UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

KARINA TORRES POMINI ROCHA

Applicability of homologous fibrin composite in the bone repair process associated or not with laser photobiomodulation therapy

Aplicabilidade de compósito de fibrina homólogo no processo de reparo ósseo associado ou não a terapia por fotobiomodulação a laser

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Orientador: Prof. Dr. Rogério Leone Buchaim

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DEDICATÓRIA

“Esta é a confiança que temos ao nos aproximarmos de Deus: se pedirmos alguma coisa de acordo com a vontade de Deus, ele nos ouvirá. E se sabemos que ele nos ouve em tudo o que pedimos, sabemos que temos o que dele pedimos”.

1 João 5:14-15

Agradeço a Deus por esse momento tão esperado em minha vida.

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Valeu a pena acreditar!!!

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“Assim como os perfumes alegram a vida, a amizade sincera dá ânimo para viver”.

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"E não vos conformeis com este mundo, mas transformai-vos pela renovação do vosso entendimento, para que experimenteis qual seja a boa, agradável e perfeita vontade de Deus".

Romanos 12:2

ABSTRACT

Applicability of homologous fibrin composite in the bone repair process associated or not with laser photobiomodulation therapy

Aim: In the search for improvement in the results of reconstructive surgical interventions that require bone repair, several therapeutic modalities have been researched. However, studies that elucidate the combined use of three-dimensional composite scaffold and non-invasive extra-operative therapies are still scarce. Thus, the objective of the first article was to develop a systematic review to compile results of scientific studies with clinically relevant in vivo test models, in order to investigate the applicability of homologous fibrin sealant as a cellular support in the bone regeneration process. The second article sought to elucidate the effects of the association of the homologous fibrin sealant incorporated with the deproteinized bovine bone matrix and laser photobiomodulation therapy in a model of critical bone defect in rat calvaria. Material and methods: Article 1 - The studies were selected from the PubMed/Medline databases, according to the PRISMA statement, through a combination of keywords, fibrin sealant and bone repair. The search filters were built using the parameters: articles over 10 years old *(n = 175)*; without access to the full text *(n = 5)*; not english language *(n = 4)*. Article 2 - Thirty-six rats were divided into four groups: BC *(n = 8)* defect filled with blood clot, BC^{PBMT} *(n = 8)* defect filled with blood clot and laser photobiomodulation, FSB *(n = 10)* defect filled with deproteinized bovine bone matrix plus fibrin sealant, FSB^{PBMT} *(n = 10)* defect filled with deproteinized bovine bone matrix plus fibrin sealant and laser photobiomodulation. The animals were killed after 14 and 42 days and the specimens subjected to microtomographic, histological and histomorphometric analyzes. Results: Article 1 - After selecting the inclusion and exclusion criteria, 12 articles were selected from 313 initially identified. Studies have reported that the fibrin sealant is capable of providing a favorable microenvironment for the proliferation and differentiation of osteoprogenitor cells and combined with other bone grafts form multifunctional scaffolds with greater mechanical resistance, greater graft stability in the surgical bed, and longer support time throughout the bone repair process. Article 2 - In the histological and microtomographic analysis, new bone formation was observed in all groups, limited to the defect margins, and without complete wound closure. In the FSB group, bone formation increased

between periods (4.3±0.46 to 6.01±0.32), yet with lower volume density when compared to the FSB^{PBMT} (5.6±0.45 to 10.64±0.97) group. Conclusion: Article 1 - The studies analyzed pointed out that homologous fibrin sealants are capable of building a three-dimensional framework that provides support for cell growth. Article 2 - It is concluded that the support system formed by fibrin sealant derived from human blood and the deproteinized bovine bone matrix associated with the proposed laser photobiomodulation protocol was able to promote greater speed in the deposition of mineralized bone matrix.

Keywords: Biocompatible materials. Bone regeneration. Low-level light therapy. Bone substitutes. Fibrin tissue adhesive. Heterografts.

RESUMO

Aplicabilidade de compósito de fibrina homólogo no processo de reparo ósseo associado ou não a terapia por fotobiomodulação a laser

Objetivo: Na busca pela melhora nos resultados das intervenções cirúrgicas reconstrutivas que necessitam de reparo ósseo, várias modalidades terapêuticas têm sido pesquisadas. Entretanto, ainda são escassos estudos que elucidam o emprego combinado de arcabouços compostos tridimensionais e terapias extra operatórias não invasivas. Assim, o objetivo do primeiro artigo foi desenvolver uma revisão sistemática para compilar resultados de trabalhos científicos com modelos de testes *in vivo* clinicamente relevantes, a fim de investigar a aplicabilidade do selante de fibrina homólogo como suporte celular no processo de regeneração óssea. O segundo artigo buscou elucidar os efeitos da associação do selante de fibrina homólogo incorporado a matriz óssea bovina desproteinizada e a terapia por fotobiomodulação a laser em modelo de defeito ósseo crítico em calvária de ratos. Material e métodos: Artigo 1 – Os estudos foram selecionados nas bases de dados PubMed/Medline, de acordo com a declaração do PRISMA, por meio da combinação das palavras-chave, selante de fibrina e reparo ósseo. Os filtros de pesquisa foram construídos usando os parâmetros: artigos acima de 10 anos *(n = 175)*; sem acesso ao texto completo *(n = 5)*; não idioma inglês *(n = 4)*. Artigo 2 – Trinta e seis ratos foram divididos em quatro grupos: BC *(n = 8)* defeito preenchido com coágulo sanguíneo, BC^{PBMT} *(n = 8)* defeito preenchido com coágulo sanguíneo e fotobiomodulação a laser, FSB *(n = 10)* defeito preenchido com matriz óssea bovina desproteinizada mais selante de fibrina, FSB^{PBMT} *(n = 10)* defeito preenchido com matriz óssea bovina desproteinizada mais selante de fibrina e fotobiomodulação a laser. Os animais foram eutanasiados após 14 e 42 dias e as espécimes submetidas as análises microtomográficas, histológica e histomorfométrica. Resultados: Artigo 1 - Após a seleção dos critérios de inclusão e exclusão foram selecionados 12 artigos em 313 identificados inicialmente. Estudos relataram que o selante de fibrina é capaz de proporcionar um microambiente favorável para a proliferação e diferenciação de células osteoprogenitoras e combinado a outros enxertos ósseos formam *scaffolds* multifuncionais com maior resistência mecânica, maior estabilidade do enxerto no leito cirúrgico, e maior tempo de suporte celular durante todo processo de reparo ósseo. Artigo 2 – Nas análises

histológica e microtomográfica foi observada nova formação óssea em todos os grupos, limitada às margens dos defeitos e sem fechamento completo da ferida. No grupo FSB, a formação óssea aumentou entre os períodos (4.3±0.46 para 6.01±0.32), porém com menor densidade de volume quando comparada ao grupo FSB^{PBMT} (5.6±0.45 para 10.64±0.97). Conclusão: Artigo 1 – Os estudos analisados apontaram que os selantes de fibrina homólogos são capazes de construir um arcabouço tridimensional que proporciona suporte ao crescimento celular. Artigo 2 - Conclui-se que o sistema de suporte formado por selante de fibrina derivado de sangue humano e a matriz óssea bovina desproteinizada associado ao protocolo proposto de fotobiomodulação a laser foi capaz de promover maior celeridade na deposição de matriz óssea mineralizada.

Keywords: Materiais biocompatíveis. Regeneração óssea. Bioestimulação a Laser. Substitutos ósseos. Adesivo tecidual de fibrina. Xenoenxertos.

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1 INTRODUCTION

1 **INTRODUCTION**

The management of large bone defects has still been a challenging problem for medical and dental specialties in terms of the complexity of the treatments available, the significant morbidity and the high incidence of late complications (KAMAL; SIAHAAN; FIOLIN, 2019).

Combined with an increasing prevalence of traumas, congenital anomalies, and degenerative diseases that can compromise the restoration of bone architecture, tissue engineering and regenerative medicine seek to develop new reconstructive therapies in order to regenerate lost bone and restore its function (MUSCHLER et al., 2010).

Among the various reconstructive methods, autogenous grafting provides the most favorable conditions for repair, as it aggregates all the characteristics required in bone regeneration in terms of osteoconduction, osteoinduction and osteogenesis. However, there are several factors that limit its application, including the risk of morbidity at the donor site and limited availability of tissue, which provides advances in the development and improvement of new biomaterials (MCGOVERN; GRIFFIN; HUTMACHER, 2018).

In view of this, in the last decades, bone substitutes have been the object of intense research, in order to overcome the limitations resulting from graft harvesting or the use of bone banks, and thus assist and accelerate the regenerative process, repairing the injury by a new fabric with native morphofunctional characteristics (GHIASI et al., 2017).

Given the great diversity of commercially available biomaterials, previous studies have presented scientific evidence and predictability of clinical success in the use of bovine xenografts (SHI et al., 2018).

Deproteinized xenograft or also known as deproteinized bovine bone matrix is a bone substitute of natural origin, in which the bovine bone is subjected to chemical and thermal extraction processes below 300° C (unsintered), in order to remove the components organic matter remaining only hydroxyapatite crystals. Thus, the chemical composition and crystallinity remains unchanged, giving 70-75% porosity, naturally similar to human bone, and with a large specific surface area (GUÉHENNEC; LAYROLLE; DACULSI, 2004; JANG et al., 2010).

Thus, the physical-chemical properties of these xenografts give the biomaterial remarkable osteoconductive characteristics for providing biomechanical support to cells, ensuring bone growth (SPONER; STRNADOVÁ; URBAN, 2011).

Although this method is clinically established, a new therapeutic approach to bone regeneration is currently being employed with the use of specialized tissue constructions, in order to achieve synergistic effect and better resulting general properties when compared to conventional grafting techniques (BORIE et al., 2015).

Among the constructions of tissue engineering for bone repair, the association of three-dimensional scaffolds is based on the attempt to mimic the native bone microstructure, facilitating the recruitment of osteogenic cells, growth factors in situ and promoting the synthesis of a new mineralized bone matrix (KIM et al., 2014).

It is in this context that natural biopolymers, such as homologous fibrin sealants have become an ideal candidate for use combined with particulate bone grafts (ASAAD et al., 2016). This since it allows the manufacture of multifunctional compound scaffolds that stop bleeding by homeostatic mechanisms, increase the mechanical resistance, the stability of the graft in the surgical bed, a major factor in the prevention of micromotion, providing longer cell support during the entire bone repair process, increasing the graft success rate (AHMED; DARE; HINCKE, 2008).

Fibrin, fibrinogen and thrombin sealant precursors, derived from human plasma, interact in the final stages of the blood clotting cascade resulting in a reticulated matrix of fibrin, a temporary structure necessary to support the tissue repair and remodeling process (WEISEL; LITVINOV, 2017). In addition, fibrin specifically binds to various proteins and growth factors released in response to injury, playing an active role in the repair process, through interactions with specific cell surface receptors (NOORI et al., 2017).

In the search for improvement in the results of reconstructive surgical interventions that require tissue repair, several postoperative therapeutic modalities have been researched. Among non-invasive treatments, laser photobiomodulation has been widely used in several clinical conditions, in order to accelerate tissue regeneration and modulate inflammatory processes of cells with functional deficit (DE FREITAS; HAMBLIN, 2016).

Not unlike what occurs in bone tissue, photobiomodulation has been shown to be effective in modulating biochemical reactions, increasing the supply of adenosine triphosphate (ATP), and the permeability of the cell membrane, enabling calcium influx,

stimulating differentiation and cell proliferation, regulate growth factors and inflammatory cytokines, induce collagen synthesis and remodeling, and angiogenesis (HAMBLIN, 2017).

This sum of cellular events stimulated by photobiomodulation causes the injured bone tissue to restore its homeostasis, that is, the normalization of its shape and function, leading to its repair (KAZANCIOGLU; EZIRGANLI; AYDIN, 2013).

However, despite the evidenced potential of the mentioned therapeutic resources, there is still a limited understanding about the simultaneous use of particulate bone grafts associated with fibrin sealant and photobiomodulation to improve bone healing.

Having the knowledge that the combination of intra and extra operative technologies can constitute an optimized treatment to improve bone regeneration, the present study aimed to analyze the effects of laser photobiomodulation therapy in a model of cranial bone defects in rats filled with bone matrix deproteinized bovine incorporated with fibrin sealant derived from human blood.

2 ARTICLES

2 **ARTICLES**

The articles presented in this thesis were written according to the instructions and guidelines for submitting articles from the corresponding journals.

1. **Article 1 -** Applicability of homologous fibrin sealant in bone repair: An integrative review (Published - International Journal of Advanced Engineering Research and Science, IJAERS);

2. **Article 2 -** Fibrin Sealant Derived from Human Plasma as a Scaffold for Bone Grafts Associated with Photobiomodulation Therapy (Published - International Journal of Molecular Sciences, IJMS).

