UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

MARCELIE PRISCILA DE OLIVEIRA ROSSO

Use of photobiomodulation therapy associated with bovine bone matrix and heterologous fibrin biopolymer in the process of bone defect reconstruction

Utilização da terapia por fotobiomodulação associada a matriz óssea bovina e ao biopolímero heterólogo de fibrina no processo de reconstrução de defeitos ósseos

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Orientador: Prof. Dr. Rogério Leone Buchaim

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ERRATA

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Albert Einstein

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“Amizades verdadeiras se baseiam numa visão comum – a de que nossas vidas são melhores porque certas pessoas fazem parte delas.”

Bradley Trevor Greive

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“Ensinar não é transferir conhecimento, mas criar as possibilidades para a sua própria produção ou a sua construção.”

Paulo Freire

“Se eu vi mais longe, foi por estar sobre ombros de gigantes.”

Isaac Newton

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"Se um dia tiver que escolher entre o mundo e o amor lembre-se: se escolher o mundo ficará sem o amor, mas se escolher o amor com ele você conquistará o mundo."

Albert Einstein

ABSTRACT

Use of photobiomodulation therapy associated with bovine bone matrix and heterologous fibrin biopolymer in the process of bone defect reconstruction

In view of the challenges encountered after a tissue loss, methods that help in the reconstruction of bone tissue arouse interest in health research. The interaction between photobiomodulation therapy (PBMT) and bioproducts creates new perspectives on the success of tissue repair, such as, for example, the heterologous fibrin biopolymer (HFB) that can be associated with lyophilized bovine bone matrix (BM) in bone defects. Objective: In article 1, the objective was to systematically review the literature regarding the interaction of PBMT with bovine bone and, in article 2, to evaluate the effect of PBMT on the reconstruction of tibial defects with lyophilized bovine bone associated or not with the heterologous fibrin biopolymer. Materials and methods: In article 1, PubMed / MEDLINE, Web of Science and Scopus databases selected articles resulting from the combination of the keywords: “Bovine bone AND low-level laser therapy”, “Bovine bone AND photobiomodulation therapy”, “Xenograft AND low-level laser therapy” and “Xenograft AND photobiomodulation therapy”. In article 2, thirty male Wistar rats (Rattus norvegicus) were randomly separated into three groups, of 10 animals each, which after anesthesia were submitted to a 2 mm non-critical tibial defect. Groups received the following treatments: Group 1: BM + PBMT, Group 2: BM + HFB and Group 3: BM + HFB + PBMT. Animals in Groups 1 and 3 were submitted to PBMT in the immediate postoperative period and every 48 hours until the day of euthanasia that occurred at 14 and 42 days. Results: In article 1, the search in the three databases retrieved 240 articles, 18 of which met the established inclusion criteria. In studies in research animals (17 articles), PBMT assists in osteoconduction related to biomaterials, formation of new bone, bone healing, expression of immunomarkers, increase in collagen fibers and reduction of local inflammation. A human study demonstrated that the association of PBMT with bovine bone was effective for periodontal regeneration. In article 2, histomorphometric analysis showed a statistical difference in the percentage of bone formation between groups 3 (BM + HB + PBMT) and 2 (BM + HFB) (26.4% ± 1.03% and 20.0% ± 1.87%, respectively) at 14 days and at 42 days (38.2% ± 1.59% and 31.6% ± 1.33%,

respectively) and at 42 days there was bone with mature characteristics, and organized connective tissue. Computed microtomography (µCT) demonstrated BM particles filling the defect and depositing new bone in the superficial region of the ruptured cortex. Conclusion: The association of PBMT with HFB and BM improves the process of reconstruction of bone defects. In addition, the HFB favors the manipulation, insertion and permanence in the defect of the xenogenic graft.

Keywords: Fibrin Tissue Adhesive. Biocompatible Materials. Bone Transplantation. Low-Level Light Therapy. Laser Therapy. Heterografts.

RESUMO

Diante dos desafios encontrados após uma perda tecidual, os métodos auxiliadores na reconstrução do tecido ósseo despertam interesse nas pesquisas em saúde. A interação entre a terapia por fotobiomodulação (PBMT) e bioprodutos criam novas perspectivas no sucesso do reparo tecidual como, por exemplo, o biopolímero heterólogo de fibrina (HFB) que pode ser associado com matriz óssea bovina liofilizada (BM) em defeitos ósseos. Objetivo: No artigo 1, o objetivo foi revisar sistematicamente a literatura acerca da interação da PBMT com osso bovino e, no artigo 2, avaliar o efeito da PBMT na reconstrução de defeitos em tíbias com osso bovino liofilizado associado ou não ao biopolímero heterólogo de fibrina. Materiais e métodos: No artigo 1, nos bancos de dados PubMed/MEDLINE, Web of Science e Scopus foram selecionados artigos resultantes da combinação das palavras-chaves: “Osso bovino E terapia por laser de baixo nível”, “Osso bovino E terapia por fotobiomodulação”, “Xenoenxerto E terapia por laser de baixo nível" e “Xenoenxerto E terapia por fotobiomodulação”. No artigo 2, trinta ratos machos Wistar (Rattus norvegicus) foram separados aleatoriamente em três grupos, de 10 animais cada, que após anestesia foram submetidos a um defeito tibial não crítico de 2 mm. Os grupos receberam os seguintes tratamentos: Grupo 1: BM + PBMT, Grupo 2: BM + HFB e Grupo 3: BM + HFB + PBMT. Os animais dos Grupos 1 e 3 foram submetidos à PBMT no pós-operatório imediato e a cada 48 horas até o dia da eutanásia que ocorreu aos 14 e 42 dias. Resultados: No artigo 1, a busca nos três bancos de dados recuperou 240 artigos, sendo que 18 atenderam aos critérios de inclusão estabelecidos. Nos estudos em animais de pesquisa (17 artigos), a PBMT auxilia na osteocondução relacionada aos biomateriais, formação de osso novo, cicatrização óssea, expressão de imunomarcadores, aumento de fibras colágenas e redução da inflamação local. Um estudo em humanos demonstrou que a associação de PBMT com osso bovino foi eficaz para a regeneração periodontal. No artigo 2, a análise por histomorfometria demonstrou diferença estatística na porcentagem de formação óssea entre os grupos 3 (BM + HB + PBMT) e 2 (BM + HFB) (26,4% ± 1,03% e 20,0% ± 1,87%, respectivamente) aos 14 dias e aos 42 dias (38,2% ± 1,59% e 31,6% ± 1,33%, respectivamente) e aos 42 dias houve presença de osso com características maduras e tecido conjuntivo organizado. A microtomografia computadorizada (µCT) d

demonstrou partículas de BM preenchendo o defeito e a deposição de novo osso na região superficial da cortical rompida. Conclusão: A associação de PBMT com HFB e BM melhoram o processo de reconstrução de defeitos ósseos. Além disso, o HFB favorece a manipulação, inserção e permanência no defeito do enxerto xenogênico.

Palavras-chave: Adesivo tecidual de fibrina. Materiais biocompatíveis. Transplante ósseo. Terapia por luz de baixa intensidade. Terapia por laser. Xenoenxertos.

SUMMARY

1 Introduction

1 **INTRODUCTION**

Tissue injuries, depending on their extent, can lead to numerous changes in the context of daily life such as functional, work, biopsychosocial, emotional and self-esteem implications. Consequently, they generate exorbitant expenses with public health, resulting in improvements in the scientific literature for cases of bone tissue reconstruction. The complex process of bone reconstruction by means of application of chemical stimuli such as biomaterials compounds, bone morphogenetic proteins (1), in addition to electromagnetic methods of physiotherapy such as ultrasound and low-level laser therapy are the subject in current perspective.

In view of the demand to assist in the bone reconstruction process, due to the aforementioned influences, the lesion may have on patients' lives; photobiomodulation has been growing in this context. The characteristics of directionality, coherence and monochromatic light beams, which provide a high energy density are the differential between lasers and other radiation sources, emerging as attractive for clinical applications (2,3). In addition to the effects already observed in the literature, the highlight of biomodulation with the use of light stands out due to its accessibility and low cost.

The association of low-intensity, athermic and non-destructive energy with cellular effects is supported by the relationship over the mitochondrial respiratory chain, more specifically in cytochrome C oxidase associated with tissue renewal (4,5), increasing metabolism and causing biostimulation or biomodulation. However, the interest on bone tissue emerges about the application of the laser as an influence on the reconstructive process, the literature highlights its potentiation on the time of bone repair, maturation and development, reduction of pain, edema and inflammation (6,7), vascular increase, of fibroblasts and calcium, activity of macrophages and pre-osteogenic cells (8), stimulus on osteoblastos (9), in addition to mechanical properties in fractures and osteointegration in implants (10).

Due to the search for increasingly effective means for bone reconstruction, laser therapy has also acted as an adjunct to grafts and biomaterials, these are a high point of tissue engineering in order to increase bone quality and quantity in strategic locations. Bone substitutes of synthetic or natural origin depend particularly on biocompatibility, osteoconductive and inductive effects, as well as mechanical support for the tissue to be reconstructed, since the objective must be to focus on the recovery of local functionality (11,12). As clinical material, the autologous graft is chosen for osteoinduction, osteoconduction and osteogenesis, however its quantity is limited (13). Thus, as an alternative, the xenograft is the main choice, already analyzed by the world literature, standing out in the question of not needing a second surgical bed (donor area) that increases the cost of the procedure, besides that when it is inorganic, it is highly osteoconductive and with slow degradation (14).

The demineralized bone matrix of bovine origin has its physical-chemical formula closely comparable to human bone, proving to be of high biocompatibility and osteoconductive capacity (13). Regarding graft porosity, which is linked to vascularization, multiplication and differentiation of osteoblasts and development of newly formed bone, the biomaterial used in this research with 42% porosity and already confirmed in previous studies its effectiveness (14,15).

