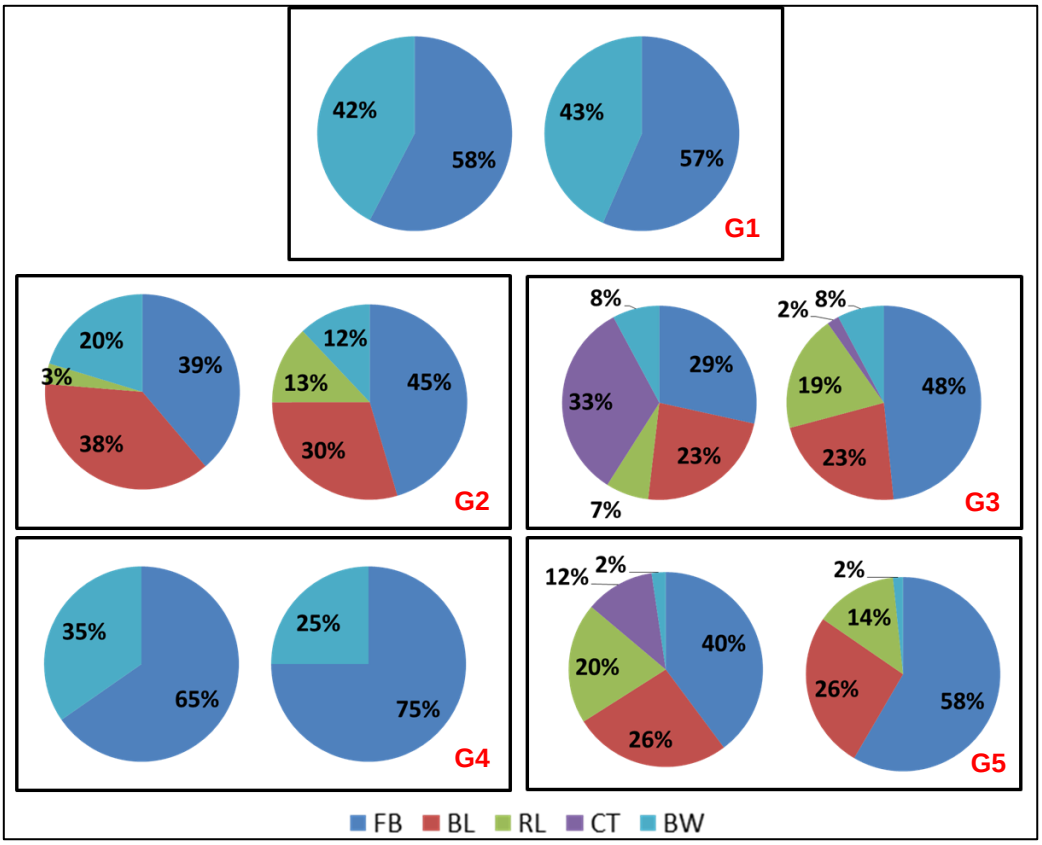

Figure 13. Relative quantification of the defect site of **all groups** 30 and 60 days after procedure observed on histological sections. **FB**: newly formed bone. **BL**: calcium phosphate material. **RL**: resorption lacunae **BW**: bone marrow **CT**: cellular connective tissue.