2.1 ARTICLE 1 - **“Applicability of Homologous Fibrin Sealant in Bone Repair: An integrative Review”*.** (Appendix A and Annex 1).

- International Journal of Advanced Engineering Research and Science, IJAERS.

Abstract - The repairing of bone defects is still a challenge for researchers and clinicians. Nonetheless there are many procedures that use different biomaterials such as scaffolds for bone regeneration however the results are often still unsatisfactory. As a result, the fibrin sealant derived from the interaction between proteins participating in the final blood coagulation cascade, is one of the most promising biopolymers in the tissue engineering field due to its unique characteristics. The present study aimed to perform a systematic review on homologous fibrin sealants highlighting its applicability as a three-dimensional framework in the process of bone regeneration. The database used for search strategy was the PubMed (Medline) and followed the guidelines provided in the PRISMA statement. From an initial 313 articles, only 12 articles between 2009 to 2019 were selected for this review after checking all inclusion and exclusion criterias. Due to this background, it is notable that fibrin sealant is one of the promising biopolymers used for tissue engineering and bone regeneration applications.

Keywords — Fibrin Sealant, Bone Repair, Tissue engineering, Biopolymer, Scaffold.

2.1 ARTICLE 1 – **"Applicability of Homologous Fibrin Sealant in Bone Repair: An integrative Review".** This manuscript was published in the International Journal of Advanced Engineering Research and Science (IJAERS) at https://ijaers.com/detail/applicability-of-homologous-fibrin-sealant-in-bone-repair-an-integrative-review/July 2019. It is authorized by the journal to be used in the defense of thesis, according to the terms and conditions of the University of São Paulo (The Digital Library of Theses and Dissertations of the University of São Paulo; https://www.tese.usp.br/index.php?lang=en).

Applicability of Homologous Fibrin Sealant in Bone Repair: An integrative Review

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I. INTRODUCTION

Bone is a highly dynamic tissue that undergoes a continuous renovation process to maintain its architectural bone structure, mechanical properties and metabolic capacities and when injured is able to reestablish the lost tissue morphofunctional characteristics without compromising the function (SEAL; OTERO; PANITCH, 2001; LOI et al., 2017).

However, this mechanism may or may not occur in large defects, such as tumor resections, unconsolidated fractures, congenital malformations, and the loss or surgical removal of bone fragments (HONMA et al., 2008; SPICER et al., 2012; HETTIARATCHI et al., 2017), in addition, it may require reconstructive operative procedures whose bone grafting is the main treatment technique (POUNTOS; GIANNOUDIS, 2016; BAI et al., 2018).

Among the available bone grafts, the autogen is still considered the gold standard in the bone regeneration techniques because it has osteogenic, osteoinductive and osteoconductive properties combined. Although its use is associated with limited supply, possible complications in the donor site and the unpredictability of bone resorption, which may negatively influence postoperative outcomes (POLLOCK et al., 2008; PILIPCHUK et al., 2015).

Because of these limitations, research is being conducted in order to a new treatment approach for bone regeneration, aiming at the development of biologically active natural materials (GHIASI et al., 2017).

As a result, the fibrin sealant derived from the interaction between proteins participating in the final blood coagulation cascade, is one of the most promising biopolymers in the tissue engineering field due to its unique characteristics (NOORI et al., 2017).

For instance, its excellent biocompatibility, controllable biodegradability, and multi-functional three-dimensional structure that provides support, cell proliferation and differentiation, anchoring surrounded molecules and growth factors and therapeutic agents transport, makes fibrin sealants have remarkable advantages over other biomaterials, besides a candidate with potential to assist in engineering of bone tissue (SHIU et al., 2014; SPOTNITZ, 2014; BORIE et al., 2015).

Although, all fibrin sealants contain fibrinogen and thrombin, qualitatively and quantitatively the exact composition varies, such as the velocity of rate of hemostasis, clot biochemistry, viscosity, adhesive strength, durability, fibrin polymerization rate and

the three-dimensional structure of the clot, and can directly influence its use (WOZNIAK, 2003; DIETRICH et al., 2013; CUNHA et al., 2015). For this purpose, the present study aimed to perform a systematic review on homologous fibrin sealants highlighting its applicability as a three-dimensional framework in the bone regeneration process.

II. MATERIALS AND METHODS

This review followed the guidelines provided in the PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). Medical subject heading (MeSH) terms were used in this study. The database used for search strategy was the PubMed (Medline). The search string used was following these terms “fibrin sealant AND bone repair”.

It has been included all articles in English at periods between 2009 and 2019, with access to the full text, either openly or by signatures available at the University of São Paulo (Brazil).

The titles and abstracts of the articles were evaluated and those that did not meet each inclusion criteria were removed from the review. After second analysis, only articles that used fibrin sealant as a three-dimensional scaffold for the lodging of biologically active cells and molecules in the bone regeneration process were selected for detailed review.

Inclusion Criteria:

- Periods between 2009 – 2019;
- Articles types: full articles available;
- English language;
- In vivo research model;
- Fibrin sealant used as a scaffold for tissue engineering applications.

Exclusion Criteria:

- Any article that did not meet the inclusion criteria listed earlier.

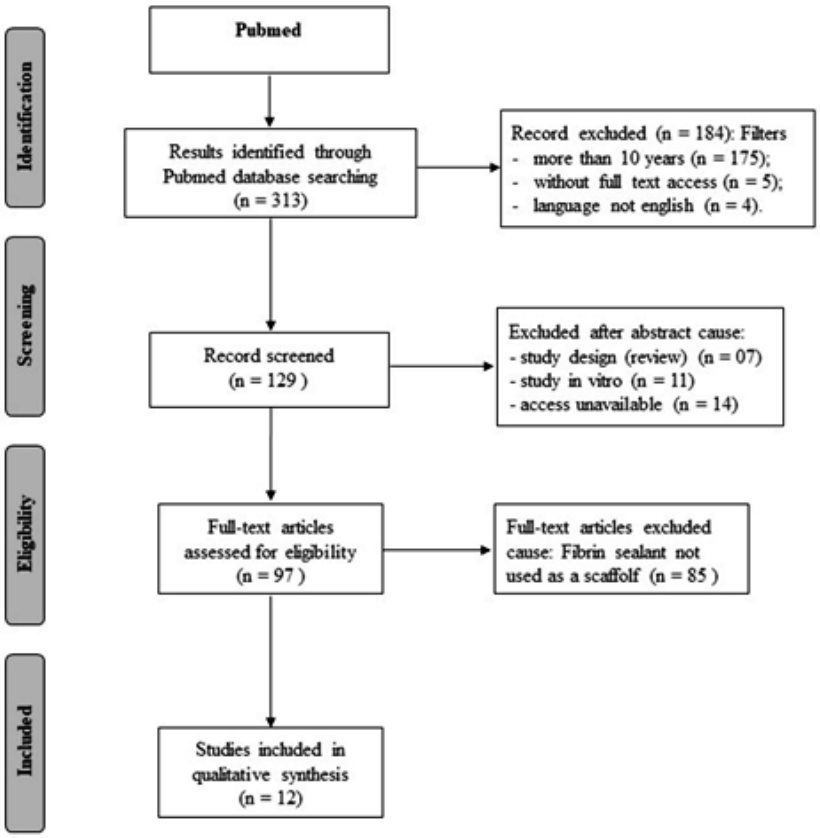

Fig.1: PubMed (Medline) Keywords combination

III. RESULTS

In total, from an initial 313 articles, only 12 articles were selected for this review (see Table 1) after checking all criterias listed earlier such as periods between 2009 to 2019, full articles available, English language, fibrin sealant used as a scaffold for tissue engineering and in vivo research model. For full search process see Fig. 1.

Table 1 Summarizes of the selected articles about tissue engineering applications of fibrin scaffolds.

Author (Date)	Objective	Component origin and/or Trade name	Implantation Site	Mixture with biomaterials or cells	Conclusion
McDuffee et al. (2012)	To compare the efficacy of osteoprogenitors in fibrin glue to fibrin glue alone in bone healing of surgically induced	Homologous (non-commercial)	Metacarpal bone in horses	Osteoprogenitor cells or fibrin sealant alone	Injection of periosteal-derived osteoprogenitors in a fibrin glue carrier into surgically

Author (Date)	Objective	Component origin and/or Trade name	Implantation Site	Mixture with biomaterials or cells	Conclusion
	ostectomies of the fourth metacarpal bones in an equine model.				created ostectomies of MC4 does not accelerate bone healing.
Reppenhagen et al. (2012)	To assess the safety and efficiency of a bone substitute as an alternative for autologous bone in the treatment of benign bone tumours and tumour-like lesions.	Homologous Tissucol™ Duo S	Distal tíbia; calca neal; glenoid in human	Biphasic calcium phosphate granules	The biomaterial represents an easy-to-handle alternative to autologous cancellous bone grafts for the treatment of benign bone tumours and tumour-like lesions.
Zhang et al. (2012)	To clarify whether it could be efficient to reconstruct the alveolar bone by the combination of bone marrow stem cells (BMSCs) without pre-osteoinduction in vitro with fibrin glue (FG).	Homologous (non-commercial)	Rat alveolar bone	Bone marrow stem cells	The results suggest that the strategy of combing BMSCs with FG is effective in the repair of alveolar bone defects. Its clinical application is promising.
Streckbein et al. (2013)	To evaluate the efficacy of bioactive implants (ADSC in fibrin glue) for repair of critical-size mandibular defects in athymic rats.	Homologous Beriplast™ P	Mandibular defects in rats	Adipose-derived stem cells	Fibrin sealant is a suitable biological scaffold for cell transplantation.
Xuan et al. (2014)	To compare the potentials of PRF-mixed Bio-Oss™	Tisseel ™	Canine sinus model.	Demineralized bovine bone	The findings from this study suggest that

Author (Date)	Objective	Component origin and/or Trade name	Implantation Site	Mixture with biomaterials or cells	Conclusion
	and Tisseel™-mixed Bio-Oss™ to enhance bone regeneration in a canine sinus model.				when platelet-rich fibrin is used as an adjunct to Bio-Oss™ particles for bone augmentation in the maxillary sinus, bone formation in the graft sites is significantly greater than when Tisseel™ is used.
Lappalainen et al. (2015)	To evaluate ossification of cranial bone defects comparing the healing of a single piece of autogenous calvarialbone representing a bone flap as in cranioplasty compared to particulated bone slurry with and without fibrin glue to represent bone collected during cranioplasty.	Tisseel™	Rabbit calvarial	Autologous particulate bone	Autogenous bone grafts in various forms such as solid bone flaps or particulated bone treated with fibrin glue were associated with bone healing which was superior to the empty control defects.
Santos et al. (2015)	To compare the potential of bone repair of collagen sponge with fibrin glue in a rat calvarial defect model.	Tissucol™	Rat calvaria	Fibrin sealant alone	Results have shown the benefits of using collagen sponge and fibrin glue to promote new bone formation in rat calvarial

Author (Date)	Objective	Component origin and/or Trade name	Implantation Site	Mixture with biomaterials or cells	Conclusion
					bone defects, the latter being discreetly more advantageous.
Zazgyva et al. (2015)	To establish an experimental model and assesses the effect of glass granules fixed with fibrin compared to fibrin alone as fillers of the osteochondral defects created in the weight-bearing and partial weight-bearing regions of the distal femur in six adult rabbits.	Tisseel TM Lyo	Rabbit distal femur	Bioactive glasses	A commercially available fibrin sealant can be successfully used to retain bioactive glass granules in the defects, offering a fast intra-operative and a subsequently stable fixation.
Hao et al. (2016)	To evaluate the efficacy of local injection of bone mesenchymal stem cells (BMSCs) and fibrin glue in the treatment of atrophic nonunion in an animal model.	Homologous (non-commercial)	Rat distal femur	Allogeneic bone mesenchymal stem cells	The analyzes demonstrated that local injection of BMSCs-seeded fibrin glue promoted atrophic nonunion repair.
Mehrabani et al. (2018)	To investigate the healing and regenerative effects of fibrin glue associated with adipose-derived stem cells (ADSCs) and fibrin glue scaffold alone with autologous	Autologous	Rabbit mandible	Adipose-derived stem cells	The healing process had a significant increase in the thickness of new cortical bone when fibrin glue scaffold associated with Adipose-derived

Author (Date)	Objective	Component origin and/or Trade name	Implantation Site	Mixture with biomaterials or cells	Conclusion
	bone grafts in experimental mandibular defects of the rabbit.				stem cells was used.
Pomini et al. (2019)	To evaluate the support system formed by a xenograft fibrin sealant associated with photobiomodulation therapy of critical defects in rat calvaria.	Tisseel TM Lyo	Rat calvaria	Demineralized bovine bone	The support system formed by the xenograft fibrin sealant associated with the photobiomodulation therapy protocol had a positive effect on the bone repair process.
Rezaei et al. (2019)	To evaluate the effects of PRP and canine BM-MSCs (marrow-derived mesenchymal stem cells - cBM-MSCs) in combination with a suitable carrier (fibrin glue) on periodontal regeneration.	Autologous	Dog class II furcation defects	PRP, cBM-MSCs and alone	More studies should be done in order to recommend an effective therapeutic approach that induces endogenous regenerative processes, such as cell homing.