In recent years, scientific research has applied the different types of bone grafts combined with other compounds, aiming to relate therapeutic methods that assist in the reconstructive process, such as the morphogenetic bone protein (BMP) (16), the collagen matrix, the platelet-rich plasma (17), anionic tendon (18) and fibrin sealant derived from human blood (19). All of these materials are characterized with their interactions in the tissue reconstructive process, with emphasis on the scaffold function for the new bone formation (13,20).

Fibrin sealants composed of fibrinogen and thrombin, act as adhesives and hemostatic agents, however they have a high cost and, in the case of homologous sealants, they may not be viable in emergency surgeries. The heterologous fibrin biopolymer (HFB) can be highlighted as an innovator, with applicability in soft tissues of animals such as nerves (21), cornea (22), uterus (23), in humans such as gingival grafts (24) and skin with venous ulcers (25). Composed of a fraction of gyroxin extracted from snake venom (*Crotalus durissus terrificus*) associated with a cryoprecipitate abundant in buffalo fibrinogen (*Bubalus bubalis*), in order to provide an alternative to conventional sutures, HFB showed itself with properties of a healing agent, sealer and with promising results, mainly due to biocompatibility and because they are heterologous components and, being of Brazilian manufacture presenting the possibility of cost reduction (26–28).

This study aimed to evaluate the impact of PBMT on bone reconstruction in rat tibiae, using bovine bone associated with heterologous fibrin biopolymer by means of computed microtomography and histomorphometric analyzes. In view of the need observed in the literature, a systematic review was also carried out on the association of PBMT and bovine bone matrix.

In view of the above and the objective of this experiment, the hypothesis formulated was that low-level laser photobiomodulation therapy may assist in bone reconstruction associated with HFB.

2 Articles

2 ARTICLES

The two articles that make up this doctoral thesis are formatted following the specific instructions for submission in each journal.

- Article 1: Photobiomodulation Therapy (PBMT) Applied in Bone Reconstructive Surgery Using Bovine Bone Grafts: A Systematic Review. (Published on journal Materials).

- Article 2: Photobiomodulation therapy associated with heterologous fibrin biopolymer and bovine bone matrix helps to reconstruct long bones. (Published on journal Biomolecules).

2.1 Article 1:

Photobiomodulation Therapy (PBMT) Applied in Bone Reconstructive Surgery Using Bovine Bone Grafts: A Systematic Review. (Appendix 1 and Annex 1).

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materials

MDPI

Review

Photobiomodulation Therapy (PBMT) Applied in Bone Reconstructive Surgery Using Bovine Bone Grafts: A Systematic Review

Marcelie Priscila de Oliveira Rosso [1], Daniela Vieira Buchaim [2,3], Karina Torres Pomini [1], Bruna Botteon Della Coletta [1], Carlos Henrique Bertoni Reis [2], João Paulo Galletti Pilon [2], Getúlio Duarte Júnior [2] and Rogério Leone Buchaim[1,2,*]

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Abstract: The use of low-level laser therapy (LLLT) with biomodulatory effects on biological tissues, currently called photobiomodulation therapy (PBMT), assists in healing and reduces inflammation. The application of biomaterials has emerged in bone reconstructive surgery, especially the use of bovine bone due to its biocompatibility. Due to the many benefits related to the use of PBMT and bovine bones, the aim of this research was to review the literature to verify the relationship between PBMT and the application of bovine bone in bone reconstruction surgeries. We chose the PubMed/MEDLINE, Web of Science, and Scopus databases for the search by matching the keywords: "Bovine bone AND low-level laser therapy", "Bovine bone AND photobiomodulation therapy", "Xenograft AND low-level laser therapy", and "Xenograft AND photobiomodulation therapy". The initial search of the three databases retrieved 240 articles, 18 of which met all inclusion criteria. In the studies concerning animals (17 in total), there was evidence of PBMT assisting in biomaterial-related conduction, formation of new bone, bone healing, immunomarker expression, increasing collagen fibers, and local inflammation reduction. However, the results disagreed with regard to the resorption of biomaterial particles. The only human study showed that PBMT with bovine bone was effective for periodontal regeneration. It was concluded that PBMT assists the process in bone reconstruction when associated with bovine bone, despite divergences between applied protocols.

Keywords: bone repair; bovine bone; low-level laser therapy; photobiomodulation therapy; tissue regeneration; xenograft

1. Introduction

Low-level laser therapy (LLLT) has been of interest to the scientific community since 1967, when Mester et al. [1] reported its effects on hair growth in rats. It was later verified that this therapy not only stimulated cellular components, but also modulated them, establishing photobiomodulation therapy

(PBMT). Further, regenerative medicine has emerged in recent decades to develop adjuvant and assistive means in pathological processes, highlighting PBMT in relation to the anti-inflammatory, anti-allergic, healing and stimulating effects of tissue growth factors [2–5].

PBMT features electromagnetic energy technology with a wavelength spectrum of 600–1100 nm, with low energy density from a constant beam (0.04–60 J/cm²). Laser light sources include helium–neon (HeNe) and gallium–aluminum arsenide (GaAlAs), as these sources have excellent tissue penetration [4,6]. The therapeutic effects of PBMT are based on photochemical, photoelectric and photoenergetic reactions that affect cells by altering their metabolic functions. The modulatory effect is mainly related to cytochrome C oxidase, which, via photon absorption with mitochondrial reactions, generates increased adenosine triphosphate (ATP) [7,8]. The literature points to the effects of PBMT on tissues by its modulation of biological processes for cell differentiation and proliferation [9]. Its effects are related to the repair of muscle [10], nerves [11], bone [12], and burn injuries [13], besides the reduction of inflammatory cytokines and bacterial load due to photosensitive agents and biostimulation of blood vessels [10,13–15].

Most experimental and clinical studies describe that PBMT aids in the process of tissue regeneration, demonstrating biological modulatory effects on cell differentiation [9,16,17]. Photobiomodulation of bone tissue seems to increase the results of fracture repair [18], periodontal tissue [19], implant osseointegration [20] and bone reconstruction with or without biomaterials [15,21–25]. Its application in clinical practice with the purpose of assisting healing after bone graft reconstruction surgery [26] is still poorly described in the scientific literature.

Bone lesions with tissue loss can lead to changes in quality of life, especially when it concerns the face [27]. Patients requiring reconstructive surgery typically describe functional loss and physical, emotional, social and labor disturbances, as well as a financial change associated with these challenges [28]. The physiological bone remodeling process is naturally coordinated; however, imbalances may occur between bone deposition and removal. In extensive tissue defects, repair can become a challenge, requiring the use of bone grafts, implants or biomaterials. At this time, tissue engineering comes into play in helping the development of components that can lead to or assist in the reconstruction of lost tissue [29–31].

Bone graft material, regardless of its origin (autografts, allografts, alloplastic materials or xenografts), must have the biological, physical and chemical properties necessary for the tissue repair process. Emphasis is given to those materials that have osteointegration, osteoinduction, osteoconduction and osteogenesis capacities; however, only autologous material is capable of covering all four of these properties [32,33].

In instances of large bone loss, the need for a graft is imminent. In such a scenario, autologous bone is the first choice, but there are difficulties associated with potential morbidity of the donor site. In these cases, however, grafts tend to be absorbed before osteogenesis is complete [34]. The literature cites as necessary in the reconstruction of bone defects three simultaneous conditions: (i) osteoconductive properties; (ii) inductive properties; and (iii) the presence of bone-forming cells [35]. As an alternative material, bovine bone graft [15,20,36–39] is the most widely used due to its biocompatibility characteristics, as indicated in reconstructive areas related to traumatology, cranio-maxillary surgery, facial prosthetic rehabilitation, skeletal aging and esthetic aging [28]. Physical methods, such as low-intensity ultrasound (LIPUS) [40] and photobiomodulation therapy (PBMT), have the potential to improve the bone reconstruction process, acting or not in combination with bone grafts.

However, gaps still exist in explaining the mechanisms of PBMT and its relationship with the widely-used bovine bone. In this context, this systematic review research was based on the PICO [41,42] strategy, P: animals or humans with bone defects, I: The use of bovine bone as a scaffold and PBMT for bone defect repair, C: comparison to non-use of these components, and O: effect on bone repair. This PICO strategy was used to verify the relationship between PBMT and the use of bovine bone in bone reconstruction surgeries in different animals, based on the results presented by scientific studies already published in the PubMed/MEDLINE, Web of Science and Scopus databases.

2. Materials and Methods

This systematic review was conducted in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist, as well as previously published systematic reviews [43,44].

For this study, we searched three databases, PubMed/MEDLINE, Web of Science, and Scopus, during September 2019, using the following terms as keywords: "Bovine bone AND low level laser therapy", "Bovine bone AND photobiomodulation therapy", "Xenograft AND low-level laser therapy" and "Xenograft AND photobiomodulation therapy", with no restriction on publication time.

The search results were initially screened by title and then abstract to sort articles into included and excluded folders. Eligibility criteria were applied impartially by the authors regardless of the results presented by each article.

Eligibility criteria:

Inclusion criteria were:

- Use of bovine bone as a scaffold and PBMT in bone reconstructions;
- Human or animal studies;
- Publications in the English language only and which allowed full access to the text.
- Each included article should present data regarding: wavelength, output power, energy density, application protocol (points, frequency and days).

Exclusion criteria were:

- Duplicate articles;
- Excluded because title was not related to aim;
- Did not use bovine bone;
- Use of other languages (not English);
- No access;
- Literature review;
- Data absence: wavelength (nm), output power (mW); energy density (J/cm^2); quantity of radiation.