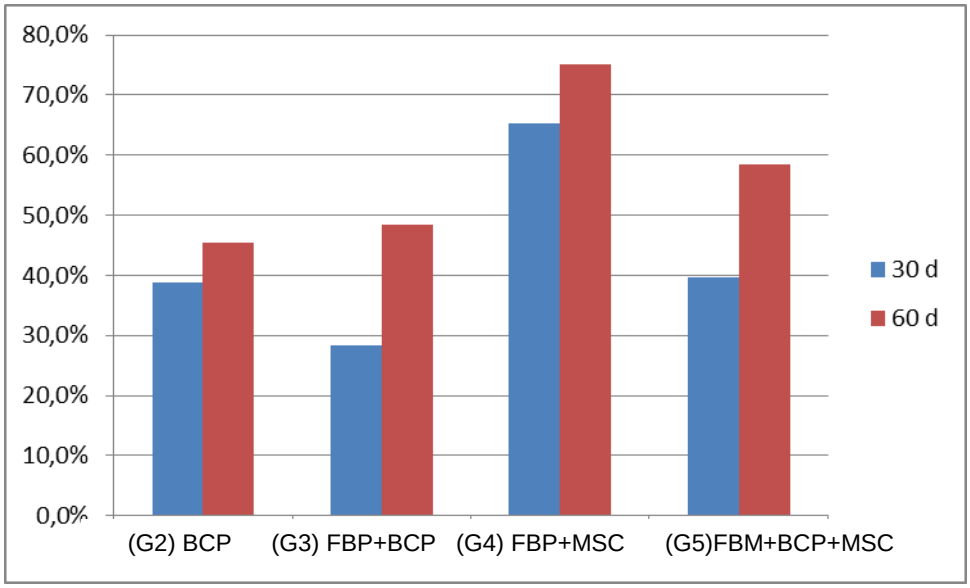

Figure 14. Relative quantification of progression of bone formation of **treated groups** 30 and 60 days after procedure observed on histological sections.

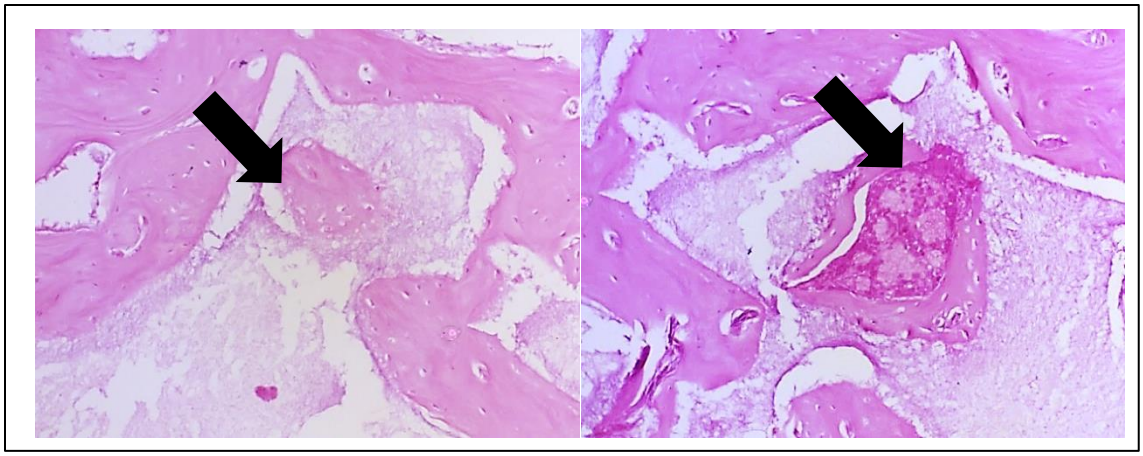

Figure 15. Bone formation inside biphasic calcium phosphate granules in receptor site.