IV. DISCUSSION

The aim of the present study was to perform a systematic review of the homologous fibrin sealants unique properties as a support material for cell adhesion, migration, proliferation and differentiation, and to enhance the physical and biological osteoconductive biomaterials properties.

The advances achieved in reconstructive surgical techniques combined with the development and natural biopolymers improvement by tissue engineering have

attracted the attention of several research groups because it is a promising alternative to existing treatments (CHEN; LIU, 2016).

Among the available sealants, fibrin sealants are the most promising in this field due to the combination of excellent biocompatibility, biodegradability and intrinsic bioactivity. Additionally, over the last few decades emphasis has been placed on the importance of fibrin sealant properties in the repair of bone defects in different anatomical regions (NOORI et al., 2017).

As a consequence, it has been searched for alternative methods to obtain blood components, for this reason, a group of researchers from Center for the Study of Venoms and Venomous Animals (CEVAP-Unesp-Botucatu-SP-Brazil) has developed a fibrin adhesive derived from the snake Crotalus durissus terrificus venom and the buffalo blood. In its composition, the cryoprecipitate containing fibrinogen and coagulation factors are derived from the buffalo blood (Bubalus bubalis), and the functional thrombin by gyroxin, a thrombin-like protein derived from the snake (FERREIRA, 2014; BISCOLA et al., 2017; FERREIRA et al., 2017; MOZAFARI et al., 2018).

Likewise, fibrin biopolymer is a clinically useful tool due to flexibility and applications diversity such as nerve injury repair, chronic ulcer treatment, and bone repair (BUCHAIM et al., 2015, 2016, 2017; DE OLIVEIRA GONÇALVES et al., 2016; ROSSO et al., 2017).

Hence several studies have been reported the use of fibrin sealants as a support for mesenchymal stromal cells (MSCs) and stem cells derived from adipose tissue to facilitate cell attachment, growth and differentiation, allowing enhancement of expansion and survival in the area implanted (RYU et al., 2005; KALBERMATTEN et al., 2008; VADALÀ et al., 2008).

In the same time, the insertion of these cells into the three-dimensional fibrin network has presented promising results in the process of bone repair in several models (ZHANG et al., 2012; STRECKBEIN et al., 2013; HAO et al., 2016; MEHRABANI et al., 2018; REZAEI et al., 2019). In addition, these researchers suggested that fibrin sealant is able to lead a stem cells microenvironment, without deforming its structure, increasing cell survival time and therefore being effective in repairing bone defects.

However, previous results from McDuffee et al. (2012) contradict the previously mentioned results, since they affirm that osteoprogenitor cells inserted in the three-

dimensional network formed by fibrin sealant is not able to accelerate the process of bone consolidation.

Despite, the fibrin sealants have beneficial characteristics in the bone regeneration, it is still not possible to have precise control over the microarchitecture of these materials and good tensile strength (GUÉHENNEC; LAYROLLE; DACULSI, 2004). Consequently, is necessary to associate with materials that have great scaffolding potential in many tissue engineering applications in order to minimize or eliminate these limitations. In this way, it allows the manufacture of multifunctional scaffolds with greater resistance mechanics, the graft greater stability in the surgical site, and longer time of cellular support throughout the process of bone repair (AHMED; DARE; HINCKE, 2008).

Several experimental and clinical trials have demonstrated the synergistic characteristics of fibrin sealant associated with materials that have great scaffolding potential led to satisfactory results (REPPENHAGEN et al., 2012; XUAN et al., 2014; LAPPALAINEN et al., 2015; ZAZGYVA et al., 2015; POMINI et al., 2019).

V. CONCLUSION

Due to this background, it is notable that fibrin sealant is one of the promising biopolymers used for tissue engineering and bone regeneration applications. Indeed, the combination with different types of bone grafts, biomolecules and stem cells make this scaffold unique and attractive feature for futures studies.

Nevertheless, there is a necessity for additional studies, for evaluation the concentrations of the components, as a fibrinogen and thrombin, which directly interfere in the density of the network, allowing or not the cellular migration and consequently the bone consolidation.

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2.2 ARTICLE 2 **–** "**Fibrin Sealant Derived from Human Plasma as a Scaffold for Bone Grafts Associated with Photobiomodulation Therapy".** (Appendix B - C and Annex 2-3).

- International Journal of Molecular Sciences, IJMS.

Abstract: Fibrin sealants derived from human blood can be used in tissue engineering to assist in the repair of bone defects. The objective of this study was to evaluate the support system formed by a xenograft fibrin sealant associated with photobiomodulation therapy of critical defects in rat calvaria. Thirty-six rats were divided into four groups: BC (*n* = 8), defect filled with blood clot; FSB *(n* = 10*),* filled with fibrin sealant and xenograft; BC^{PBMT} (*n* = 8*)*, blood clot and photobiomodulation; FSB^{PBMT} *(n* = 10*)*, fibrin sealant, xenograft, and photobiomodulation. The animals were killed after 14 and 42 days. In the histological and microtomographic analysis, new bone formation was observed in all groups, limited to the defect margins, and without complete wound closure. In the FSB group, bone formation increased between periods (4.3±0.46 to 6.01±0.32), yet with lower volume density when compared to the FSB^{PBMT} (5.6±0.45 to 10.64±0.97) group. It was concluded that the support system formed by the xenograft fibrin sealant associated with the photobiomodulation therapy protocol had a positive effect on the bone repair process.
Keywords: bone regeneration; bone repair; fibrin sealant; biomaterial; photobiomodulation therapy; low-level laser therapy

2.2 ARTICLE 2 – **"Fibrin Sealant Derived from Human Plasma as a Scaffold for Bone Grafts Associated with Photobiomodulation Therapy".** This manuscript was published in the International Journal of Molecular Sciences (Int. J. Mol. Sci.) at https://www.mdpi.com/1422-0067/20/7/1761/April 2019. It is authorized by the journal to be used in the defense of thesis, according to the terms and conditions of the University of São Paulo (The Digital Library of Theses and Dissertations of the University of São Paulo; https://www.tese.usp.br/index.php?lang=en).

Fibrin Sealant Derived from Human Plasma as a Scaffold for Bone Grafts Associated with Photobiomodulation Therapy

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1. Introduction

There are currently available treatment options for the repair of bone defects, but their effectiveness is limited in large defects, and the influence of extrinsic factors such as smoking and alcohol exposure are unfavourable in this process [1–3]. Annually more than two million bone grafts are performed worldwide, the second most frequent tissue transplantation, being surpassed only by blood transfusion [4,5].

Among all available types, the autologous graft is still considered the gold standard, since all the necessary properties in bone regeneration in terms of osteoconduction, osteoinduction, and osteogenesis are combined [6,7]. However, its availability is limited, and morbidity at the donor site has led to the development of new bone substitutes that restore, ameliorate, or prevent aggravation of compromised tissue function [8,9].

In order to solve this problem, tissue engineering has developed xenografts that are skeletal derivatives of other species, mainly bovine, with satisfactory osteoconductive properties and widely used in reconstructive procedures with greater scientific evidence among biomaterials [4,10].

To form a graft material mouldable to the surgical bed, facilitate its insertion and agglutination, and prevent its dispersion and collapse of soft tissue into the defect, biodegradable polymers known as scaffolds are used as three-dimensional supports for the lodging of cells and biologically active molecules, providing a favourable environment for tissue regeneration [10].

Among the scaffolds, fibrin sealants derived from human blood may have the potential to guide this process of bone remodelling, because it has compatible physiological characteristics to human tissue and thus is readily colonised by the surrounding cells. Thus, they allow surgeons to influence and improve the cellular microenvironment in vitro or in vivo, increasing the success rate of the bone graft [11].

Other attempts have been studied to minimise the time of bone healing and to reduce the chance of possible complications arising from the abnormal regeneration process. Among them, low-intensity pulsed ultrasound [12] and laser photobiomodulation therapy have been highlighted by their satisfactory effects on bone metabolism and repair, due to their possible osteogenic effect [13,14].

Laser photobiomodulation therapy is a non-invasive treatment method with relatively low cost [15]. However, there are controversies regarding the best

parameters to be used to obtain an effective result in the process of bone repair of critical size defects filled with biomaterials [5].

Despite the growing interest in blood-derived biomaterials in the reconstruction of bone defects, in the literature reviewed, no studies were found on the effects of the combination of sealant with bone grafts and alternative methods such as photobiomodulation. Thus, this study evaluated the support system formed by a xenograft fibrin sealant associated with the protocol of photobiomodulation therapy in critical size defects in rats.

2. Results

2.1. Microtomographic Analysis

In microtomographic images, at 14 days, it was observed that in all the defects, the new bone formation occurred centripetally from the critical defect margins towards the centre. All groups exhibited a continuous increase of new bone formation during the analysed periods; however, in no specimen was there a complete closure of the defect, and the formed bone was restricted to the defect borders (blue arrow—Figure 1A).

In the groups where the defects were filled with fibrin sealant associated with the xenogeneic graft (FSB – without laser photobiomodulation therapy and FSB^{PBMT} - with laser photobiomodulation therapy), the images showed the surgical cavity filled with the materials implanted and fine bone trabeculae adjacent to the border of the defect and under the dura mater. In animals biostimulated with a low-power laser, a more evident formation of the bone tissue was observed in FSB^{PBMT} compared to the FSB group (Figure 1A).

In the subsequent period, at 42 days, an increase in the amount of bone tissue, interweaving the biomaterial, in a more organised configuration, especially in the FSB^{PBMT} group was observed. The xenograft particles were still evident (red arrow), with some areas of remodelled tissue at the defect margins (Figure 1B).

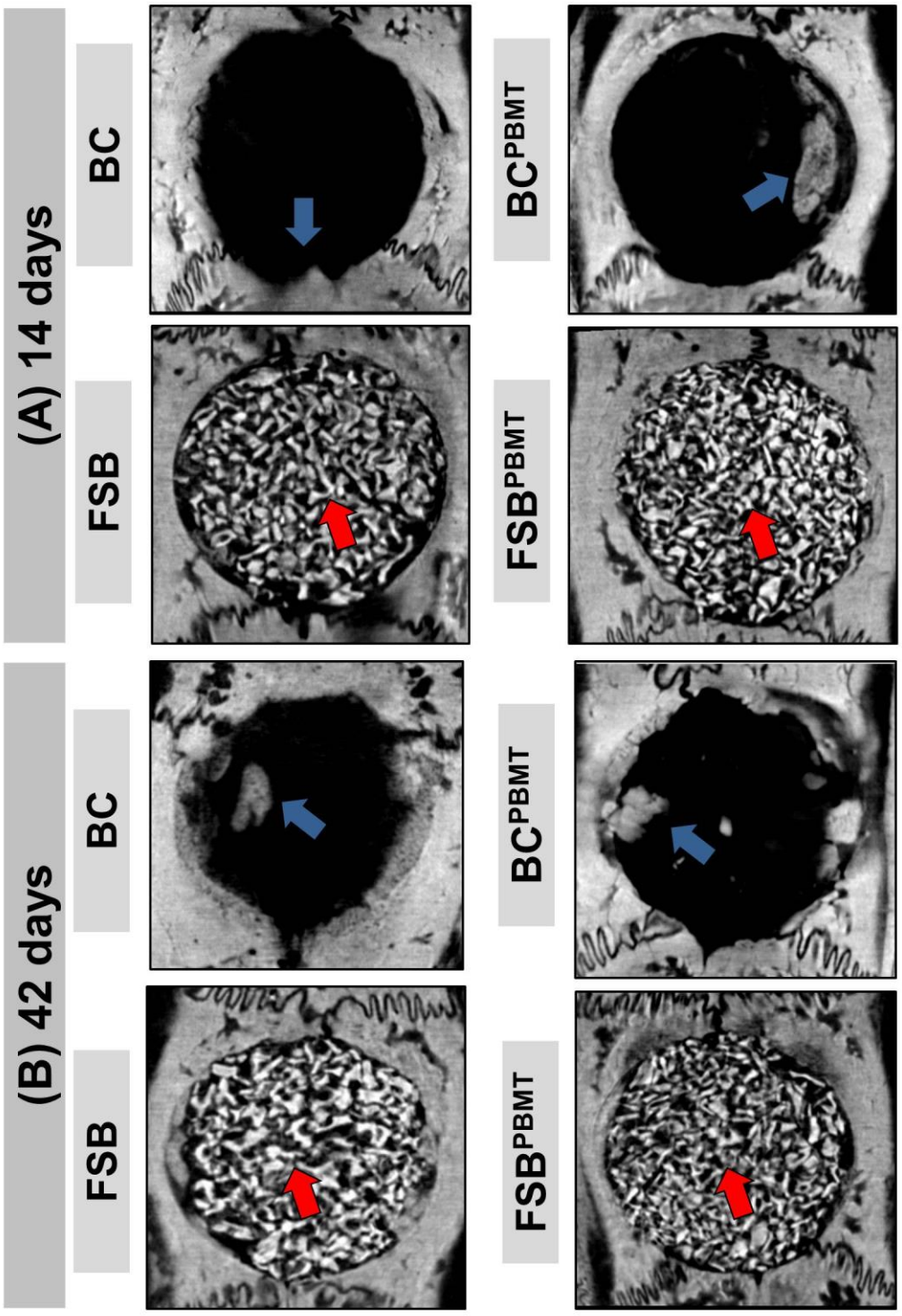

Figure 1
Microtomographic images showing the evolution of the repair of defects filled with clot and fibrin sealant plus xenograft (biomaterial) with or without low-level laser biostimulation therapy. Biomaterial particles (red arrow) and newly formed bone tissue (blue arrow). Two-dimensional trans-axial cuts at (A) 14 days; and (B) 42 days, respectively.