First, we verified the works that presented titles and abstracts that related to the theme of the initial research, using the two variables: bovine bone as a scaffold and PBMT. The next step was to evaluate and restrict those articles that used bovine bone as a scaffold in animals or humans. The methodology, results and relevance were considered to list the selection of articles.

Analysis and integration of reflective and consistent texts on the subject were performed. The search scheme is presented in Figure 1, according to the PRISMA flow diagram [42,44].

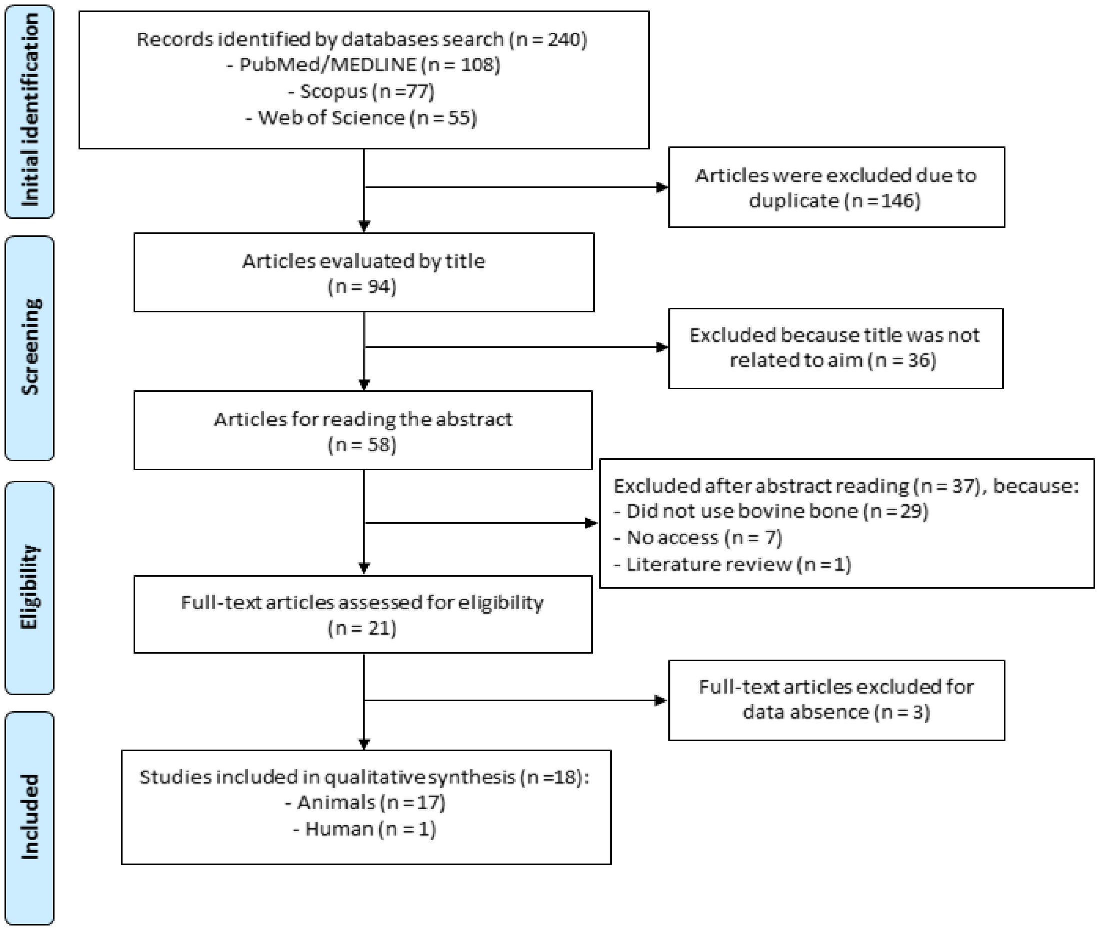

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram delineating the search performed in the PubMed/MEDLINE, Web of Science and Scopus databases.

3. Results

3.1. Inclusion of Studies, Quality of Studies, and Test Subjects

The initial search retrieved 240 articles from the three databases, after which 146 articles were excluded because they were duplicated and 36 were excluded due to their titles being unrelated to the theme. The abstracts of 58 articles were read, resulting in the further exclusion of 37 papers as they either did not use bovine bone, did not provide access or were a literature review article, and therefore did not meet the inclusion criteria. This left 21 articles elected for full analysis. After full reading of these 21 articles, three more papers were deleted due to incomplete data. Therefore, in the end, 18 articles related to the theme were included, 17 of which were related to animals and only 1 to humans.

Tables 1 and 2 present the main details of the selected animal and human studies, respectively.

Table 1. Summary of the main photobiomodulation therapy (PBMT) parameters used in animals studies.

Authors	Type of Laser (Manufacturer)	Wavelength (nm)/Spot Beam (cm²)	Output Power (mW)	Energy Density (J/cm²)	Quantity of Radiation	Bovine Bone	Therapeutic Variables	Irradiation Site (Defect)	Evaluation Time	Outcome Measures
Luca et al., 2019 [45]	GaAlAs (IRRADIA Mid-Laser Stockholm, Suécia)	808/-	450 Frequency of 3800 Hz	2/1.9 J per session	4 points around the defect plus 1 central point. 17 s/point. Started IP, repeated every 48 h, until the established sacrifice day.	BBG	CM	Rat calvaria (5 mm Ø)	14, 21 and 30 days post-surgery.	By CMS/SS-OCT quantitative analysis in 30 days, BBG + PBMT with higher volume bone formation (27.11%, $p \leq 0.05$). Histological analysis (by MT) shows new bone around the particles, osteoid lamellae delimited by osteoblasts.
Pomini et al., 2019 [12]	GaAlAs (Laserpulse IBRAMED, Amparo, SP, Brazil)	830/0.11	30	6	4 points in contact area, 24s/point. Started IP, repeated every 48 h, three times a week until euthanasia.	DBBm	FS	Rat calvaria (8 mm Ø)	14 and 42 days post-surgery.	Histomorphometric analysis quantified higher bone volume density between both periods (5.6 to 10.64, $p<0.05$) for the FS + DBBm + PBMT group and presence of the particles seen in the μCT. In the histological analysis (HE), the new bone started from the defect edges and there was more evidence of trabecular formation in the irradiated group FS + DBBm. Association of PBMT with xenograft and fibrin sealant had beneficial effects on bone repair.
Gerbi et al., 2018 [46]	GaAlAs (Thera Lase Surgery; DMC	830/0.28	40	4	4 points applied in contact around the defect and was repeated every	OmB	BMP + collagen Binder + bovine biological membrane	Rat femur (3mm Ø)	15 and 30 days post-surgery.	By histomorphometric analysis the OmB + PBMT group exhibited a larger area of newly formed

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	Equipamentos, São Carlos, SP, Brazil)				other day for 15 days, total of 7 sessions.					bone tissue (21.11%, p<0.05), demonstrating the efficacy of bone photobiomodulation in 30 days. Picrosirus and HE analysis show trabecular bone and complete cortical repair.
de Oliveira et al., 2018 [15]	GaAlAs (Therapy XT, DMC São Carlos-SP, Brazil)	808/0.02	100	354/ point Total energy 28 J	4 points in contact area, 10s/point. Started IP, repeated every 48 h for 13 days, 7 sessions in total.	DBB	HA/βTCP + Teflo capsule, peripheral ring	Rat mandibular branch (Four holes of 0.5 mm Ø were made 6 mm from each other to form the edges of a square, the region was scarified).	30, 60 and 90 days post-surgery	Quantitative analysis by μCT: 90 days, higher PBMT effect on the amount of mineralized tissue associated with DBB (±63%, p≤0.05) compared to non-biostimulated groups. Histomorphometry showed greater amount of new bone in the DBB + PBMT group (±25%, p≤0.05). Lower amount of biomaterial in the PBMT, DBB (±30%, p≤0.05). Immunohistochemi-cal analysis showed increased ALP in the irradiated DBB (45%, p<0.05) group.
Bosco et al., 2016 [47]	GaAlAs (Bio Wave; Kondortech Equipment Ltd., São Carlos-SP, Brazil)	660/0.07	35	30.85/ point total energy of 19.44 J	8 points in contact area plus 1 central point in the scanning procedure. 72	IBBG	-	Rat calvaria (10 mm Ø)	30 and 60 days post-surgery	Histomorphometric analysis showed that the IBBG/PBMT group had the largest newly formed bone area (7.39 to 9.44, p <0.05), and histological

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					s/point, 1 application IP.					analysis (HE) showed a large osteoid matrix area, osteoblasts and newly formed bone around the particles at 60 days. No statistical difference for particle resorption at 30 (21.98 ± 4.10) and 60 (27.20 ± 6.39) days. PBMT can improve bone formation, but did not speed up the resorption of biomaterial particles.
Cunha et al., 2014 [22]	GaAlAs (Thera Lase DMC São Carlos-SP, Brazil)	780/0.05	100	210 6J per point	4 points in contact area plus 1 central point, 60 s/point. Application IP.	IBBG	-	Rat calvaria (5 mm Ø)	30 days post-surgery	Histomorphometric analysis showed that the group (IBBG + PBMT) presented the largest area of bone neoformation with 48.57% ($p < 0.05$) and smallest area of remaining particles (16.74%, $p < 0.05$). In the histological analysis (HE) presence of osteoid matrix with bone formation leading to the center of the defect, and parallel collagen fibers around the particles. PBMT benefited bone healing and particle resorption.