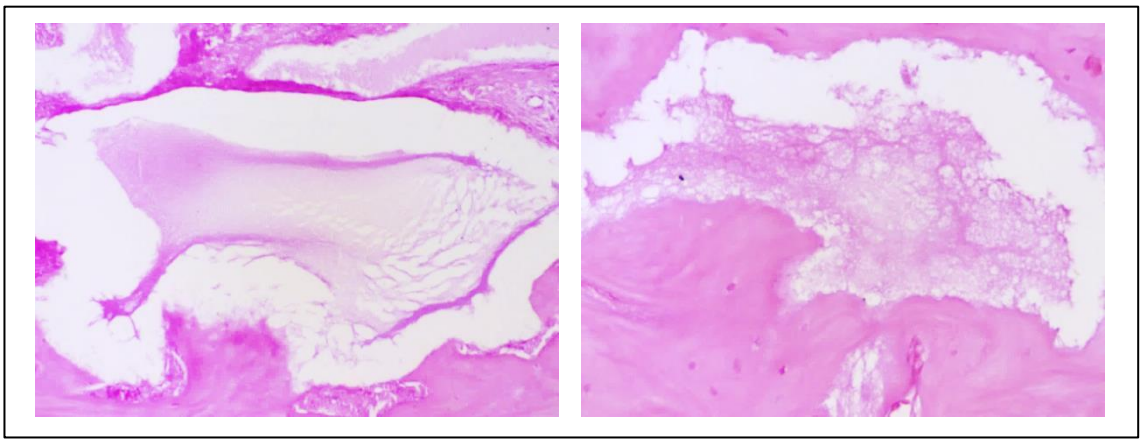

Figure 16. Progressive resorption of calcium phosphate granules in receptor site. **a)** 30 and **b)** 60 days after implantation.

4. Discussion

This study evaluated for the first time the application and repair potential of the fibrin biopolymer as a biological scaffold to the biphasic calcium phosphate biomaterial associated with the mesenchymal stem cells in a critical defect of the femurs of rats.

Prior to the materials application the mesenchymal stem cells were characterized by flow cytometry and presented positive expression for the CD90, CD44 and ICAM1 markers, and negative for CD45, CD11b and RT1-Aw2. These results are in accordance with the standardized data for surface markers of this cell line [49,54].

At the performing of surgical procedure, the addition of the calcium phosphate material and mesenchymal stem cells to the fibrin biopolymer scaffold improved the handling and application of the association due to the formation of a moldable composite, reducing the time of surgical procedure [55-58]. This was probably due to the malleability of the dense fibrin network formed during the stabilization of the clot [30.] Previous studies applying the FBP also reported as advantages the shortening of the procedure time [38,39], the flexibility of the scaffold [38] and the easy handling of this material [37]. Gonçalves et al [42] highlighted the stability at the receptor site provided by this scaffold. During the collect of femurs, no inflammatory infiltrates or rejection at the graft site and surrounding muscular tissue were observed, demonstrating the biocompatibility of the applied associations, corroborating previously described data in literature [43,59,60,41].

The visualization of the defect sites through the images obtained by tomography indicated that the total consolidation had not yet occurred after 60 days, requiring a longer period to determinate the outcome of the repair process. It was verified that opacification was greater in the groups where the biphasic calcium phosphate was applied, which was expected due to its similarity with the mineral phase of the bone [61,62]. However, that also indicates that a micro tomography analysis, using bone density as the pa-

rameter, is not indicated as a single approach to determinate the repair progression of injured bone when calcium base materials are applied. This is because it can indicate a false overview of recovery process. The UH value is directly related to the density of the tissue in formation, and the higher bone density found in the treated groups can indicate the presence of a more compact tissue formation and, therefore, more resistance [63,64], or differences in the rate of calcium phosphate resorption, that is, only this parameter cannot answer if the density was due to the presence of a new formed bone or presence of implanted material. In order to clarify these questions, a qualitative analysis of the defect site was required. It is known from the literature that, after bone injury, the process of resorption is initiated, followed by bone proliferation and consolidation, which may result or not in adequate remodeling [52,65]. The qualitative parameters observed on micro-CT images indicated poor cell proliferation and remodeling at the control group, indicating that the applied associations acted as a guide for the repair process, and the application of the tested combinations improved the level of cellular migration to the lesion site, expected action of the bioactive materials [66.]

The approximation of the borders of the observed macroscopically and by SEM from 30 to 60 days follows the expected remodeling pattern [67,43]. The characteristic granular morphology of the material could also be observed [67,68]. Also was observed apparent non-resorption at the central region of the defect site, and apparent covering on the defect area at control group and the only group without calcium phosphate (group 4). However, we emphasize that SEM analysis illustrates only the superficial view of the region of interest.

Thus, additionally to observation of the surface of the region of interest by electron microscopy, the histological images provided an overview of the tissue formation morphology. The progressive deposition indicates the osteoconductive potential of all the materials applied [59,69,70,71].