2.2. Histological Evaluation

In all groups, the repair of bone defects occurred centripetally, with the absence of necrotic tissue, the presence of bone cells, osteoid matrix, and budding of new blood vessels at the site.

At 14 days, all BC (defect filled with blood clot without laser photobiomodulation therapy) and BC^{PBMT} (defect filled with blood clot with laser photobiomodulation therapy) animals presented incomplete bone repair both in the height and in the conformation of the newly formed bone, which was irregular along the dura mater (Figure 2A(i)–A(ii) and Figure 3A). In the animals in the BC group, the central area of the defect was predominantly filled by loose connective tissue with small loci of new bone formation at the defect border, but in the BC^{PBMT} animals, the defects were filled by immature bone and more obvious blood vessels (Figure 2A(i)–A(ii) and Figure 3A).

In all animals of the FSB and FSB^{PBMT} groups, in the same experimental period, the defects presented with large amounts of the biomaterial. The new bone formation also occurred from the defect border, with trabecular conformation, being more pronounced in the FSB^{PBMT} group. The presence of inflammatory infiltrate was identified in both groups, diffusely distributed in the interstitial space (Figure 2A(iii)–A(iv) and Figure 3A).

At 42 days, in the BC group, the formed connective tissue filled the entire extent of the defect, maintaining a seemingly smaller thickness in relation to the remaining (original) bone, and the new bone formed was limited to the proximities of the injured borders. In the BC^{PBMT} animals, biostimulated with low power laser, the defect was still filled by a large amount of connective tissue, exhibiting a thin layer of bone tissue (asterisk) with diploe characteristics, and in some cases partial closure of the defect, but without recovery of its height (Figure 2B(i)–B(ii) and Figure 3B).

In the same period, in all FSB and FSB^{PBMT} animals, the surgical area was almost completely filled by biomaterial particles, without any significant changes in relation to the previous period. The bone formation remained limited to the edges, but with a denser and lamellar arrangement. The tissue reaction appeared to be in the resolution phase, with the most fibrotic interstitial space. In the FSB^{PBMT} group, the reduction of oedema was more evident resulting in the formation of a denser stroma with more cells and with concentric collagen fibres forming a capsule around the biomaterial (Figure 2B(iii)–(iv) and Figure 3B).

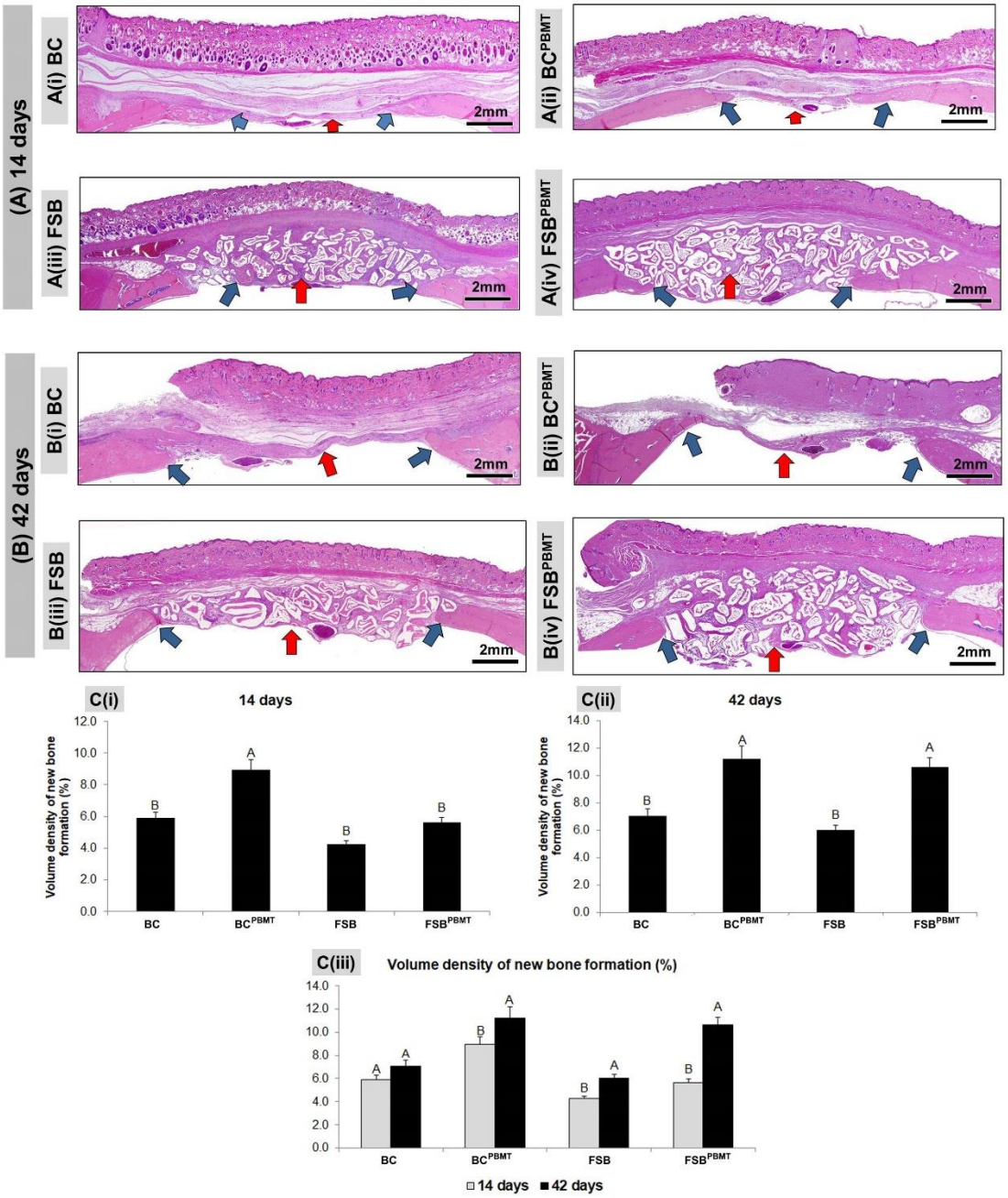

Figure 2

Panoramic histological views at (A) 14 days; and (B) 42 days, respectively; (C) graphs of volume density of newly formed bone in skull defects filled with a blood clot or fibrin sealant plus xenograft and with or without laser photobiomodulation therapy. (A) A(i)–A(ii) bone formation (blue arrows) occurring at the defect border and under the dura mater surface. A(iii)-A(iv): the defect showed trabecular bone formation (blue arrows) adjacent to the defect border, in a more advanced stage of bone maturation. (B) B(i)–B(ii) both groups showed similar bone formation limited to the defect border and a large region filled with fibrous connective tissue (red arrows); B(iii)– B(iv) a large part of the defect was filled by connective tissue and biomaterials (red arrows), but in the FSB^{PBMT} group, greater bone formation defect could be observed compared to the FSB group; (C) Graphs of newly formed bone showed smaller bone formation in the non-biostimulated group (BC and FSB) than the biostimulated group (BC^{PBMT} and FSB^{PBMT}). (BC and BC^{PBMT}: *N* = 4/group and

periods), (FSB and FSB^{PBMT}: *N* = 5/group and periods). C(i) and C(ii) where different letters (A≠B) indicate a statistically significant difference between groups in the same period and C(iii) where the different letters (A≠B) indicate a statistically significant difference in the same group in the two periods analysed ($p < 0.05$). (HE; original magnification × 4; bar = 2 mm).

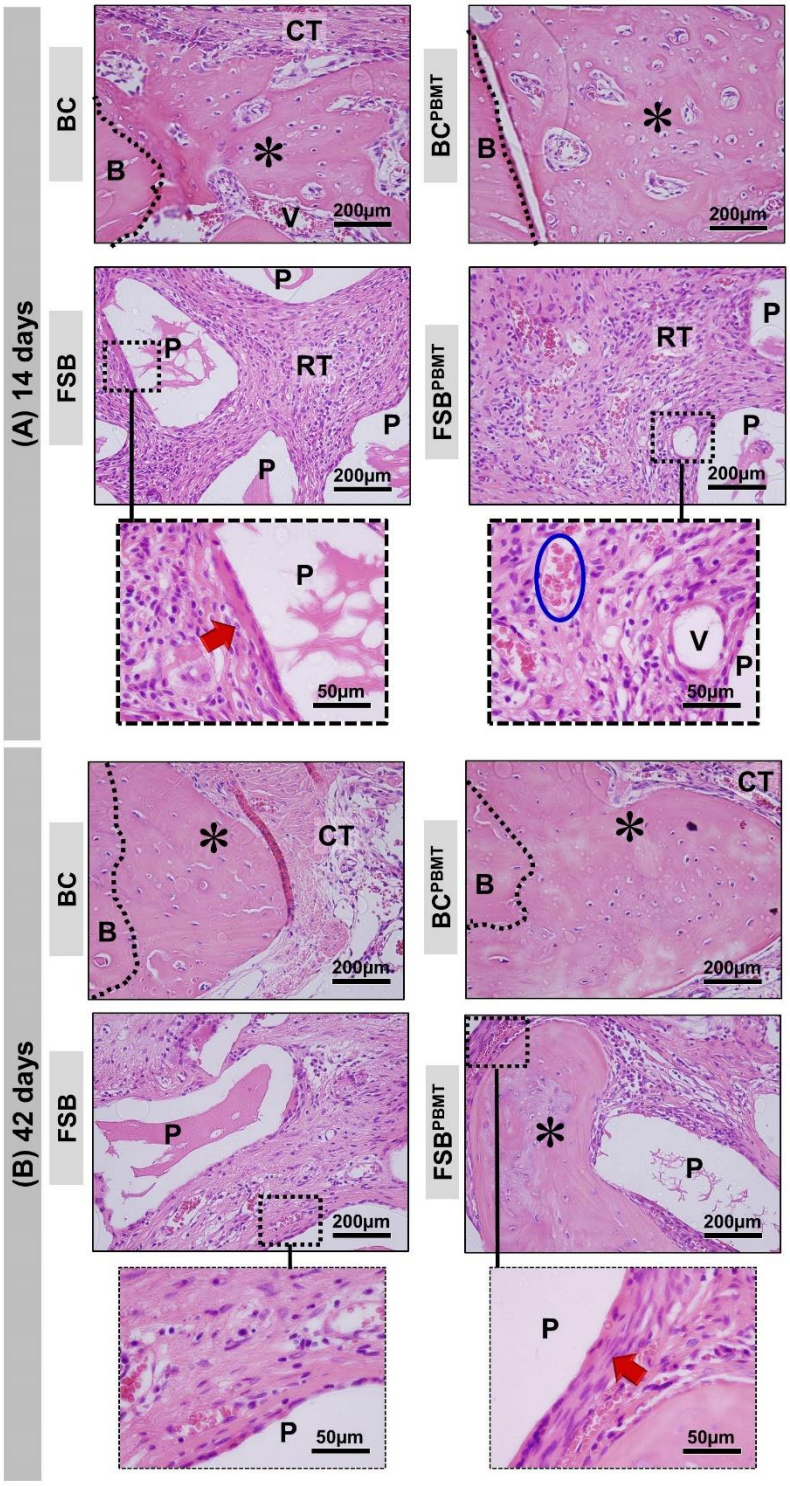

Figure 3

Details of the evolution of the bone healing of the skull defects filled with a blood clot or fibrin sealant plus xenograft (biomaterial) with or without low-level laser biostimulation therapy. (A)At 14 days, BC and BC^{PBMT}: the defect shows the trabecular bone formation (asterisks) adjacent to the defect border and spaces between the trabeculae filled by connective tissue. FSB: the defect was filled by particles of the biomaterial (P) surrounded by connective tissue with some inflammatory cells (RT - reactional tissue). Collagen fibres surrounding

the xenograft particles (red arrow). FSB^{PBMT}: the defect was filled by particles of the biomaterial (P) surrounded by connective tissue with some inflammatory cells (RT—reactional tissue). Presence of many red blood cells (inside the blue lined area) and blood vessels (V) permeating connective tissue; (B) At 42 days, BC and BC^{PBMT}: the new bone shows a gradual increase in thickness of the trabeculae leading to a compact structure. FSB: the new bone formation increases, becoming compact, there is a presence of xenograft particles and a decrease in inflammatory response. In FSB^{PBMT}, the collagen fibres are arranged in more layers surrounding the particles. (HE; original magnification × 40; bar = 200 µm; and Insets, magnified images × 100; bar = 50 µm).