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Havlucu et al., 2014 [37]	LED OsseoPulse (Biolux Research Ltd, Vancouver, Canada)	618/-	20 mW/cm^2	24 total/ session	20 min of total application in contact with the area. Started 24 h after surgery and followed in this interval for 7, 14 and 21 days.	DBB	-	Rat femur (two defects of 3 mm Ø each)	8, 15 and 22 days post-surgery	By histomorphometric analysis in the DBB + PBMT group, all animals presented new bone tissue average >60% ($p<0.01$), less inflammation (<30%, $p<0.01$) and remaining particles less than 30%, $p<0.05$) at 3 weeks. Histologically (HE), newly formed bone trabeculae with active osteoblasts were around the particles and reconstructed the defect.
Rasouli Ghahroudi et al., 2014 [33]	Diode laser (Giga com, China)	810/-	300	4	Applied around the surgical area IP and followed by ten applications (every other day) for the next 20 days.	IBB	-	Rabbit calvaria (Four defects 8 mm Ø each)	28 and 56 days post-surgery	A histomorphometric group of DBB + PBMT group had the highest mean of new bone formation, 41.83 and 47% at weeks 4 and 8, respectively, with statistically significant differences ($p<0.05$) and an inflammation index <25% in 66.7% of the animals. Coinciding with the bone tissue presented in histology (HE), altering the auxiliary PBMT in bone healing.

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Lopes et al., 2010 [18]	Diode Laser Unit, (Kondortech, São Carlos-SP, Brazil)	790/0.5	40	4/point	4 points applied transcutaneously around the area. Started IP, repeated every 48 h, per 15 days	LOBB	IRF + Biomaterial (LOBB + Collagen + BMP + Decalcified cortical osseous membrane)	Rabbit tibia (complete bone fracture, 5 mm)	30 days post-surgery	Raman spectroscopy demonstrated that biomaterial associated PBMT was effective in improving bone healing due to increased CHA levels. Highest group average IRF + biomaterial + PBMT (9316%, p=0.05). PBMT was effective in improving bone healing.
Kim et al., 2009 [20]	GaAlAs (500DPSS, LVI Technology, Seoul, Korea)	808/0.01	96 power density of 830 mW/cm^2	8.3/point	3 points applied in contact, 10 s/point. Started IP, repeated every 24 h, per 7 days.	DBB	-	Rat calvaria (2.7 mm Ø)	7, 14 and 21 days post-surgery.	The results of immunohistochemical analysis showed that RANKL expression (>50%, p=0.199), OPG expression (>75%, p=0.035) and RANK expression (<50%, p=0.020) in the experimental group had a significant increase from 7 to 21 days. At 21 days of expression in osteoid formation and bone density in histology (Goldner's trichrome).
Gerbi et al., 2008 [48]	Thera Lase, DMC Equipamentos, São Carlos, SP, Brazil	830/0.28	40	4/point	4 points applied in contact around the defect, begun immediately after suturing and was repeated every	OLDBB	Biomaterial (OLDBB + collagen gel + BMP)	Rat femur (2 mm Ø)	15, 21 and 30 days post-surgery.	Qualitative analysis (HE and Sirius red) showed an increased collagen fibers (at 15 and 21 days) and amount of well-organized bone trabeculae at 30 days in laser irradiated

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					other day, for 15 days.					animals. PBMT associated with biomaterial showed positive biomodulatory. effects.
Márquez Martínez et al., 2008 [49]	Thera Lase, DMC Equipamentos/ São Carlos, SP, Brazil,	830/0.28	40	4/point	4 points applied in contact around the defect and was repeated every other day, for 2 weeks.	OBB	-	Rat femur (3 mm² cavity)	15, 21 and 30 days post-surgery.	Qualitative analysis (HE and Picrosirus) at 30 days—higher amount of collagen fibers, evident osteoblastic activity and mature bone formation, with complete repair of the defect in group OBB + PBMT.
Pinheiro et al., 2008 [50]	DMC Equipamentos, São Carlos, SP, Brazil	830/0.28	40	4/point	4 points applied in contact around the defect and was repeated every other day, for 15 days.	OLDBB	Biomaterials (Collagen gel + BMP + bone resorbable decalcified cortical bone membrane)	Rat femur (2 mm² cavity)	15, 21 and 30 days post-surgery.	Qualitative analysis (HE and Sirus red) showed that biomaterials + membrane-associated PBMT developed collagen fibers, accelerated cortical bone repair, and developed the Haversian system.
Marquez de Martinez Gerbi et al., 2003 [51]	DMC Equipamentos, São Carlos, SP, Brazil	830/0.28	40	4/point	4 points applied in contact around the defect and was repeated every other day, for 15 days, total of 7 sessions.	OBB	Decalcified cortical osseous membrane	Rat femur (3mm² cavity)	15, 21 and 30 days post-surgery.	Qualitative histological analysis (HE and Picrosirus) showed positive effect of PBMT at 15 days with evident amounts of collagen fibers, osteoblastic activity and evident bone neoformation and complete repair of the defect. Positive effects of PBMT independent of

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										organic bone or membrane.
de Assis Limeira Júnior et al., 2003 [52]	Thera Lase, DMC Equipamentos, São Carlos, SP, Brazil	830/0.28	40	4/point	4 points applied in contact around the defect and was repeated every other day for 15 days, total of 7 sessions.	IBB	Decalcified bovine cortical osseous membrane	Rat femur ($3mm^2$ cavity)	15, 21 and 30 days post-surgery.	Qualitative analysis (HE and Picrosirius) showed that the level of bone neoformation did not change much until day 30 in most groups except for the PBMT + IBB + membrane group, where bone neoformation was most evident between days 21 and 30, with dense and well organized neoformed bone trabeculae and the conclusion of cortical repair.
Pinheiro et al., 2003 [53]	Thera Lase, DMC Equipamentos, São Carlos, SP, Brazil	830/0.28	40	4/point	4 points applied transcutaneouslyS tarted IP, repeated every 48 h, total of 7 sessions.	IBB	-	Rat femur ($3mm^2$ cavity)	15, 21 and 30 days post-surgery.	Histological qualitative analysis (HE and Picrosirius) showed that IBB + PBMT at 21 days obtained increased amount of bone neoformation and collagen fibers around the graft. At 30 days still presence of dense collagen fiber graft. PBMT had beneficial effects associated with inorganic bovine bone.

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Authors	Type of Laser (Manufacturer)	Wavelength (nm)/Spot Beam (cm²)	Output Power (mW)	Energy Density (J/cm²)	Quantity of Radiation	Bovine Bone	Therapeutic Variables	Irradiation Site (Defect)	Evaluation Time	Outcome Measures
Pinheiro et al., 2003 [54]	Thera Lase, DMC Equipamentos, São Carlos, SP Brazil	830/0.28	40	4	4 points applied transcutaneously Started IP, repeated every 48 h, total of 7 sessions.	IBB	Decalcified cortical osseous membrane	Rat femur (3mm² cavity)	15, 21 and 30 days post-surgery.	Histological qualitative analysis (HE and Picrosirius) showed that at 30 days IBB + membrane + PBMT there was more pronounced, well-organized bone formation with dense trabeculae around the graft particles, the cortical repair was complete. All groups irradiated with more collagen fibers. PBMT accelerated bone repair.

Abbreviations: Immediate postoperative (IP); Bovine bone graft (BBG); Collagen membrane (CM); Diameter (Ø); Complex master slave enhanced swept source optical coherence tomography imaging instrument (CMS/SS-OCT); Masson trichrome (MT); Demineralized bovine bone matrix (DBBm); Fibrin sealant (FS); Microtomographic (μCT); Organic matrix bovine (OmB); Bone morphogenetic proteins (BMP); Deproteinized bovine bone (DBB); Hydroxyapatite/β-tricalcium phosphate (HA/βTCP); Alkaline phosphatase (ALP); Inorganic bovine bone graft (IBBG); Inorganic bovine bone (IBB); Hematoxylin and Eosin (HE); Lyophilized organic bovine bone (LOBB); Calcium hydroxyapatite (CHA); Internal Rigid Fixation (IRF); Receptor activator of nuclear factor-κB ligand (RANKL); Osteoprotegerin (OPG); Receptor activator of nuclear factor –κB (RANK); Organic lyophilized decalcified bovine bone (OLDBB); Organic Bovine Bone (OBB).

Table 2. Summary of the main PBMT parameters used in the human study.

Authors	Type of Laser (Manufacturer)	Wavelength (nm)/Spot Beam (cm²)	Output Power (mW)	Energy Density (J/cm²)	Quantity of Radiation	Bovine Bone	Irradiation Site	Evaluation Time	Outcome Measures
Bhardwaj, 2016 [36]	GaAlAs	810/-	100	4/point	5 min in contact with the internal margins of the flap and then 10 min without contact on the defect. Application for 5 days consecutively (outer surfaces of buccal and lingual flaps)	DBM	Treatment of intraosseous defects. Alveolar bone between 44 and 45.	30, 60 and 90 days post-surgery	By radiological measurement PBMT + DBM showed good results in clinical insertion level (CAL) gain of 4 mm, linear bone gain of 2.5 mm, bone filling of 37% and reduction of defect angle from 68° to 32°, showing a positive treatment result. Safe treatment to approach periodontal regeneration.

Abbreviations: Demineralized bone matrix (DBM).

Evaluating the 17 articles that involved animal experiments, the total population of test subjects was 663. This total population was made up of 27 rabbits and 636 rats, divided into control groups with a total of 157 animals and intervention groups with 506 animals. The control group animals were always characterized as "empty cavity" or "clot", while the intervention groups contained animals that underwent treatment. Nine studies used male animals [12,15,18,20,22,33,37,46,47] and seven used male and female animals [48–54], while only one study did not describe the gender of the subjects [45].

The periods chosen for analysis ranged from a minimum of 7 days [20] to a maximum of 90 days [15]. There appeared to be a preference seems for studies conducted up to 30 days, with 13 articles falling into this category [18,20,22,37,45,46,48–54], while four articles [12,15,33,47] evaluated results after this period.