Characteristics such as regeneration from the edges of the lesion, bone formation around the applied materials and more gaps of resorption at 60 days are observations similar to those found on other studies about this tissue [43,56,62,71-74]. The deposition of bone matrix was higher in group 4, which received the association of fibrin biopolymer and mesenchymal stem cells. Besides the apparent similarity of covering at group 4 and control group showed by SEM images, histological analysis showed that the formation on control group presented a less organized tissue and more infiltrated bone marrow in formed bone. In agreement with the SEMs analysis, no notable resorption was observed inside of the calcium phosphate granules on histological sections, but resorption lacunae indicates that this was occurring, nevertheless at a slow rate.

Due to the characteristic deposition of the bone tissue in formation around the calcium phosphate materials when they were implanted [75], and the linear pattern of formation in the absence of these, the quantification by stereological method [53] allowed to more precise measure of the proportion of bone formed and other constituents in the sections obtained from the region of the defect. The quantitative analysis corroborate that group 4 (fibrin biopolymer and cells association) presented greater deposition of bone matrix, in agreement both with studies with traditional sealants [47,76-79] and previous studies of our research group [30,45].

Although the association between fibrin biopolymer, cells and calcium phosphate presented less bone formation than fibrin biopolymer and cell, this group presented less bone marrow infiltrated in the tissue in formation, indicating the stability provided for the association between fibrin based scaffolds and other materials [80,81]. In addition, this group presented more bone formation than the association containing only the biopolymer and calcium phosphate, once again showing the osteogenic improvement provided by mesenchymal stem cell transplantation [12,30,77.]

As described, in the control group the bone matrix still presented a disorganized pattern and was permeated by bone marrow, indicating a lower bone density and lower resistance. In addition, it was not observed evolution of the deposition in the bone matrix from 30 to 60 days; this data highlights the importance of the application of bioactive materials to stimulate osteoprogenitor cell proliferation[82].

The low cell proliferation in the internal of calcium phosphate granules indicated slow reabsorption of this. The reabsorption of the material is dependent on variables such as particle size and porosity [83], and in the case of biphasic compounds, also on the proportion of its components [55]. The porosity of this material has to allow vascularization, diffusion of fluids and cell migration [84] and its gradual resorption by osteoclasts [85,86]. Bose [87] recommends that the ideal particle diameter is between 200-350 μm. In the present study, the highest percentage of calcium phosphate porosity is 10 to 100 μm, which may have impeded the diffusion of liquids and the absorption of the material in the defect produced. The resorption rate is also influenced by particle size [88,89]. In the cranial defect implantation of rabbits, Kon et al [90] found that particles of 150-400 μm showed adequate resorption proportional to the speed of bone remodeling, but offered little stability compared to 1.0-2.0 mm particles. Thus, gradual resorption may not be totally negative where grafts need more stability and mechanical strength at the first moment of remodeling process [62,91]. In these cases, the adding of fibrin biopolymer may improve the mechanical strength of the site, helping in the incorporation of the graft to the injured area [62,72,92]. In view of this characteristics, a longer observation period is necessary to determinate the efficacy of FBP, calcium phosphate and MSC association.

On the other hand, the fibrin biopolymer could not be visualized after 30 days of implantation, what may indicate reabsorption proportional to the new bone formation. In accordance with the principle that an ideal biological framework should offer hemostasis and be degraded in the same

proportion as a new tissue is formed [47,93], FBP is a suitable scaffold for bone repair.

We highlight that in the groups where phosphate and fibrin biopolymer was used, with or without addition of stem cells, was observed at 30 days a high presence of highly cellularized connective tissue. Gasparotto et al. [30] described the ability of the fibrin biopolymer to capture and maintain in its site the stem cells applied, due to the dense fibrin network formed, and other studies have found that the presence of fibrin increased cell proliferation and recruitment of mesenchymal stem cells to such scaffolds [94]. However, due to the unique characteristics of fibrin biopolymer of this study, further analysis is needed to elucidate cell behavior in the presence of this scaffold. Nevertheless, the bioactive characteristic of this compound was validated, in addition to the advantages of being a biodegradable scaffold [77] with good integration capacity with or autologous [42] or synthetic biomaterials [43], don't present toxic or cytotoxic compounds [44] and shows biocompatibility with bone tissue [41].