2.3. Histomorphometric Evaluation

At 14 days of the repair process and after a quantitative evaluation of the volume density of the newly formed bone, it was observed that animals of the BC^{PBMT} group presented the highest means (8.9±0.64) with significant difference in relation to the other experimental groups BC, FSB, and FSB^{PBMT} (5.9±0.38; 4.3±0.46; 5.6±0.45, respectively), that were not significantly different to each other (Figure 2C(i)).

In the 42-day period, the groups biostimulated with a low-power laser (BC^{PBMT} and FSB^{PBMT}) presented the highest means, but without significant difference between them (11.22±0.94; 10.64±0.97, respectively). However, the animals in the aforementioned groups showed a significant difference when compared to the non-biostimulated animals BC and FSB (7.06±0.49; 6.02±0.32, respectively), but did not show any significant difference when compared to each other (Figure 2C(ii)).

The evaluation of the volume density of the newly formed bone within the same group in the two experimental periods (14 and 42 days) revealed that bone formation was higher in all groups in the 42-day period, with a significant difference between periods except for the BC group (Figure 2C(iii)).

3. Discussion

The existing scientific evidence in the field of tissue engineering indicates promising results in the treatment of bone defects with the use of fibrin sealants derived from human plasma as scaffolds for cellular development [11,16–19]. However, there is no data in the literature which reports its effect on the bone repair process when associated with xenograft and alternative therapeutic methods. Thus, the results in this

study show that the association of these treatments favoured the repair process of critical bone defects in the calvaria of rats.

The bone calvarium defect rat model is the most used among others in the scientific literature since it provides a clinically relevant evaluation of regenerative therapies and bone substitute materials, allowing for more effective clinical interventions. The defect produced is perfectly reproducible, fast, and does not require fixation for stabilisation, as compared to long bones [20].

The search for noninvasive methods, such as low intensity ultrasound (LIPUS), electromagnetic fields, and laser photobiomodulation therapy, has been increasing exponentially in recent years to improve the bone healing process [12]. As a consequence, there are numerous clinical and experimental studies with low-level laser photobiomodulation therapies, but so far without consensus on the optimal parameters for the bone repair process [5].

This study used a wavelength of 830 nm, a power density of 258.6 mW/cm^2, and mode of continuous operation, corroborating previous studies that presented satisfactory results in the process of bone repair [21].

Therefore, with the knowledge that the biomodulation effects of the laser are intrinsically related to the wavelength and that the loss of intensity may compromise its function, the right choice of the spectral band has become of extreme importance in the treatment. Thus, the wavelength in the infrared spectrum became widely used due to its lower loss, which can reach up to 37% of its intensity after a depth of 2 mm [22]. Knowing previously that the pre-calvarial tissue thickness in the rat has small dimensions, it is assumed that the loss is minimal. In situations exceeding 2 mm, there may be a maximum loss of 162.92 mW for each cm^2 of tissue, with the same protocol used in this study.

In addition, the infrared spectrum, between 780 and 1100 nm, is based on non-thermal mechanisms, which do not generate a significant increase in tissue temperature (up to 37.5°C). In excitation states, a fraction of energy is converted into heat, which causes local and transient increases in the temperature of absorbent chromophores, without heating the total cell [23].

To evaluate the potential of fibrin sealants derived from human blood, this study comprised microtomographic, histological, and histomorphometric analyses. In the microtomographic analysis, at 14 days it was possible to observe the formation of new bone at the margins of the surgical wound in all groups, probably via the stimulation of

growth factors released after craniotomy. The growth remained limited to this region until the end of the experiment, as reported in other experimental studies [24,25].

In the groups where the defects were filled with the fibrin sealant associated with the xenogeneic graft (FSB and FSB^{PBMT}), the particles remained at the site of implantation without dispersion, corroborating with studies that report on the mechanical stability and the binding effects provided by the fibrin sealant to bone grafts [26,27].

Histologically, at 14 days, the BC and BC^{PBMT} groups exhibited new bone formed in the defect margins, overlapping the dura, with a trabecular and immature arrangement. This can be attributed to the action of growth factors in this region after vascular rupture due to craniotomy and the presence of the underlying periosteum, which is the main source of osteoprogenitor cells and osteoinductive factors [28]. At 42 days, the newly formed bone became lamellar and compact. These findings are generally observed in repair procedures in lesions similar to those performed in this study [3,29,30]. However, none of the defects presented complete closure, with a large part being filled by fibrous connective tissue, in agreement with studies that reported that this is a critical defect according to Gosain et al. [31], An et al. [32], and Maciel et al. [30].

In the two analysed periods, the animals biostimulated with the laser presented greater evidence of new bone formation and greater tissue organisation at the end of the experiment [33]. These results are consistent with the literature that indicates the positive photobiomodulatory effects of the laser in the initial phases of bone repair, when, among several events, there is a proliferation of osteoblasts and differentiation of mesenchymal cells [34,35].

The defects filled with fibrin and xenograft (FSB and FSB^{PBMT}) sealers showed intense angiogenesis as early as 14 days, as well as the presence of reactional tissue at 42 days of resolution [36]. The tissue reaction observed in these groups did not trigger a foreign body type granulomatous reaction, which suggests that the grafts used were biocompatible [26,37,38] and the biological response was consistent with the inflammatory process after implantation of the biomaterial [14,35,39–43].

Histomorphometric analysis of the clot-treated and biostimulated laser defects, BC^{PBMT}, revealed a gradual and significant increase in bone volume during the experimental periods (8.9±0.64 to 11.22±0.94) in relation to the animals of group BC (5.9±0.38 to 7.06±0.49) in periods of 14 to 42 days, respectively [44]. The biological

mechanisms involved in improving the growth of bone tissue irradiated by a low-power laser are still not clearly understood. Studies suggest that laser energy can excite intracellular chromophores, especially the cytochromes of mitochondria, stimulating the cellular activity and consequently increasing ATP concentration, calcium, protein synthesis, and signalling pathways actively interconnected with the differentiation of stem cells into osteoblasts [45,46].

In the group with defects treated with sealant and xenograft, FSB, the bone formation was increasing between the periods (4.3±0.46 to 6.01±0.32), but in lower volume density compared to the animals of the FSB^{PBMT} group (5.6±0.45 to 10.64±0.97), supporting the positive influence of laser photobiomodulation in the repair process. Similar results were reported by De Oliveira et al. [21] in calvarial defects of autogenous graft-filled rats treated with low-power laser, in which a higher bone formation was also observed in all analysed periods.

The results obtained in this experiment provide evidence that defects filled with fibrin sealant and xenograft, and treated with low-power laser presented an evolution in the tissue repair process, with a better response compared to the other groups investigated, suggesting that there was a photobiomodulatory action in the inflammatory process, with a more organised deposition of collagen fibres in the defect area and consequently with a more homogenous bone conformation.

3.1. Strengths

The present research is a pioneer experimental study on the use of a fibrin sealant derived from human blood and xenograft associated with the protocol of photobiomodulation therapy with the use of low-power laser demonstrating effective repair of nerve and bone lesions. In addition, the association provided ease of insertion, local haemostasis, and maintenance of the implanted materials in the surgical bed, allowing the accomplishment of procedures in a shorter operative time.

3.2. Limitations

One limitation of this study is the absence of a quantitative evaluation of the microtomographic images due to the similar radiopacity between the newly formed bone and the xenograft, which makes it difficult to quantify [47].

For prospective studies requiring repair of bone defects, analysis of other fibrin sealants may be proposed, such as a promising fibrin biopolymer free of human blood components [16], and associations with complementary therapies that present osteogenic potential as pulsed ultrasound (LUPUS) and ultralaser [12].

4. Materials and Methods

4.1. Blood-Derived Biomaterials—Fibrin Sealant

Tisseel Lyo™ (Baxter Healthcare Ltd., Norfolk, United Kingdom; Ministry of Health Registration nº: 1.0683.0182) is a two-component fibrin sealant that contains two of the proteins that make the blood clot, fibrinogen and thrombin. Tisseel Lyo is prepared as two solutions which mix when applied. When prepared, 1 mL of each solution contains human fibrinogen (as a clotting protein), 91 mg/mL in 3000 UIC/mL protein; aprotinin (synthetic) and human thrombin, 500UI/mL, in 40 µmol/mL calcium chloride.

Initially, the vials containing lyophilised sealer protein concentrate and aprotinin solution, lyophilised human thrombin, and calcium chloride solution were preheated for approximately three minutes in a water bath at a temperature of 33–37 °C, with the aid of a mercury thermometer (Termometros Labor™, São Paulo, Brazil). Thereafter, the sealant protein concentrate was dissolved with the aprotinin solution to form the sealant solution. Simultaneously, the lyophilised human thrombin was dissolved with the calcium chloride solution to form the thrombin solution. The two solutions were kept in the water bath until use.

4.2. Biomaterial—Xenograft

The commercial demineralised bovine bone matrix (Bio-Oss™; Geistlich Pharma AG, Wolhusen, Switzerland; Ministry of Health Registration nº: 806.969.30002) is a natural biomaterial available as granules of cancellous bone (0.25–1mm granule size; 2.0 g vial). The highly purified osteoconductive mineral structure is produced from natural bone in a multi-stage purification process and sterilisation is carried out by γ-irradiation. Thus, it is chemically as well as structurally comparable to the mineralized human bone. Bio-Oss™ contains pores of different sizes: macropores (300–1500 µm), micropores (size of Haversian and vascular marrow canals), and intracrystalline

spaces (3–26 nm), resulting in an overall porosity of 70–75% and a wide internal surface area of almost 100 m^2/g [48].

4.3. Experimental Design

Thirty-six adult male Wistar rats (Rattus norvegicus), 90 days old, weighing around 400 g, were obtained from the animal laboratory of the Ribeirão Preto campus of the University of São Paulo.

The animals were housed in conventional cages initially containing four animals each (alteration according to the animal weight recommended by the Animal Laboratory of Bauru School of Dentistry - University of São Paulo), with feeders and drinkers "*ad libitum*" (irradiated feed – Nuvilab rodents and filtered water), in an air-conditioned environment, air exhaustion, light-dark period 12L/12D, temperature 22°C ± 2°C, humidity 60% ± 10, lighting 150lux/1 m floor, maximum noise 70 dB (decibel - SPL, Sound Pressure Level). All experimental procedures in the animals were conducted with the approval of the Institutional Review Board in Animal Studies of the Bauru School of Dentistry, University of São Paulo (Protocol: CEEPA-019/2016).

Initially, the animals were randomly divided into two groups: BC, *n* = 16 (Blood Clot, the defect was filled with a blood clot) and FSB, *n* = 20 (the defect was filled with a mixture of xenograft and fibrin sealant). After the surgical procedures, four subgroups were preformatted according to the treatment: BC, *n* = 8 (the defect was filled with blood clot without photobiomodulation), BC^{PBMT}, n = 8 (the defect was filled with blood clot and photobiomodulation), FSB, *n* = 10 (the defect was filled with a mixture of fibrin sealant and biomaterial without photobiomodulation) and FSB^{PBMT}, *n* = 10 (the defect was filled with a mixture of fibrin sealant and biomaterial and photobiomodulation) (Figure 4A).