Considering all the articles included in this review, the application of PBMT in bone lesions was verified in rats in 15 articles, five of which involved the calvaria [12,20,22,45,47], nine the femur [37,46,48–54] and one the mandibular branch [15]. The two articles employing rabbits involved the calvaria [33] and tibia [18]. The only article in humans [36] was on the alveolar bone due to periodontal disease. The use of bovine bone in its inorganic phase was observed in 10 studies [12,15,20,22,33,37,47,52–54] versus six using the organic phase [18,46,48–51]; only one study [45] did not offer a distinction. Bovine bone was associated with another component in 11 articles: fibrin sealant [12]; bone morphogenic proteins (BMP), collagen binder and bovine biological membrane [46,50]; hydroxyapatite/β-tricalcium phosphate (HA/βTCP) [15]; internal rigid fixation (IRF), BMP, collagen bone and decalcified cortical osseous membrane [18]; BMP and collagen gel [48]; decalcified cortical osseous membrane [51,52,54]; and collagen membrane [45].

The wavelength parameter employed in the studies covered a wide range of values, from 618 to 830 nm. This included one study for each of 618 nm [37], 660 nm [47], 780 nm [22] and 790 nm [18], three studies with 808 nm [15,20,45], two studies with 810 nm [33,36], and nine studies with 830 nm [12,46,48–54], as shown in Figure 2.

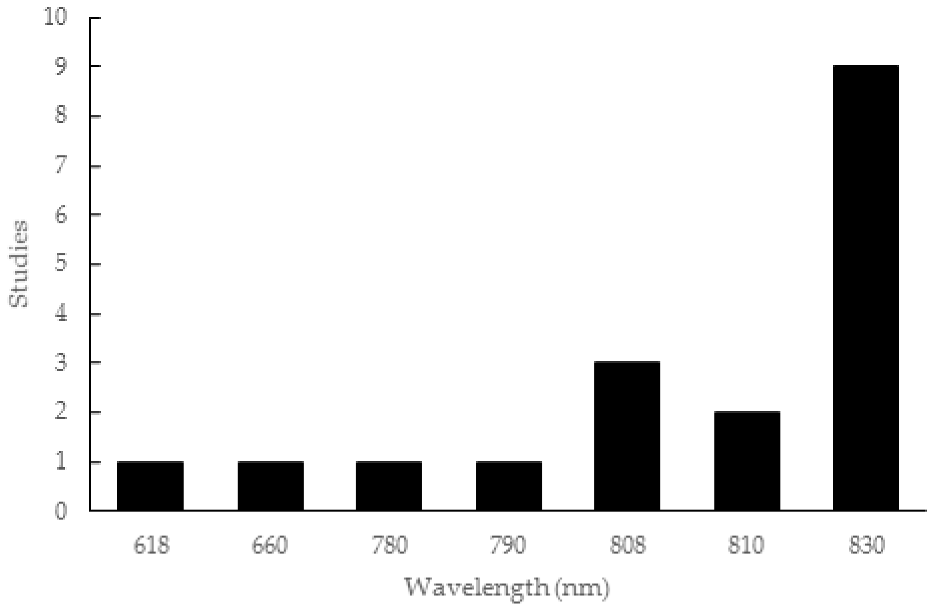

Figure 2. Wavelength parameters used in the articles included in this review.

Regarding the type of laser used in the studies, eight studies employed GaAlAs lasers (44.44%), one study cites the use of a light-emitting diode (LED) (5.55%), two specified the use of a diode laser (11.11%) and 7 researches did not mention the type of laser (38.88%). The seven studies that did not mention the type of laser used describe the application of the 830nm wavelength, which corresponds to the infrared range (Figure 3).

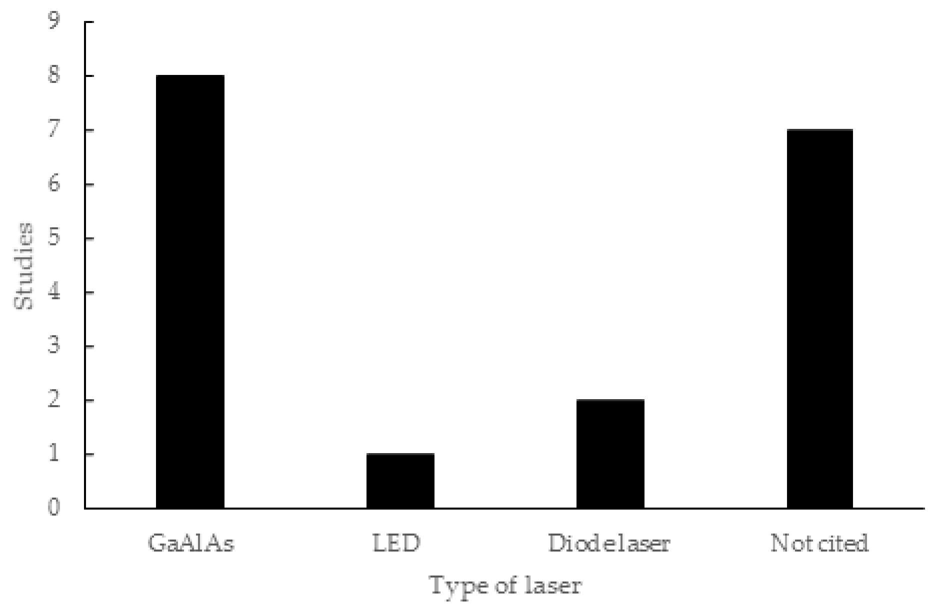

Figure 3. Laser types used in the articles included in this review.

The energy density employed in the studies ranged from 2 to 354 J/cm², with one study only citing the total energy (24 J/cm²) without specifying the energy per point. Eleven studies used 4 J/cm² and two used 6 J/cm², while energy densities of 8.3 J/cm², 30.85 J/cm² and 354 J/cm² were applied in one study each (Figure 4).

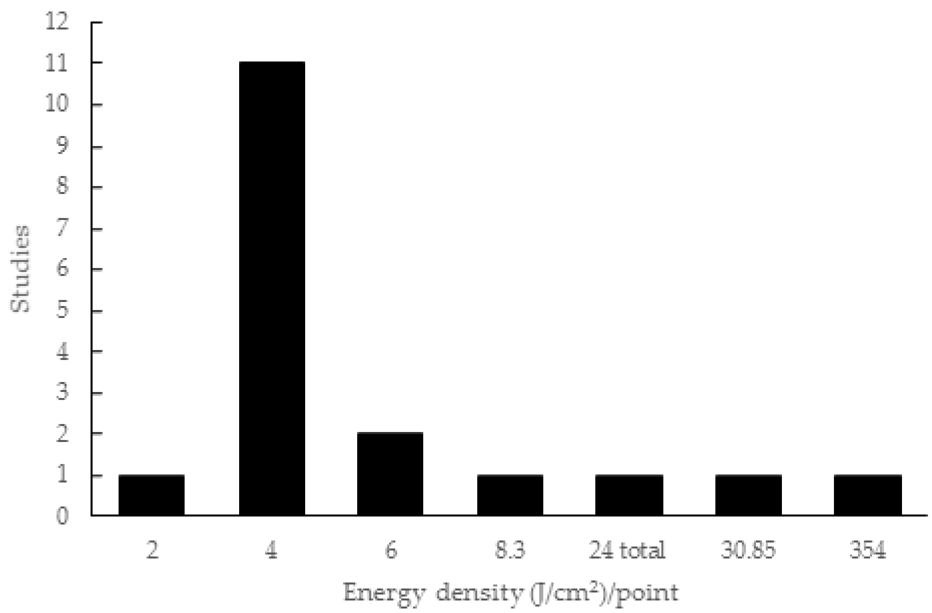

Figure 4. Energy density parameters used in the articles included in this review.

3.2. Outcome Measures Used in the Included Studies

Table 3 presents the outcome measures, characteristics of the test subjects, and results obtained from the studies included in this review. Ten studies evaluated the primary outcome measure of bone density using four major methods: μCT, histological analysis of percent volume density of bone (v/v), plain X-rays and the multimodal CMS/SS OCT system. Five studies evaluated the secondary outcome measure of expression of markers, most commonly examining expression of receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG) and receptor activator of nuclear factor-κB (RANK), through histopathological analysis, inflammatory process detection and Raman spectroscopy, and measurement of hydroxyapatite deposition.

Table 3. Data from included studies regarding outcome measures, subject attributes, and results.

Authors	Quantitative Analyzis	Measurements Results
Luca et al., 2019 [45]	CMS/SS-OCT	Bone volume formation: 27.11%
Pomini et al., 2019 [12]	Histomorphometric	Bone volume density: 10.64%
Gerbi et al., 2018 [46]	Histomorphometric	Bone volume density: 21.11%
de Oliveira et al., 2018 [15]	Histomorphometric μCT Immunohistochemistry	Bone volume density: ±25% Mineralized tissue: ±63% ALP (45%)
Bosco et al., 2016 [47]	Histomorphometric	Bone volume density: 9.44%
Cunha et al., 2014 [22]	Histomorphometric	Bone volume density: 48.57%
Havlucu et al., 2014 [37]	Histomorphometric Histopathological	Bone volume density: >60% Inflammation: <30%
Ghahroudi et al., 2014 [33]	Histomorphometric Histopathological	Bone volume density: 47% Inflammation: <30%
Lopes et al., 2010 [18]	Raman spectroscopy	CHA level: 9316%
Kim et al., 2009 [20]	Immunohistochemistry	RANKL (>50%), OPG (>75%), RANK (<50%)
Bhardwaj, 2016 [36]	Radiological for CAL	Linear bone gain: 2.5 mm and reduction of defect angle: 32°

Abbreviations: Complex master slave enhanced swept source optical coherence tomography imaging instrument (CMS/SS-OCT); Microtomographic (μCT); Alkaline phosphatase (ALP); Receptor activator of nuclear factor-κB ligand (RANKL); Osteoprotegerin (OPG); Receptor activator of nuclear factor-κB (RANK); Calcium hydroxyapatite (CHA); Clinical attachment level (CAL).