The highly satisfactory results obtained by the association of the fibrin biopolymer with mesenchymal stem cells evidence the clinical potential of this association. Sixty days after implantation, a mean of 70% of observed histological sections was occupied by formed bone. For the present study, no differentiation on mesenchymal stem cells into osteogenic lineage was performed, indicating that this process may not be required to achieve satisfactory bone repair, what could short the time between cells collect and application and, consequently, reduce the cost of the cell expansion.

Finally, further studies applying differentiated lineage of mesenchymal stem cell and longer observational periods are required to reinforce the obtained results.

5. Conclusions

All applied biomaterials are biocompatible and presented good interaction between each other and the adjacent tissue, showing connection interfaces and osseointegration. There was progressive bone matrix deposition in all treated groups, which was greater in the group that received fibrin biopolymer in association with mesenchymal stem cells. Fibrin biopolymer proved to be an excellent candidate for scaffold in bone repair therapies due to facilitate the application of biomaterials and cells in the region of interest and present biocompatible, biodegradable and bioactive properties. The association between this biopolymer with mesenchymal stem cells showed satisfactory results, what may provide a more effective and less costly new approach for clinical applications on bone tissue engineering.

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Conflicts of interest

All authors declare no conflict of interests.

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APPENDIX I – Surface cell markers used on mesenchymal stem cells characterization by flow cytometry.

MARKER	EXPRESSION	TRADEMARK	REF.CODE	DILUTION	FLUOROCHROME
CD 45	Negative	ABCAM	AB123522	1/100	FITC
CD 11b	Negative	ABCAM	AB33816	1/100	PE
MHC- II	Negative	ABCAM	AB111825	1/100	PE
RT1- Aw2	Negative	ABCAM	AB119770	1/100	FITC
CD 90	Positive	ABCAM	AB226	1/100	FITC
ICAM1	Positive	ABCAM	AB23835	1/100	FITC

APPENDIX II- Specifications of tomography Shimadzu® SCT-7800TC – helical single channel and obtained scans

- Electric current of source: 80 mA
- Exposure time: 50 sec
- Field of view (FOV): 80
- Matrix: 512x512
- Rotation: 1sec
- Speed: 1mm/s
- Pitch: 1
- Sections: 1 mm x 1 mm
- Tension: 120 kV

APPENDIX III- Equipment and software used on histological analysis

- Leica DM500® microscope coupled to a Leica DMC2900® camera;
- Software Leica LAS v4.5®;
- Zeiss Stemi 2000® magnifier coupled to Dino Eye AM7025X® camera
- Software Dino Capture 2.0®

APPENDIX IV – qualitative analysis of images obtained by micro-computed tomography

30 days

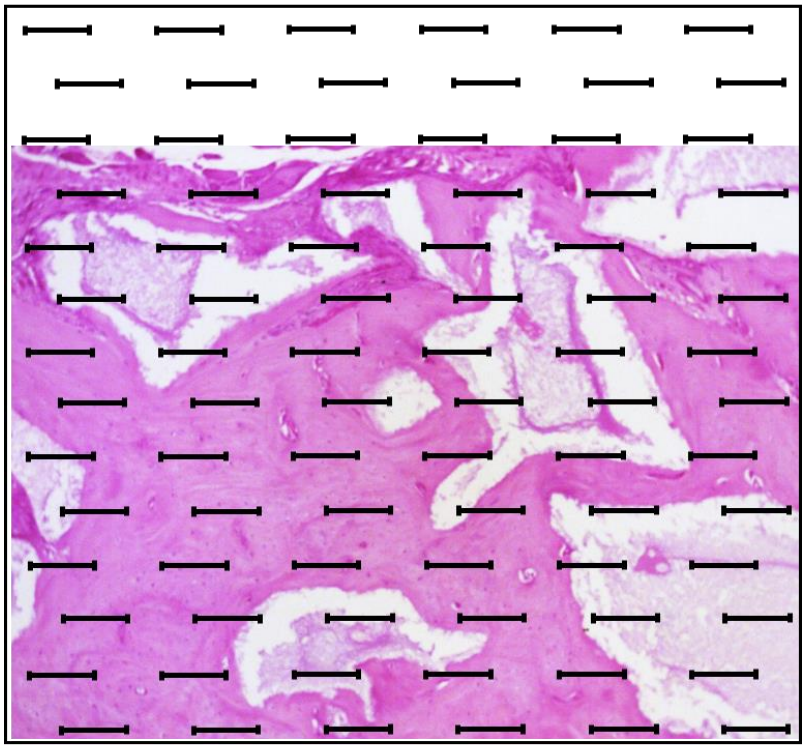
	Group 1	Group G2	Group G3	Group G4	Group G5
Proliferation	Absent	Discreet bone proliferation with irregular surface	Discreet	Discreet with bone proliferation of lamellar aspect	Discreet bone proliferation with irregular surface
Remodeling	Discreet remodeling with irregular surface	Discreet remodeling with irregular surface	Remodeling with irregular surface	Cortical on remodeling phase. Presence of mineralized thin connection	Remodeling with irregular surface