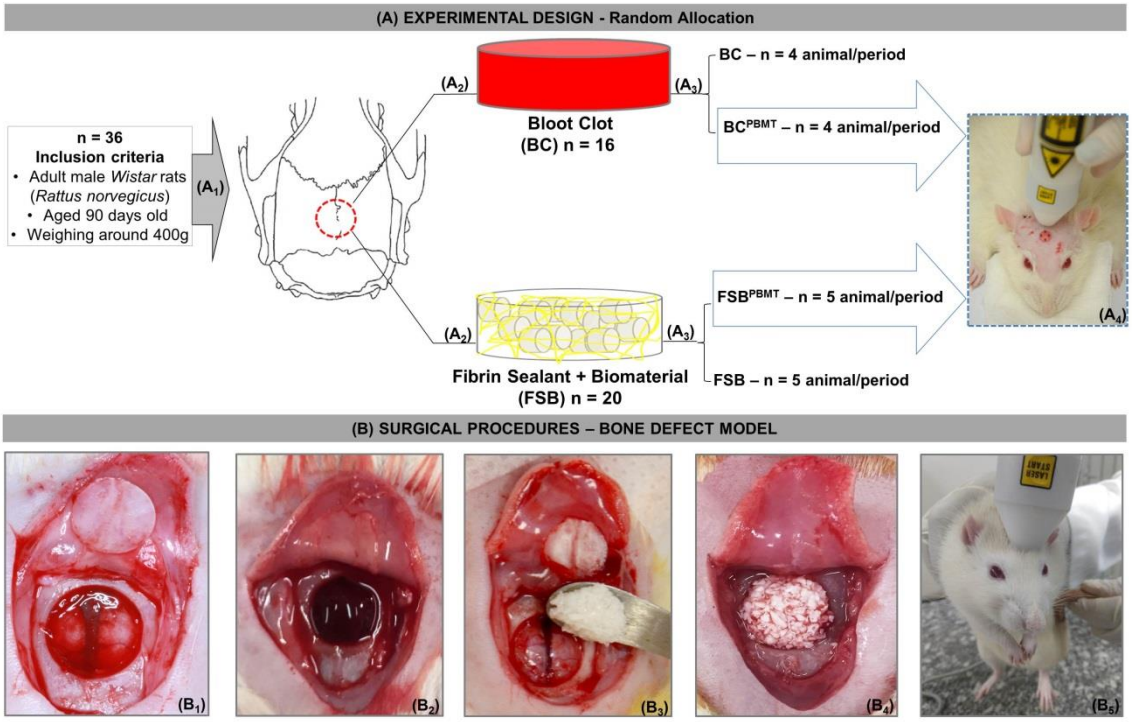

Figure 4
(A) Experimental design (A_1) Random allocation: Thirty-six rats were divided into two groups; (A_2) BC (*n* = 16)—Blood Clot and FSB (*n* = 20)—Fibrin Sealant + Biomaterial; (A_3) After surgical procedures, two subgroups were preformatted according to treatment: BC, *n* = 8 (Blood Clot, the defect was filled with blood clot and without photobiomodulation), BC^{PBMT}, *n* = 8 (Blood Clot, the defect was filled with blood clot and photobiomodulation), FSB, *n* = 10 (the defect was filled with a mixture of biomaterial and fibrin sealant and without photobiomodulation), and FSB^{PBMT}, *n* = 10 (the defect was filled with a mixture of biomaterial and fibrin sealant and photobiomodulation); (A_4) illustration of the four points of cross-application of the low-level laser on rat calvarium; (B) Surgical procedures—bone defect model; (B_1) Osteotomy using an 8 mm trephine bur with exposure of the fragment removed from the parietal bones; (B_2) defect filled with blood clot; (B_3) deposition of the mixture fibrin sealant + biomaterial in the defect; (B_4) defect filled with mixture; (B_5) low-level laser therapy (photobiostimulation).

4.4. Surgical Procedures

All surgical procedures were performed at the Mesoscopic Laboratory—discipline of Anatomy (Bauru School of Dentistry, University of São Paulo, Brazil) by the same team of professionals.

The animal surgeries were performed under general anaesthesia with an intramuscular injection of Ketamine (50 mg/kg i.m. (Dopalen ™, Ceva, Paulínia, SP, Brazil) and Xylazine (10 mg/kg i.m. (Anasedan ™, Ceva, Paulínia, SP, Brazil) followed

by fronto-parietal trichotomy and disinfection with 10% povidone-iodine (PI). With a scalpel blade n. 10, a half-moon incision was made in the cranial tegument and folded to expose the calvarium. Then, a defect was created in the centre of the parietal bone using an 8 mm diameter trephine bur, under continuous irrigation with saline, exposing the dura mater [49,50] (Figure 4B_1). The defects in the BC group were filled with 0.25 mm^3 of cardiac puncture blood [51] (Figure 4B_2).

In the FSB group, the defects were filled with 0.1 mm^3 of xenograft incorporated into 40 µL of the reconstituted fibrinogen solution in aprotinin and 40 µL of reconstituted human thrombin solution in sodium chloride (proportion 1:1, according to the manufacturer's recommendations) (Figure 4B_3–B_4). The amounts of xenograft and fibrin sealant used were previously established in a pilot study.

The periosteum and tegument were repositioned and sutured with nylon 5-0 (Mononylon™ Somerville S.A, New Jersey, USA) and silk 4-0 (Ethicon™ Johnson & Johnson Company, New Orleans, USA), respectively, to provide stability to the graft, decreasing the risk of soft tissue collapse [49,52].

The postoperative care consisted of a single oral administration of acetaminophen at a dose of 200 mg/kg (Paracetamol, Medley, São Paulo, Brazil) dissolved in water, available in the cages.

4.5. Photobiomodulation Therapy Protocol

The animals of Groups BC^{PBMT} and FSB^{PBMT} underwent laser irradiation (Laserpulse IBRAMED, Amparo, SP, Brazil) with continuous pulse GaAlAs (gallium–aluminium–arsenide). The following parameters were used for photobiomodulation therapy [21] — Table 1 below (Figures 4A_4 and B_5):

Table 1
Therapeutic parameters of the photobiomodulation therapy used in this study.

Parameter	Unit/Explanation
Optical Power	30 mW
Wavelength	830 nm
Density of Power or Irradiance	258.6 mW/cm^2
Fluency or Density of Energy or Dosimetry	6 J/cm^2
Beam Area	0.116 cm^2
Total Power	2.9 J
Type of Beam	Positioned for laser irradiation at perpendicular incidence to the skull
Emission Mode	Continuous (laser power remains constant at all times)

Form of Application	Four points surrounding the surgical area, north, south, east, and west
Duration of Irradiation	24 s/point
Total Time of each Application	96 s
Treatment Time	Immediately after surgery and three times a week until euthanasia

Laser beam emissions were self-calibrated by the device during all applications.

4.6. Collection of Samples and Histological Procedures

All animals were killed with an overdose of a Ketamine/Xylazine mixture following the guidelines of the Brazilian College of Animal Experimentation after 14 and 42 days, BC and BC^{PBMT} groups—4 animals/period and FSB and FSB^{PBMT}—5 animals/period. The cranial vaults with the lining skin were collected and fixed in 10% phosphate-buffered formalin for 48 h, and later, for examination in the microtomography.

4.7. MicroCT Scan (µ-CT)

The specimens were subjected to an X-ray beam scan in the computed microtomograph machine SkyScan 1174v2 (µ-CT—Bruker microCT, Kontich, Belgium). Initially they were packaged in an acrylic, cylindrical sample holder, (diameter 18.3 mm; height 10.9 mm), with exo- and endocranial aspects of the parietal bones in the vertical position. The images were captured with 13.76 µm voxel, 0.73° at each pace, and further reconstructed using the NRecon® v.1.6.8.0, SkyScan, 2011, Bruker microCT, with the same reconstruction parameters for all specimens. Then, the reconstructed images were realigned using the DataViewer® 1.4.4.0 software.

4.8. Histotechnical Processing

The specimens were washed in tap water for 24 h and immersed in 10% ethylenediaminetetraacetic acid (EDTA—a solution containing 4.13% Titriplex™ III Merck KGaA, Darmstadt, Germany and 0.44% sodium hydroxide Labsynth, São Paulo, Brazil), for a period of approximately 60 days [53]. Then, the collected bone fragments underwent successive standard histological staging and were finally included in Histosec™ (Merck KGaA, Darmstadt, Germany). Semi-serial coronal cuts of 5 µm

thickness were performed, prioritising the centre of the circular defect and stained with haematoxylin-eosin.

4.9. Histological and Histomorphometric Evaluation of Defects Bone Healing

The histological sections were analysed by light microscopy (Olympus model BX50) at approximate magnifications of × 4, 10, and × 40 in the Histology Laboratory of the Bauru School of Dentistry, the University of São Paulo (São Paulo, Brazil). To standardise and avoid bias, a training session was performed with an experienced pathologist.

Histological analysis of sections stained with HE consisted of an evaluative description of the healing events such as inflammation, granulation tissue, new bone formation, and remodelling, and the interaction among the biomaterial bone graft and the newly formed bone.

For morphometric evaluation, two central sections stained with HE were used for quantification of newly formed bone areas using an image capture system (DP Controller 3.2.1.276 – 2001–2006, Olympus Corporation, Tokyo, Japan). Initially, the total area to be analysed was established as every area of the surgical defect. The limits of this area were determined from the external and internal surfaces of the original calvarium on the right and left margins of the surgical defect. Then, the drawn lines were connected following their respective curvatures. Considering the total length of the defect, its centre point, measuring from this point, was 4 mm to the right and left to the edges of the surgical wound to determine the limits of the original surgical defect.

Morphometric analysis under light microscopy allowed the determination of the volumetric density (%), defined as the volume fraction of the entire graft filled by a given component/structure (newly formed bone). The volume density (Vvi) that is equal to the area density (AAi) was determined by AxioVision Rel. 4.8 Ink (Carl Zeiss MicroImaging GmbH, Jena, Deutschland), Vvi = AAi [50]. The area of graft filled by each structure (Ai) and the total area examined (A) were determined in pixels, and the volume density (Vvi) of each type of structure was calculated according to the relation Vvi = AAi = Ai/A × 100.

4.10. Statistical Analysis

An analysis of variance (ANOVA) was applied to the data obtained for the percentage of newly formed bone to verify the effect of the different groups tested in each evaluated period. The homogeneity of variances and normality of residues and the necessary assumptions for the conduction of ANOVA, were tested, respectively, by the Shapiro–Wilk and Bartlett tests, both at 5% probability. Subsequently, the means were compared by the Tukey test at 5% probability. The effect of the period evaluated in each group was compared by Student's *t*-test at 5%. All analyses were conducted with R (R Core Team, 2017).

5. Conclusion

It was concluded that the support system formed by the xenograft fibrin sealant associated with the photobiomodulation therapy protocol had a positive effect on the bone repair process in critical size defects in rat calvaria.

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3 DISCUSSION

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A variety of congenital and acquired conditions can affect bone tissue leading to significant bone loss, which makes bone transplantation the second most transplanted tissue in the world, behind only blood transfusion (CHEN; LIU, 2016b).

In this context, more than two million bone graft procedures are performed annually worldwide. Thus, the search for new synergistic combinations has been shown to be increasingly prevalent and widely used in the field of bone tissue engineering and in reconstructive medicine (FERNANDEZ DE GRADO et al., 2018).

The improvement of research in this area is extensive and continues to evolve. In fact, excellent biocompatibility, biodegradability, intrinsic bioactivity and many other unique characteristics make therapeutic combinations viable and attractive in the reconstruction of extensive bone defects (AMINI; LAURENCIN; NUKAVARAPU, 2012).

Despite the growing interest in blood-derived biomaterials in the reconstruction of bone defects, in the reviewed literature there are few clinical and pre-clinical studies regarding the use of fibrin sealants as three-dimensional scaffolding associated with other biomaterials. Therefore, as a first article, we developed a systematic review on the use of fibrin sealant derived from homologous human blood as a three-dimensional scaffold in bone repair in order to provide relevant information for a correct interpretation and discussion of the results of experimental research (Article 1).

That said, it was possible to observe a great variability in the results, which makes the studies, many times, with divergent estimates and difficult to compare between quantitative and qualitative analyzes (NOORI et al., 2017; POMINI et al., 2019c). This fact can be attributed, in part, to the diversity of fibrin sealant formulations, in relation to the origin and concentration of blood components, affecting the rate of hemostasis, the clot biochemistry, viscosity, adhesive resistance, durability, rate of fibrin polymerization, and consequently changes in the three-dimensional structure (KIM et al., 2014).

The data presented in this review served as a basis to conclude that combinations with fibrin sealants and different types of bone grafts are able to build a three-dimensional framework providing support for cell growth.

In view of this, our team of researchers proposed a preclinical study in a bone defect model in rat calvaria, with the aim of elucidating the effects of the homologous

fibrin sealant incorporated into the deproteinized bovine bone matrix associated with laser photobiomodulation therapy, through microtomographic analyzes in a two-dimensional transaxial section, histological in hematoxylin and eosin and the volumetric density of the new bone tissue formed (Article 2).