4. Discussion

In recent decades, there has been a significant increase in the incidence of craniomaxillofacial and orthopedic disorders, although this has been simultaneous with remarkable progress in the development of biomaterials for reconstruction of lost bone tissue [55].

However, even though there is a wide variety of bone substitutes with satisfactory bone-filling results, histological evidence and biological behavior have only been reported for bovine bone derivatives. Thus, these xenografts have transformed reconstructive surgery and significantly improved clinical outcomes [56].

In addition, noninvasive, adjuvant methods in tissue regeneration have been associated with grafting techniques in an attempt to overcome some practical limits and further improve the repair results of defects filled with biomaterial. Given this context, we performed a review of the scientific literature in order to elucidate the relationship of PBMT with bovine bone when the latter is used as scaffolding for bone reconstruction.

Scientific research related to tissue engineering aims to investigate the process of bone reconstruction using scaffolds, as these are necessary as an auxiliary means for growth of new bone tissue [57]. Efforts to minimize complications and the time needed to heal by improving the process and enhancing biocompatibility has led to the emergence of PBMT-associated biomaterial application in the world literature [12,31]. Bovine bone is listed as the most frequently used type of graft in the literature for the reconstructive bone process [15,20,36–39].

Rats accounted for 95.92% of the total animals used in the articles evaluated, showing a preference for these animals in empirical study. One advantage of using rats is their easy handling due to their size, and they are generally chosen for preclinical studies in bone reconstruction biomaterial tests—being the main choice in in vivo studies in regenerative processes [58,59].

The use of male animals in nine of the studies examined suggests a preference of gender for test subjects. This decision is supported in the literature, as it avoids the possible influence of female inhibitory hormones in relation to bone tissue, in addition to the lower risk of fracture and greater bone mass [60,61].

Concerning the use of bovine bone, a preference for its inorganic phase (10 papers) [12,15,20,22,33,37,47,52–54] was identified, although no differences in the process of bone healing when associated with a laser were reported, while six studies [18,46,48–51] used bone with an organic matrix. Bovine bone matrix has been widely used as a heterogeneous graft in orthopedic surgeries and craniofacial reconstructive procedures with satisfactory osteoconductive properties [32–34]. However, previous studies have shown differences between the effectiveness of inorganic and organic bovine matrices in the bone repair process. Some researches advocate for the use of inorganic material due to the absence of proteins and cells, which decreases the risk of immunogenic reactions. Further, this material provides a large amount of hydroxyapatite, which is a major component in normal bones [62]. Other researches elect to use organic material for the permanence of its protein scaffold, mainly comprised of type I collagen, which may initially favor formation of the extracellular matrix [15,63].

During this review, an array of different protocol elements was observed. A range of wavelength parameters—from 618 to 830 nm [12,37,46,48–54]—was used, along with variation in energy density, application time and type of laser used, even with similar types of lesions. Most articles used the infrared light spectrum [12,15,18,20,22,33,45,46,48,49,51–54], including the study on humans [36], with promotion of new (local) formations and increased protein and genes of osteoblastic factors. PBMT involves radiation from the red to infrared regions, with the latter being most cited in the literature as effective in the early stages of bone repair during the reconstruction process. This is because, at the early stages, there is a large amount of differentiating cells, and reduction of these cells at a late time of repair reduces the PBMT-related osteostimulatory potential [25,48,64].

Regarding the evaluation time of the experiments performed in the analyzed articles, a preference for periods up to 30 days was observed, as the literature shows more modulatory effects of PBMT during the early stages of the bone repair process. Specifically, effects such as greater proliferation of osteoblasts, collagen fibers, and mesenchymal cells, less inflammation, and greater expression of immunomarkers have been reported [12,15,18,20,37,65].

The therapeutic effects of PBMT is dependent on the mode of application, time, frequency and number of sessions of irradiation and dosing, as well as the biologically-dependent relationship of energy density and intensity. PBMT presents conflicting results in the literature, especially with regard to these modulatory effects, as the parameters (wavelength, power density, treatment dose, method and number of applications) are greatly diversified [66–68]. When verifying that PBMT has a major effect on mitochondria, the parameter of wavelength appears to have a major influence on the therapeutic process, with the visible (red) wavelengths activating the mitochondrial respiratory chain and the non-visible (infrared) wavelengths acting on the cell membrane. Two experiments with beneficial cellular effects of laser application can be exemplified, where greater collagen production from fibroblasts and osteoid matrix originating from osteoblasts was observed [50,66,69].

The presence of more organized collagen fibers when bovine bone grafts are associated with PBMT has been reported, relating to a biostimulatory effect on collagen production [47,48–54], as well as improving osteoblastic activity with the release of calcium hydroxyapatite [18,47]. This relationship with osteoblast activity seems to be related to an increase in alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), runt-related transcription factor 2 (Runx2) and Jagged1 differentiation genes, and osteocalcin (OCN) [15], up to a period of 30 days. Kim et al. [20] pointed to an increase of receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG) and receptor activator of nuclear factor-κB (RANK) in the first 7 days, as already mentioned in previous studies relating bovine bones and lasers [33,70].

When using PBMT with 660 nm [47] and 618 nm [37], studies mentioned that, despite the increase of new bone, there was no resorption of bovine bone particles, while at 780 nm [22] and 808 nm [15], the biomaterial resorption occurred partially. It has been reported that osteoconductive biomaterials reduce local bone formation, which, by not being absorbed eventually, replace the new bone [63]. Oliveira et al. [15] found 60% more bone in a group without a biomaterial (control); however, computed microtomography showed that, in the groups with bovine bone, there was a greater amount of mineralized tissue. This suggests that, clinically, the use of osteoconductive biomaterials is important for maintaining morphology and function, rather than for new bone formation itself.

Most studies used infrared spectrum wavelengths, with GaAlAs being cited in eight studies [12,15,20,22,36,45-47]. Seven studies [48–54] did not state which type of laser they used, but did describe the application of 830 nm in the infrared range. The infrared spectrum is the most widely used in reconstructive processes, as it shows less energy loss when penetrating tissues, with about 37% reaching 2 mm deep and, at larger thicknesses, the maximum loss can be as little as 162.92 mW per cm^2 [12,71].

Bovine biomaterial is widely used and has good results in bone reconstruction processes, such as enlargement of the maxillary sinus or preparation for dental implants [72,73]. Of the articles included in this review, 17 cite positive results regarding the association of bovine bone with PBMT. However, Bosco et al. [47] concluded that PBMT stimulates bone formation regardless of the presence of biomaterial. The presence or absence of a membrane plus a biomaterial also did not seem to have an influence on the biostimulatory effects of the laser in three other studies [51,52,54]. This is in contrast to the results reported by Ghahroudi et al. [33], wherein greater bone neoformation was found when it was associated with both a biomaterial and PBMT, and the bovine bone group alone was better than a laser alone.

A critical review of the studies elected for examination showed that PBMT was associated with the promotion of new bone at lesion sites [12,15,18,20,22,33,36,37,46-48,51–54], increased deproteinized bovine bone (DBB) and HA/βTCP osteoconduction [15], osteoblast proteins and genes [15], increased levels of calcium hydroxyapatite (CHA) [18], metabolism and expression of immunomarkers [20] and aided the treatment of periodontal disease [36], but divergent results were found regarding particle resorption.

A lack of persistence in the standardization of methodology employed by authors was observed, with instances of absence of important data, such as output power, energy density and application time, a pattern also observed in reviews relating PBMT to other types of lesions (such as nervous) [5]. It is extremely important to highlight the scarcity of publications addressing PBMT. This complementary treatment method is cited in the literature in association with the widely-used bovine bone scaffolds in bone reconstruction, with both having good results and clinical applicability.

5. Conclusions

At the end of this review, it can be verified that the data presented in recent literature shows potential to improve the bone reconstructive process using PBMT together with bovine bone as a scaffold. A variability of parameters seems to be common in studies using PBMT, as well as a lack of parameters, generating doubts regarding reproducibility and, consequently, the production of satisfactory results.