60 days

	Group 1	Group 2	Group 3	Group 4	Group 5
Proliferation	Area between the edges of the defect	Discreet with irregular surface	Discreet with irregular surface	Exuberant bone proliferation with regular surface	Discreet formation with irregular surface
Remodeling	Cortical misalignment, visualization of the defect, discreet area of density and spacing of borders	In remodeling process, irregular surface and non-visualization of full defect	In remodeling process, irregular surface, discreet area with density slightly smaller than the rest of the cortical	In remodeling process, non-visualization of full defect	In remodeling process, visualization of defect, but with medullar filling

APPENDIX V – “Weibel pattern” applyied on stereological analysis (example)

Total points= 168



Points over the histological section = X
X points – 168 = 100%

APPENDIX VI – Numerical results of stereological analysis

30 DAYS						
Group	**FB**	**BL**	**RL**	**CT**	**BW**	**rel. tot.**
G1-1	63,0%	0,0%	0,0%	0,0%	37,0%	100,0%
G1-2	51,0%	0,0%	0,0%	0,0%	49,0%	100,0%
G1-3	52,8%	0,0%	0,0%	0,0%	47,2%	100,0%
G1-4	59,4%	0,0%	0,0%	0,0%	40,6%	100,0%
MEAN	56,5%	0,0%	0,0%	0,0%	43,5%	100,0%
G2-1	39,7%	52,9%	0,0%	0,0%	7,4%	100%
G2-2	32,0%	32,8%	1,6%	0,0%	33,6%	100%
G2-3	42,7%	31,3%	6,1%	0,0%	19,8%	100%
G2-4	40,7%	33,0%	5,9%	0,0%	20,3%	100%
MEAN	38,8%	37,5%	3,4%	0,0%	20,3%	100%
G3-1	22,0%	27,2%	14,4%	33,3%	3,0%	100%
G3-2	32,6%	20,4%	2,3%	34,1%	10,6%	100%
G3-3	26,5%	21,6%	8,1%	36,3%	7,1%	100%
G3-4	32,6%	24,2%	3,8%	28,8%	10,6%	100%
MEAN	28,4%	23,4%	7,1%	33,1%	7,8%	100%
G4-1	67,0%	0,0%	0,0%	0,0%	33,0%	100%
G4-2	66,9%	0,0%	0,0%	0,0%	33,1%	100%
G4-3	68,3%	0,0%	0,0%	0,0%	31,7%	100%
G4-4	59,1%	0,0%	0,0%	0,0%	40,9%	100%
MEAN	65,3%	0,0%	0,0%	0,0%	34,7%	100%
G5-1	19,8%	28,1%	30,2%	21,8%	0,0%	100%
G5-2	36,1%	29,2%	15,5%	13,7%	5,6%	100%
G5-3	54,3%	21,7%	20,2%	3,8%	0,0%	100%
G5-4	48,9%	26,0%	14,5%	6,9%	3,8%	100%
MEAN	39,8%	26,2%	20,1%	11,5%	2,3%	100%

FB: newly formed bone. **BL**: calcium phosphate biomaterial. **RL**: resorption lacunae. **CT**: cellular connective tissue. **BW**: bone marrow. **Rel. tot:** relative total.