The results obtained showed that the support system formed by fibrin sealant derived from human blood and the deproteinized bovine bone matrix associated with the proposed laser photobiomodulation protocol was able to promote faster deposition of mineralized bone matrix (Article 2).

Bearing in mind that the selection criteria for the implantation site of the grafts in pre-clinical studies must be carefully considered, since each model employs a specific set of biological parameters that can directly influence the effectiveness of bone repair, including the amount of regenerated bone, the kinetics of bone formation and the mechanical properties of regenerated bone (HO-SHUI-LING et al., 2018). To this end, in this experiment, we used a bone defect model in rat calvaria, as it allows a reproducible defect, which does not require fixation to stabilize the skeleton, as is usually required in long bone defects and because it has a high translational potential for the reconstruction of large defects. craniomaxillofacial bones in humans (SPICER et al., 2012).

In this discussion, we will summarize the main results and demonstrate important evidence obtained, limitations and suggestions for prospective experimental studies.

Histologically, in all stains, bone defects filled with blood clot, commonly used as a negative control, showed centripetal growth, with complete closure of the wound by a thin layer of fibrous connective tissue, with no visible bone regeneration (SPICER et al., 2012).

This finding is also evidenced in the volumetric densities at 42 days and corroborated by preliminary studies that claim that critical defects without intervention result in approximately 5 to 15% of the defect volume filled with mineralized matrix (PATEL et al., 2008; AN et al., 2017).

It is evident that we observe in the scientific literature a large discrepancy in the parameters used for laser photobiomodulation in the bone repair process, making it difficult to discuss the effects of therapy and which protocol will be the most appropriate to achieve the desired therapeutic effects (KHADRA et al., 2004; ABOELSAAD et al., 2009; CRISAN et al., 2015; ESCUDERO et al., 2019).

In the face of pre-established information, we use the gallium-aluminum arsenide laser, GaAlAs, with a wavelength of 830 nm, based on successful results obtained in osteoblasts, *in vitro* and *in vivo* by different authors (KHADRA et al., 2004; LIU et al., 2007; BOUVET-GERBETTAZ et al., 2009).

It is known that infrared lasers (λ = 780-1100 nm) have greater depth of penetration into tissues compared to red light (WELCH; TORRES; CHEONG, 1989; ZEIN; SELTING; BENEDICENTI, 2017), and thus osteoblastic cells have a higher absorption coefficient in this spectral range, due to the low interference of the water chromophore (DE FREITAS; HAMBLIN, 2016).

On the other hand, different doses of energy applied to the tissue can result in different responses, since the effect of photobiomodulation has a biphasic dose-response pattern, that is, doses within a therapeutic window stimulate biological effects on the tissue, in contrast, doses below or above this therapeutic window may have null or inhibitory effects, respectively (HUANG et al., 2011).

Most studies suggest that biostimulation occurs at energy densities between 0.05 and 10 J/cm^2, while doses above 10 J/cm^2 have bioinhibitory effects (ALGHAMDI; KUMAR; MOUSSA, 2012). Thus, the fluency determined for the bone repair of cranial defects in rats in this study was 6 J/cm^2, being within the therapeutic window and already used in our preliminary studies with promising results (DE OLIVEIRA GONÇALVES et al., 2016; ESCUDERO et al., 2019; POMINI et al., 2019b).

Therefore, it can be considered that the phenomenon of laser biostimulation has shown primary effects (biochemical, bioelectric and bioenergetic) and secondary (stimulus to cell trophism and microcirculation), accelerating tissue repair. Thus, the null hypothesis that no differences were found between bone defects treated with lasers compared to a control group was rejected (ABOELSAAD et al., 2009; ABD-ELAAL et al., 2015; ZAKY et al., 2016).

With regard to the use of biomaterials, histological images allow us to associate the favorable biological response to the excellent biocompatibility property, due to the absence of foreign body type granulomas and immune granulomas, mediated by T lymphocytes (SHEIKH et al., 2015).

These findings are consistent with previous studies that affirm the absence of antigenicity or foreign body reaction in bone defects filled with deproteinized bovine bone and attribute it to this, to a multiphase purification process of the xenogenic material (PERIĆ KAČAREVIĆ et al., 2018). As for fibrin sealant, biocompatibility is

related to the combination of blood components present in these formulations, which are physiological proteins present in the final stage of hemostasis.

Since homologous fibrin sealant formulations are derived from human plasma, it is necessary to note that initially the use of these biopolymers raised concerns about the possibility of transmission of viral diseases such as Parvovirus B19, hepatitis and acquired immunodeficiency syndrome (HIV). However, after detailed screening, researchers noted that no reports of hepatitis or HIV transmission with homologous fibrin sealant appeared in more than 20 years of world literature (KAWAMURA et al., 2002).

This was made possible by extensive viral prevention methods, including viral screening, viral reduction methods, heat treatment, solvent / detergent cleaning, precipitation, pH treatment and chromatography (SPOTNITZ, 2014).

With regard to the degradation of the biomaterials used, it was observed that the particles of the bovine bone matrix remained deproteinized up to 42 days. This fact can be explained by the intrinsic characteristic of this biomaterial, that is, physiologically, after the implantation of biomaterials, there is an agglomeration of osteoclastic cells and macrophages around the particles, with intense synthesis of acids in an attempt to degrade them. Thus, the acidic pH of the microenvironment provides for the breakdown of hydroxyapatite from the bovine bone matrix, releasing calcium ions. This, in turn, progressively increases the concentration of this ion, and by a negative feedback mechanism it decreases the activity of osteoclasts and consequently delays graft reabsorption (GUARNIERI et al., 2018).

The fibrin network is dissolved in the first week after implantation, by proteolytic enzymes of the fibrinolytic system. As a result, fibrin sealant, antifibrinolytic protein formulations have been added in order to prevent the conversion of plaminogen to plasmin, responsible for fibrinolysis, thus prolonging the action of fibrin for approximately 40 to 45 days, a time sufficient for assist in the growth of bone cells and be sequentially biodegraded (GUÉHENNEC; LAYROLLE; DACULSI, 2004).

Thus, the excellent properties presented by fibrin sealants as biocompatibility, controllable biodegradability, and a multifunctional three-dimensional structure that provides cell support, proliferation and differentiation, anchoring of surrounding molecules and growth factors, makes them have remarkable advantages over other biomaterials, which makes it a potential candidate to assist in bone tissue engineering (SONG et al., 2018).

In this scenario, notably, the association of biomaterials capable of providing support and regenerative cell growth and non-invasive extra-operative therapies have played a considerable role in changing the paradigm regarding the regeneration of large bone defects (BROWN; BARKER, 2014).

However, there is a need for further evaluation on the standardization of the concentrations of blood components in sealants, which directly interfere with the density of the fibrin network, enabling or not the migration of osteogenic cells and pro-angiogenic hematopoietic cells to the center of the defect. In view of this, the lack of these cells and the low concentration of oxygen promotes the growth of fibroblasts and consequently the repair of the defect by scar tissue (COLLIGNON et al., 2017).

Therefore, it can be concluded that laser photobiomodulation associated with three-dimensional scaffolding was able to provide greater speed in the restoration of bone injury, and these are promising findings that will encourage future research in the field of bone tissue engineering and regenerative medicine in skeletal conditions compromised.

Thus, it is necessary to point out that each methodology used in experimental research has its own merits and limitations regarding specific needs. In this study, we can point out as limitations the lack of immunohistochemistry, immunofluorescence analysis and scanning electron microscopy to investigate the surface of the three-dimensional structure of fibrin in contact with bovine bone matrix.

For prospective studies that require repair of bone defects, an analysis of other fibrin sealants is suggested, such as, for example, the fibrin biopolymer free of human blood components (DE OLIVEIRA GONÇALVES et al., 2016; ROSSO et al., 2020), as well as other types of complementary, non-invasive therapies that have osteogenic potential such as pulsed ultrasound, LIPUS (POMINI et al., 2014b).

4 CONCLUSIONS

4 **CONCLUSIONS**

In conclusion, this study originally demonstrated that the support system formed by fibrin sealant derived from human blood and the deproteinized bovine bone matrix associated with the proposed laser photobiomodulation protocol provided:

- ✓ Perception of a certain scarcity of studies on the subject, demonstrating that greater knowledge is needed on the applicability of fibrin sealant as a three-dimensional support to cell growth in the bone repair process, as well as its association with other bone grafts and postoperative therapies non-invasive, such as laser biostimulation (Article 1);

- ✓ The great variability in the results of the analyzed studies may be, in part, related to the different concentrations of the blood components of the sealants, directly influencing the architecture of the fibrin network and consequently the bone repair process (Article 1);

- ✓ Formation of a composite scaffold with multifunctional properties, such as surgical hemostasis, and graft stability in the surgical bed (Articles 2);

- ✓ Biocompatibility of biomaterials (Article 2);

- ✓ Greater speed in depositing mineralized bone matrix (Articles 2).

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APPENDIXES

APPENDIX

Appendix A – Declaration of exclusive use of the article in thesis signed by the authors of the article 1**: "Applicability of Homologous Fibrin Sealant in Bone Repair: An integrative Review"**.

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the **"Applicability of Homologous Fibrin Sealant in Bone Repair: An integrative Review"** will be inclued in Thesis of the student Karina Torres Pomini was not used and may not be used in other Works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, March 24th, 2020

Karina Torres Pomini	Karina Torres Pomini
Author	Signature
Daniela Vieira Buchaim	
Author	Signature
João Vitor Tadashi Cosin Shindo	
Author	Signature
Uri Adrian Prync Flato	
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Marcelie Priscila Rosso	Marcelie Priscila de Oliveira Rosso
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Jesus Carlos Andreo	
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Rogério Leone Buchaim	
Author	Signature

Appendix B – Declaration of exclusive use of the article in thesis signed by the authors of the article 1**: "Fibrin Sealant Derived from Human Plasma as a Scaffold for Bone Grafts Associated with Photobiomodulation Therapy"**.

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the **"Fibrin Sealant Derived from Human Plasma as a Scaffold for Bone Grafts Associated with Photobiomodulation Therapy"** will be inclued in Thesis of the student Karina Torres Pomini was not used and may not be used in other Works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, March 24th, 2020

Karina Torres Pomini	Karina Torres Pomini
Author	Signature
Daniela Vieira Buchaim	
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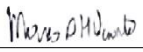
Marco Antônio Hungaro Duarte	
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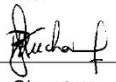
Rogério Leone Buchaim	
Author	Signature

Appendix C – Request has sent to the Committee on Ethics in the Use of Animals - FOB/USP to change the title of the doctoral research.

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Departamento de Ciências Biológicas
Disciplina de Anatomia

Of. ANAT 10/2020 Bauru, 30 março de 2020.

Comissão de Ética no Ensino e Pesquisa em Animais / FOB-USP

Ref.: Alteração do título de Pesquisa de Doutorado

Venho solicitar a alteração do título da pesquisa de doutorado de Karina Torres Pomini inicialmente intitulada "***Influência da terapia por laser de baixa intensidade no processo de reparo de defeitos ósseos preenchidos por selante de fibrina homólogo e heterólogo associados às células-tronco derivadas da polpa de dentes decíduos"***, registrada no CEEPA-Proc. sob nº 019/2016 para **"Aplicabilidade de compósito de fibrina homólogo no processo de reparo ósseo associado ou não a terapia por fotobiomodulação a laser".** A justificativa para o pedido de alteração foi a substituição das células-tronco como material a ser associado ao selante de fibrina pelo biomaterial Geistlich Bio-Oss® (substituto ósseo). Não houve nenhuma alteração no protocolo cirúrgico ou pós-operatório, assim como no número de animais. O Projeto envolveu as mesmas especificações já aprovadas pelo CEEPA, animais pertencentes ao filo Chordata, subfilo Vertebrada (exceto humanos), rato heterogênico Wistar, com aproximadamente 270g/60 dias, machos e origem Biotério da Universidade de São Paulo – Campus de Ribeirão Preto (SP). Por se tratar de uma pesquisa que envolve o doutorado da aluna Karina Torres Pomini e que está em fase de depósito de tese venho solicitar a análise do presente ofício.

Nesta oportunidade aproveito para agradecer

Atenciosamente,

Prof. Dr. Rogério Leone Buchaim
Pesquisador Responsável
Disciplina de Anatomia - FOB-USP

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
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ANNEXES

ANNEX

Annex 1 - Authorization from the editor in chief of the journal – International Journal of Advanced Engineering Research and Science.

International Journal of Advanced Engineering Research and Science
(IJAERS)
An Open Access International Journal

Certificate of Publication

The editor-in-chief is awarding this certificate to **Karina Torres Pomini** in recognition of his/her paper entitled "**Applicability of Homologous Fibrin Sealant in Bone Repair: An integrative Review**" which is published in International Journal of Advanced Engineering Research and Science (IJAERS), **ISSN: 2349-6495(P)| 2456-1908(O); Volume-6, Issue-7, July 2019, Page No. 016 – 023. Article DOI**: https://dx.doi.org/10.22161/ijaers.673.