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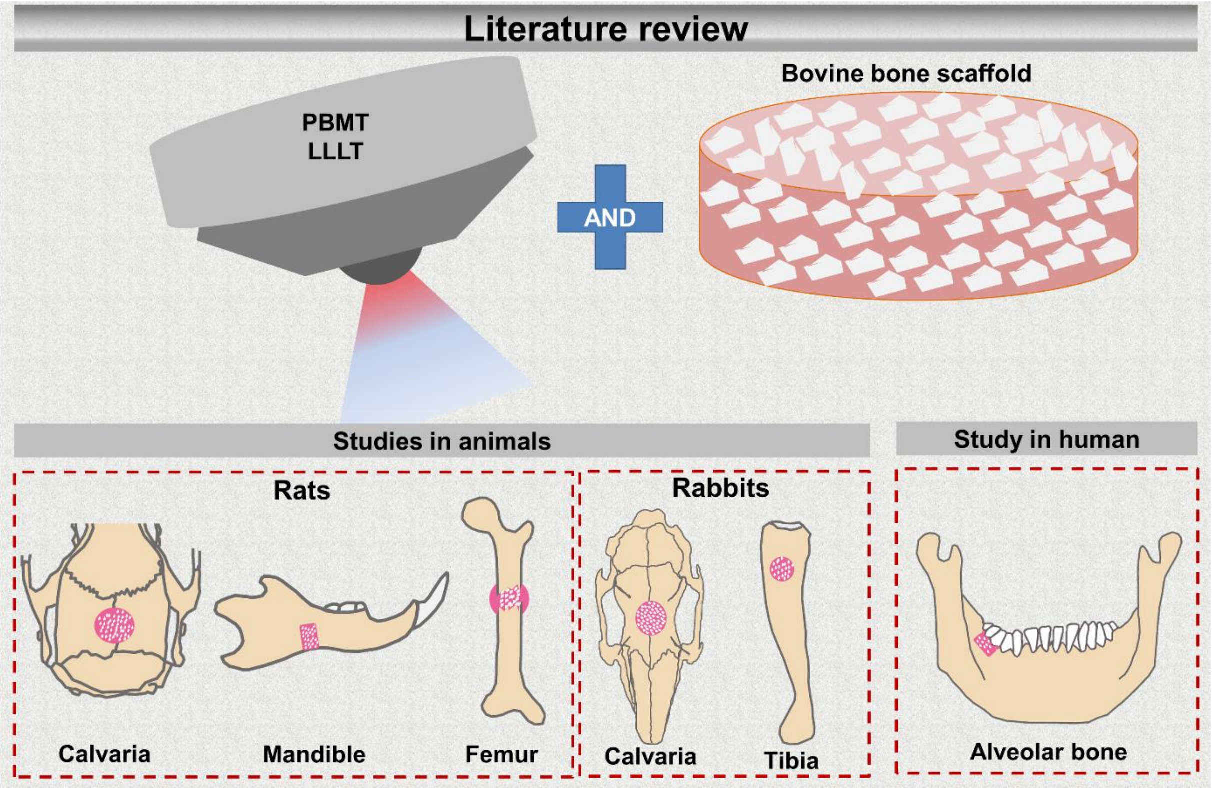
Graphical Abstract



2.2 Article 2:

Photobiomodulation therapy associated with heterologous fibrin biopolymer and bovine bone matrix helps to reconstruct long bones. (Appendix 2 and Annex 1).

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Article

Photobiomodulation Therapy Associated with Heterologous Fibrin Biopolymer and Bovine Bone Matrix Helps to Reconstruct Long Bones

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Abstract: Bone defects cause aesthetic and functional changes that affect the social, economic and especially the emotional life of human beings. This complication stimulates the scientific community to investigate strategies aimed at improving bone reconstruction processes using complementary therapies. Photobiomodulation therapy (PBMT) and the use of new biomaterials, including heterologous fibrin biopolymer (HFB), are included in this challenge. The objective of the present study was to evaluate the influence of photobiomodulation therapy on bone tibial reconstruction of rats with biomaterial consisting of lyophilized bovine bone matrix (BM) associated or not with heterologous fibrin biopolymer. Thirty male rats were randomly separated into three groups of 10 animals. In all animals, after the anesthetic procedure, a noncritical tibial defect of 2 mm was performed. The groups received the following treatments: Group 1: BM + PBMT, Group 2: BM + HFB and Group 3: BM + HFB + PBMT. The animals from Groups 1 and 3 were submitted to PBMT in the immediate postoperative period and every 48 hours until the day of euthanasia that occurred at 14 and 42 days. Analyses by computed microtomography (μCT) and histomorphometry showed statistical difference in the percentage of bone formation between Groups 3 (BM + HB + PBMT) and 2 (BM + HFB) (26.4% ± 1.03% and 20.0% ± 1.87%, respectively) at 14 days and at 42 days (38.2% ± 1.59% and 31.6% ± 1.33%, respectively), and at 42 days there was presence of bone with mature characteristics and organized connective tissue. The μCT demonstrated BM particles filling the defect and the deposition of new bone in the superficial region, especially in the ruptured cortical. It was concluded that the association of PBMT with HFB and BM has the potential to assist in the process of reconstructing bone defects in the tibia of rats.

Keywords: biomaterials; bone regeneration; fibrin biopolymer; low-level laser therapy; photobiomodulation therapy.

1. Introduction

Traumas, congenital anomalies and surgeries are morbid conditions that can lead to transient or permanent bone defects, often subject to reconstruction. These conditions influence the patient's life, affecting the social, psychic, aesthetic and work spheres with consequent increase of the financial costs to the health system [1,2].

Preclinical and clinical research has already shown that autogenous bone grafting (ABG) is the standard material for bone grafting in larger defects, which do not repair completely spontaneously. However, the limitations of supply, involvement of two surgical areas and morbidity mainly related to the collection of the autograft can be considered disadvantages of this technique. In this way, several bone substitutes are tested and used in surgeries, both dental (on facial bones) and orthopedic (on long bones) [3].

Biomaterials used as bone substitutes must be biocompatible, biodegradable and also form a scaffold for osteoconductivity, in addition to having porosity similar to the natural bone of the recipient bed and allowing the growth of osteoinductivity factors. Among the products available commercially in dentistry and orthopedic medicine, Geistlich Bio-Oss® is noteworthy due to the several published scientific works and wide use in the world [4,5].

In addition, researchers are looking for an ideal scaffold to be used in conjunction with bovine bone matrix (BM). In this context, fibrin sealant stands out as an aid in granule adhesion, in the formation of a stable fibrin network and in the maintenance of the medium as a support for cell growth [6–9]. Commercial sealants are all homologous, that is, made from human blood components, which makes them extremely expensive due to the scarcity of raw materials for production. To overcome these challenges, the Center for the Study of Venoms and Venomous Animals (CEVAP) from São Paulo State University (UNESP), Sao Paulo, Brazil, has been studying and developing heterologous fibrin biopolymer (HFB) from animal origin materials since the 1990s. This heterologous biomaterial is biocompatible, having hemostatic, adhesive, sealant, scaffold and drug delivery properties [9–11]. This bioproduct is being successfully tested in reconstruction processes when applied to various tissues [6,12–15].

In addition to specific treatments, rehabilitation therapy by techniques complementary to therapeutic treatment has been increasingly applied, especially photobiomodulation therapy (PBMT), with the use of low intensity laser [8,13,16–18]. Its photon energy reaches the cell nucleus increasing the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and consequent protein synthesis [19]. Furthermore, it increases the adenosine triphosphate (ATP) and cellular mitotic activity [20]. Defined as the application of energy directly from noncoherent (light emitting diode) or coherent (lasers) light, with varying wavelengths between 405 and 1100 nm, it is capable of producing photochemical effects by modulating cells [21,22]. This new technology is the current focus of studies of the scientific community for presenting reparative mechanisms of action. However, there is no consensus on its effective parameters, because if parameters are not properly delineated, they do not have beneficial effects on tissue regeneration, making this a current focus of research [19,21,23–25].

Considering the absence of previous studies that have used the unique heterologous fibrin biopolymer derived from snake venom in the world, (HFB) [9], associated with bone substitute of proven grafting quality [26,27], with results similar to the gold standard (ABG) [28], it was decided to conduct this research in noncritical defects of rat tibia in order to evaluate the therapeutic potential of the combination of these products, associating a recent PBMT protocol, already demonstrated in the literature as promising to treat bone defects [8,16], but not yet explored along the lines of this research.

In view of the problem and the objective previously presented, the hypothesis of the present study is that the combination of photobiomodulation therapy with bone substitutes improves the repair process of bone defects.

2. Materials and Methods

2.1. Animal Maintenance

All experimental procedures are in accordance with the Ethical Principles on Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA), and this project was approved under No. 006/2019.

Thirty adult male Wistar rats (*Rattus norvegicus*) were used (90 days), weighing an average of 470 grams, provided by the Central Biottery of the Ribeirão Preto School of Dentistry, University of São Paulo (FORP-USP). The animals were kept in an animal house in appropriate cages, four animals per box, receiving water and food "ad libitum," without restrictions on movement, respecting 12-hour light cycles, acclimatized by air extractors and air conditioning.

2.2. Experimental Design

All animals underwent experimental surgery, with defect filled with freeze-dried bovine bone matrix (BM) biomaterial, and were randomized into three groups:

Group 1: Lyophilized bovine bone matrix + photobiomodulation therapy (BM + PBMT), n = 10 (biomaterial and biostimulated);

Group 2: Lyophilized bovine bone matrix + heterologous fibrin biopolymer (BM + HFB), n = 10 (biocomplex composed of biomaterial mixed to heterologous fibrin biopolymer);

Group 3: Lyophilized bovine bone matrix + heterologous fibrin biopolymer + photobiomodulation therapy (BM + HFB + PBMT), n = 10 (biocomplex composed of biomaterial mixed to heterologous fibrin biopolymer and biostimulated).

2.3. Lyophilized Bovine Bone Matrix (BM)

The bone matrix biomaterial used in the present research was bovine demineralized bone, commercially called Bio-Oss® (Geistlich Pharma AG, Wolhusen, Switzerland; Ministry of Health Registration No. 806.969.30002). It is characterized as porous bovine bone matrix with particle size between 0.25–1 mm, packed in 2 gram flasks with corresponding porosity of 75%–80% of the total volume between 10 nm to 100 μm [29].

2.4. Heterologous Fibrin Biopolymer (HFB)

In the Group 2 (BM + HFB) and 3 (BM + HFB + PBMT) animals, the biomaterial was mixed with the heterologous fibrin biopolymer forming a biocomplex to act as drug delivery. This mixture formed a biological framework for the biomaterial particles, favoring their introduction into the defect and at the same time maintaining them in the surgical bed. The HFB used in the research was kindly provided by CEVAP itself and had in its composition three vials: one vial with fraction 1 (thrombin-like), one vial with fraction 2 (fibrinogen-rich cryoprecipitate obtained from buffalo blood) and one vial of diluent (calcium chloride). This product is under patent under Registration Numbers BR1020140114327 and BR1020140114360.