60 DAYS						
Group	**FB**	**BL**	**RL**	**CT**	**BW**	**rel. tot.**
G1-1	68%	0%	0%	0%	32%	100%
G1-2	42,0%	0,0%	0,0%	0,0%	58,0%	100%
G1-3	57,0%	0,0%	0,0%	0,0%	43,0%	100%
G1-4	63,7%	0,0%	0,0%	0,0%	36,3%	100%
MEAN	57,6%	0,0%	0,0%	0,0%	42,4%	100%
G2-1	52,0%	16,7%	21,3%	0,0%	10,0%	100,0%
G2-2	48,6%	36,4%	7,9%	0,0%	7,1%	100,0%
G2-3	27,3%	42,4%	9,8%	0,0%	20,5%	100,0%
G2-4	53,8%	22,7%	12,9%	0,0%	10,6%	100,0%
MEAN	45,4%	29,6%	13,0%	0,0%	12,0%	100,0%
G3-1	56,8%	16,4%	12,4%	4,0%	10,4%	100,0%
G3-2	47,6%	19,0%	25,5%	2,0%	5,9%	100,0%
G3-3	45,1%	26,6%	21,3%	0,0%	6,9%	100,0%
G3-4	44,1%	27,7%	18,4%	2,0%	7,8%	100,0%
MEAN	48,4%	22,4%	19,4%	2,0%	7,8%	100,0%
G4-1	71,4%	0,0%	0,0%	0,0%	28,6%	100,0%
G4-2	78,1%	0,0%	0,0%	0,0%	21,9%	100,0%
G4-3	75,5%	0,0%	0,0%	0,0%	24,5%	100,0%
G4-4	75,0%	0,0%	0,0%	0,0%	25,0%	100,0%
MEAN	75,0%	0,0%	0,0%	0,0%	25,0%	100,0%
G5-1	50,4%	32,2%	16,0%	0,0%	1,4%	100,0%
G5-2	63,5%	22,8%	12,8%	0,0%	0,8%	100,0%
G5-3	53,0%	31,3%	11,1%	0,0%	4,5%	100,0%
G5-4	66,6%	18,4%	15,1%	0,0%	0,0%	100,0%
MEAN	58,4%	26,2%	13,7%	0,0%	1,7%	100,0%

FB: newly formed bone. **BL**: calcium phosphate biomaterial. **RL**: resorption lacunae. **CT**: cellular connective tissue. **BW**: bone marrow. **Rel. tot:** relative total.

- Fim do manuscrito-

Conclusão

Ao final da presente pesquisa, a qual se propôs a avaliar o potencial regenerativo da associação entre biomaterial fosfato de cálcio bifásico, células tronco mesenquimais e o *scaffold* biopolímero de fibrina (BPF) em fêmures de ratos submetidos a um defeito crítico, constatou-se que os compostos utilizados são biocompatíveis, biodegradáveis e bioativos. Houve deposição progressiva de matriz óssea em todos os grupos tratados, a qual foi mais significativa no grupo que recebeu a associação de biopolímero de fibrina e células tronco mesenquimais. O biopolímero de fibrina mostrou-se um excelente candidato para *scaffold* em terapias de reparação óssea visto que facilitou a aplicação de biomateriais e células na região de interesse e apresentou propriedades bioativas. Os resultados satisfatórios obtidos pela associação entre o BPF e células tronco pode proporcionar uma nova abordagem clínica mais efetiva e menos onerosa para abordagens de engenharia do tecido ósseo.

Apêndice

(contendo Anexos I e II)

APÊNDICE I

Anexo I: Aprovação pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu e reconsiderações atendidas.

unesp

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

FMB 1963

Comissão de Ética no Uso de Animais
Criada através da Portaria DFM nº 611 de 13/12/2012

CERTIFICADO Nº 1164/2016-CEUA

Projeto de Pesquisa **(Protocolo CEUA 1164-2016) Selante heterólogo de fibrina como arcabouço para células-tronco mesenquimais associados ao fosfato de cálcio e aplicado em defeito crítico de fêmures de ratos**, a ser conduzido por Claudia Vilalva Cassaro, orientada pelo Prof. Dr. Benedito Barraviera, coorientado por Rui Seabra Ferreira Júnior, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal – CONCEA.