Authors Name: Karina Torres Pomini, Daniela Vieira Buchaim, João Vitor Tadashi Cosin Shindo, Uri Adrian Prync Flato, Marcelie Priscila de Oliveira Rosso, Jesus Carlos Andreo, Bruna Botteon Della Coletta, Janaina Costa Marangon Duarte, Rogério Leone Buchaim.

Principal Author Name: **Karina Torres Pomini** - Department of Biological Sciences (Anatomy), Bauru School of Dentistry, University of São Paulo (USP), Bauru (SP) 17012-901, Brazil

Note: From this certificate editor in chief also authorize the authors to use the content of this article for his/her further work and thesis submission.

Ceritifcate Issue Date: March 18, 2020.

Editor-In-Chief
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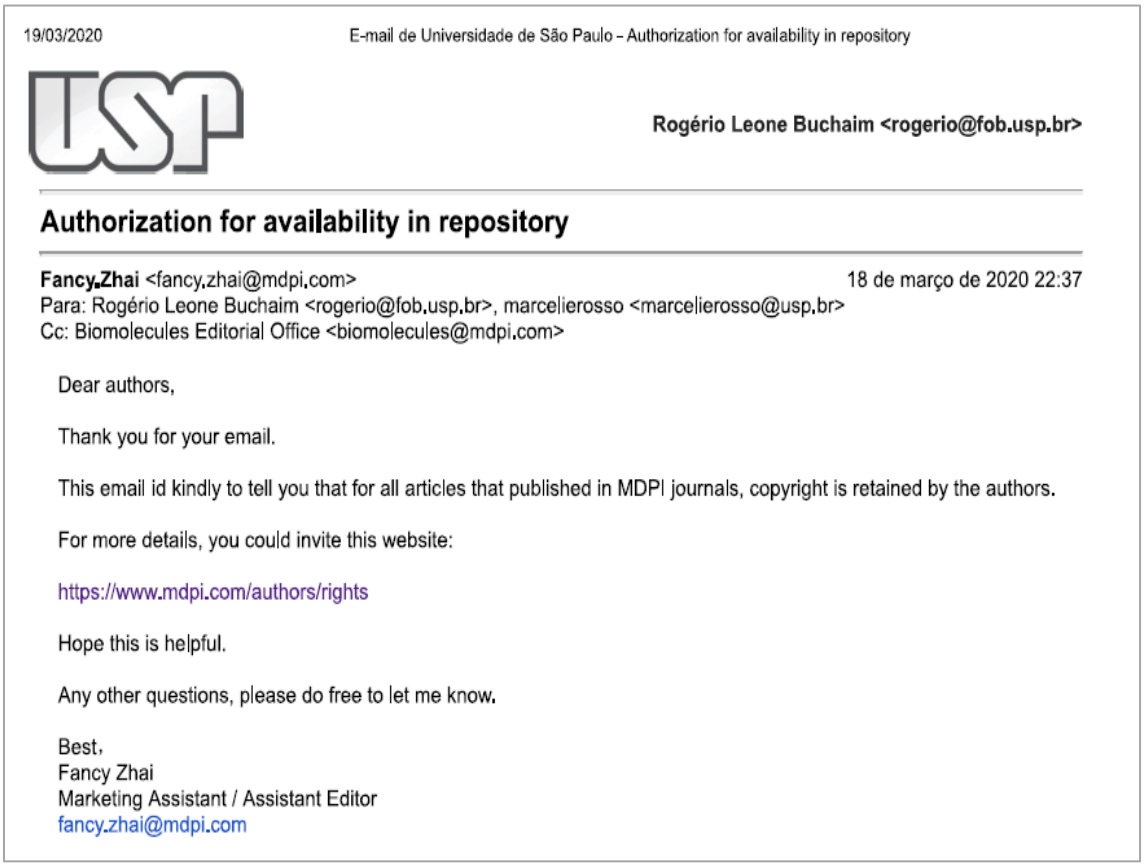
104/ 108, Sector-10, Pratap Nagar, Jaipur, Rajasthan, India, editor@ijaers.com
Website: www.ijaers.com

Annex 2: Authorization from the editor in chief of the journal – International Journal of Molecular Sciences.



19/03/2020 E-mail de Universidade de São Paulo – Authorization for availability in repository

Rogério Leone Buchaim <rogerio@fob.usp.br>

Authorization for availability in repository

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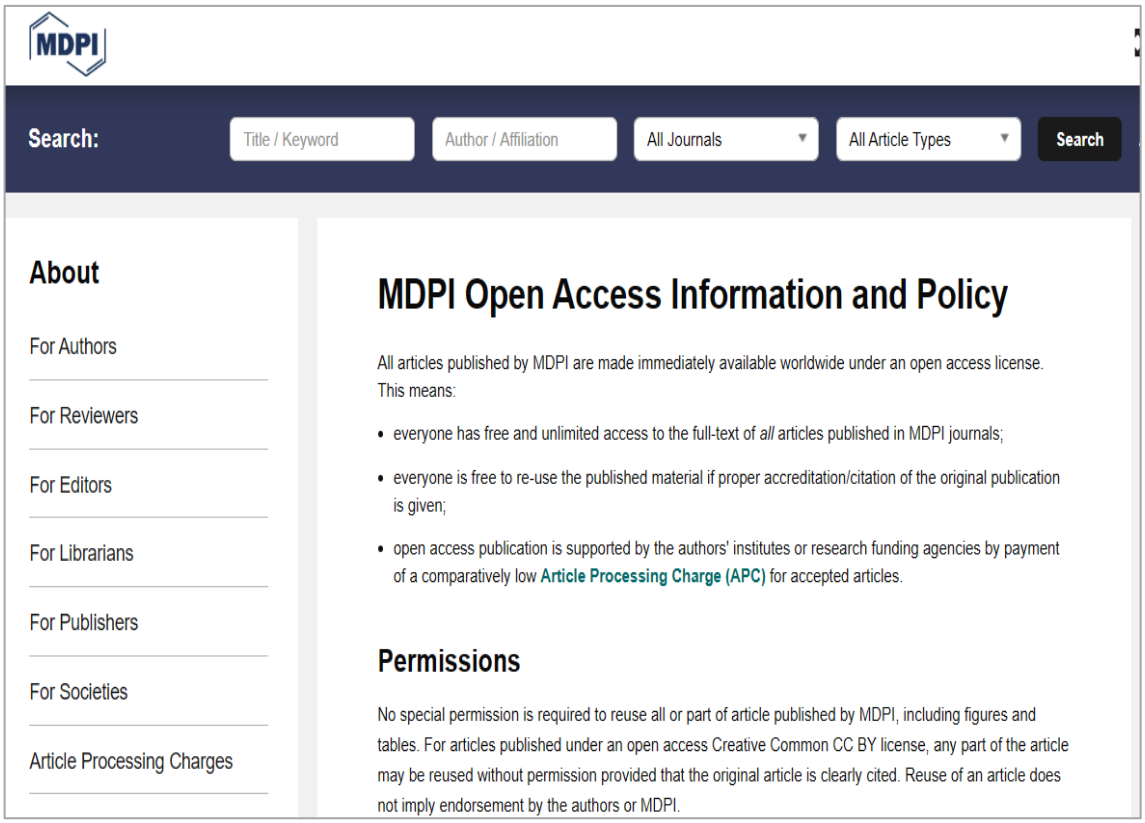
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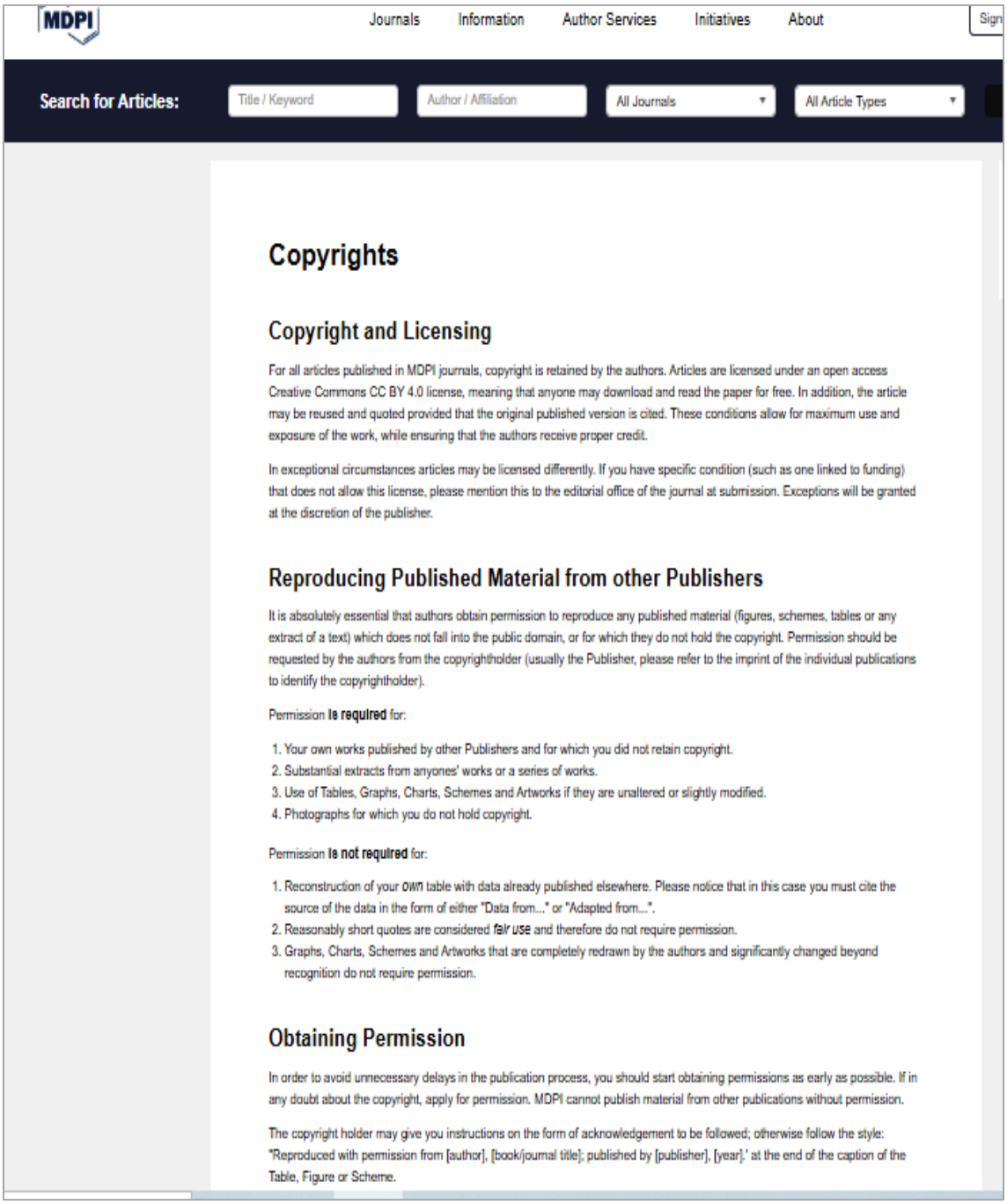

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Anenx 3: Approval of Animal Ethical Committee.

The title of the research project was changed due to the unavailability of the grafting material and will be mentioned in the final research report required by the Ethics Committee on the Use of Animals - CEUA.

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 019/2016

Bauru, 21 de outubro de 2016.

Senhor Professor,

Informamos que a proposta intitulada ***Influência da terapia por laser de baixa intensidade no processo de reparo de defeitos ósseos preenchidos por selante de fibrina homólogo ou heterólogo associados às células-tronco derivadas da polpa de dentes decíduos***, *registrada sob* ***CEEPA-Proc. Nº 019/2016,*** tendo Vossa Senhoria como Pesquisador Responsável, que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), 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 da Experimentação Animal (CONCEA), foi analisada e considerada APROVADA a sua execução nas dependências da FOB-USP, em reunião ordinária da Comissão de Ética no Ensino e Pesquisa em Animais (CEEPA), realizada no dia 21 de outubro de 2016.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização:	*Outubro/2016 a Outubro/2018*
Espécie/linhagem/raça:	*Rato heterogênico/ Wistar*
Nº de animais:	*N=56*
Peso/Idade	*270g/60 dias*
Sexo:	*Machos*
Origem:	Biotério da Universidade de São Paulo - Campus de Ribeirão Preto – SP

Esta CEEPA solicita que ao final da pesquisa seja enviado um Relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

Profª Drª Ana Paula Campanelli
Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

Prof. Dr. Jesus Carlos Andreo
Docente do Departamento de Ciências Biológicas

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