Vigência do projeto	31/03/2018
Espécie/Linhagem	Rato/Wistar
Nº de animais	70
Peso/Idade	200-300 gramas/2 meses
Sexo	Macho
Origem	Biotério UNICAMP - CEMIB

Projeto de Pesquisa aprovado em reunião da CEUA em 25/02/2016

Profª. Drª. Noeme Sousa Rocha
Vice-Presidente da CEUA

Kleber Messias de Camargo
Secretário da CEUA

Distrito Rubião Junior, s/nº - Botucatu – S.P. CEP: 18.618-970 Fone: (14) 3880-1608/3880-1609 e-mail secretaria: kleber@fmb.unesp.br

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Instituída na Faculdade de Medicina através da Portaria do Diretor nº 30 de 26/04/99

PARECER CONSUBSTANCIADO

Alterações no Projeto e Procedimentos do Projeto
Ofício 030/2016 - CEVAP

Identificação:

1. Título do Projeto: Solicitação de mudança no projeto: "Selante heterólogo de fibrina como arcabouço para células-tronco mesenquimais associado ao fosfato de cálcio e aplicado em defeitos críticos de fêmures de ratos."

2. Autor: Claudia Vilava Cassaro

3. Orientador: Prof. Dr. Benedito Barraviera

4. Solicitações de mudanças:

4.1 Alteração na procedência de animais
Solicitação de origem de animais do Biotério do Lab. de Medicina Experimental acatada.

4.2 Alteração do número de animais por grupo
Solicitação acatada e justificada pela subdivisão em dois grupo de número idêntico de animais.

4.3 Alteração nos grupos experimentais
Solicitação da adição de um grupo controle foi acatada, perfazendo um total de 80 animais a serem empregados no experimento.

CEUA, 02/05/2016.

Prof. Dr. Guilherme Antônio Moreira de Barros
Presidente da CEUA

Anexo II: Instrução Normativa para a elaboração de teses e dissertações do programa de Doenças Tropicais da Faculdade de Medicina de Botucatu (FMB).

UNESP UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Faculdade de Medicina - Campus de Botucatu

MIP Moléstias Infecciosas e Parasitárias FMB-UNESP

FMB FACULDADE DE MEDICINA 1963 BOTUCATU

PROGRAMA DE PÓS-GRADUAÇÃO EM DOENÇAS TROPICAIS

Instrução Normativa nº 008/2014-PPG/DT

ELABORAÇÃO DA DISSERTAÇÃO/TESE

Esta normativa visa regulamentar os critérios utilizados pelo Programa de Pós-Graduação em Doenças Tropicais, da Faculdade de Medicina de Botucatu / Unesp, para a elaboração da Dissertação ou Tese, com base no disposto no Regulamento do Programa (Resolução UNESP-46, de 04/09/2013).

Estrutura da dissertação/tese

- **Capa** (obrigatório)
- **Lombada** (impressão opcional)
- **Folha de rosto com ficha catalográfica no verso** (obrigatório)
- **Errata** (opcional)
- **Dedicatória** (opcional)
- **Agradecimento** (s) (opcional)
- **Epígrafe** (opcional)
- **Resumo em português** (obrigatório)
- **Resumo em inglês** (obrigatório)
- **Lista de ilustrações** (opcional)
- **Lista de tabelas** (opcional)
- **Lista de abreviaturas e siglas** (opcional)
- **Lista de símbolos** (opcional)
- **Sumário** (obrigatório)

Capítulo I

- Revisão da Literatura (obrigatório)
- Objetivo (obrigatório)
- Referências (obrigatório)

Capítulo II

- Artigo (s) Científico(s) (obrigatório)

- **Conclusão** (obrigatório)
- **Apêndice (s)** (opcional)
- **Anexo (s)** (opcional)