The HFB was handled according to the standardization proposed by Ferreira Jr. et al. [10], which describes a 1:2:1 ratio as follows: one part fraction 1 (thrombin-like), two parts fraction 2 (cryoprecipitate rich in fibrinogen) and one part diluent (fraction 3, calcium chloride), with an amount of microliters according to the size of the defect to be filled. The components of the HFB remained frozen until the moment of use, when they were thawed and mixed in the proportions previously established to generate a stable clot according to the following protocol: fraction 1 = 10 μL, fraction 2 = 20 μL and diluent = 10 μL. Fractions were dosed using Gibson® micropipettes with disposable tips. The application sequence was as follows: fraction 1 was placed over the BM in an eppendorf, followed by mixing fraction 2 with the diluent in another Eppendorf®, where they were homogenized [30] and then applied to the BM, forming a biocomplex that was inserted into the defect.

2.5. Monocortical Defect Surgery

All surgical procedures were performed at the Department of Biological Sciences at the Anatomy Mesoscopy Laboratory of the Bauru School of Dentistry, University of São Paulo, Brazil, by the same team of researchers.

For the experimental surgery, the rats were submitted to general anesthesia with intramuscular injection of ketamine (0.3 mL/kg) (Dopalen®, Ceva, Paulinia, São Paulo, Brazil) and xilasine (0.3 mL/kg) (Anasedan®, Ceva, Paulínia, São Paulo, Brazil), and trichotomy was performed in the lateral dorsal region of the left pelvic after with disinfection of the operative field by 10% povidone–iodine (PI) topical solution. Next, with a No. 15 scalpel blade, a 20 mm long linear incision was made in the craniocaudal direction, sectioning the skin and muscle fasciae, reaching the periosteum and extending it for tibial exposure. With an AR 6 steel carbide drill (Beavers Dental®, Morrisburg, ON, Canada, K0C 1×0/Ormex SA) coupled to a 1500 rpm low-speed micromotor, a 2 mm diameter cavity was prepared [31–35] in a depth reaching the bone marrow without damaging the contralateral cortical [18,36–39], with abundant irrigation of 0.9% sodium chloride solution.

For each animal in the three groups, the cavity was filled by BM in the amount of 0.012 grams (defined in a preliminary pilot study), which was weighed on an analytical balance (Micronal®, Precision Equipment, São Paulo, Brazil). In Group 1 (BM + PBMT), after weighing the biomaterial, enough saline was added to adhere the granules and facilitate deposition inside, completing the entire defect. In Groups 2 (BM + HFB) and 3 (BM + HFB + PBMT), the heterologous fibrin biopolymer was used as a scaffold for bone matrix, forming a biocomplex.

After filling the cavities, the periosteum and other tissues of the operated region were repositioned and sutured using 4–0 Ethicon® silk thread (Ethicon®, Johnson & Johnson Company, New Orleans, LA, USA). The operative acts were always performed by a single operator subjecting the animals to the same conditions, and the experimental design is detailed in Figure 1.

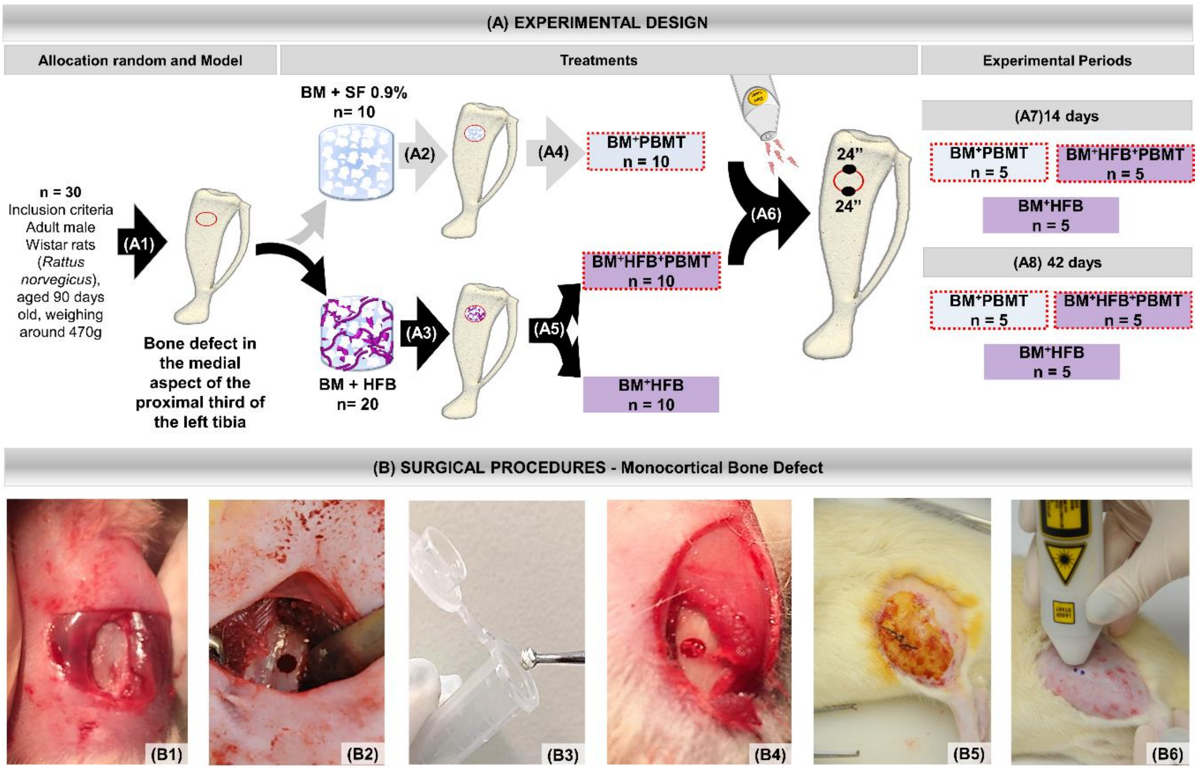
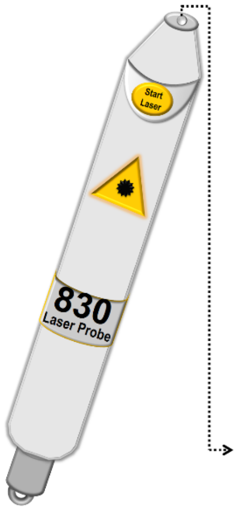


Figure 1. (**A**) Experimental design. (**A1**) Monocortical osteotomy in the medial aspect of the proximal third of the tibia. (**A2**) Defects filled with biomaterial (BM) mixed with saline (SF). (**A3**) Defects filled with biomaterial—lyophilized bovine bone matrix (BM) mixed with heterologous fibrin biopolymer (HFB). (**A4**) Group 1 (BM + PBMT): defects filled with biomaterial and photobiomodulation therapy (PBMT). (**A5**) Group 3 (BM + HFB + PBMT): defects filled with biomaterial mixed with heterologous fibrin biopolymer and photobiomodulation therapy. Group 2 (BM + HFB): defects filled with biomaterial and nonlaser biostimulated. (**A6**) Illustration of the two laser-irradiated points for 24 s each. (**A7–A8**) Euthanasia periods of 14 and 42 days: five animals from each group/period. (**B**) Surgical procedures—cortical defect bone: (**B1**) medial aspect of the proximal third of the left tibia; (**B2**) monocortical bone defect of 2 mm; (**B3**) biomaterial mixed with fibrin biopolymer; (**B4**) defects filled with biomaterial mixed with fibrin biopolymer in the surgical cavity; (**B5**) tegument suture with 4–0 silk thread. (**B6**) Schematic representation of laser application.

Immediately after the surgical procedures, the animals received paracetamol analgesic (Paracetamol®, Medley, São Paulo, Brazil) at a dose of 200 mg/kg, dissolved in the water available in the drinker for 3 days.

2.6. Photobiomodulation Therapy

For animals from Groups 1 (BM + PBMT) and 3 (BM + HFB + PBMT), the GaAlAs (gallium–aluminum–arsenide) laser (Laserpulse IBRAMED®, Amparo, São Paulo, Brazil) was applied. The design of the parameters is described in Figure 2. In all applications, the laser beam emissions were calibrated on the device itself and previously tested to certify the dose [8,16].



Parameter	Unit/Explanation
Type of laser	GaAlAs *(gallium-aluminum-arsenide)*
Emission Mode	Continuous
Type of Beam	Positioned for laser irradiation at perpendicular incidence to the tibia
Treatment Time	Immediately after surgery and three times a week until euthanasia
Form of Application	Two points surrounding the surgical area, proximal and distal
Wavelength	830 nm
Optical Power	30 mW
Fluency or Density of Energy or Dosimetry	6 J/cm^2
Beam Area	0.116 cm^2
Duration of Irradiation	24 sec/point
Total Time of each Application	48 s
Density of Power or Irradiance	258.6 mW/cm^2

Figure 2. Details of the parameters used for PBMT application.

2.7. Collection of Samples and Histological Procedures

After 14 and 42 days after surgery, five animals from each group, per period, underwent general anesthesia with an intramuscular injection of ketamine and xilazine mixture. The samples were fixed in 10% buffered formaldehyde for a period of 72 h and then computed microtomographically. The next step was decalcification in 10% ethylenediaminetetraacetic acid (EDTA) solution containing 4.13% Titriplex® III (Merck KGaA, Darmstadt, Germany) and 0.44% sodium hydroxide (Labsynth, São Paulo, Brazil) and given sequence in standardized histological processing [8,16]. Subsequently, longitudinal, semiserial sections (50 μm interval) of defects of 5 μm thickness and stained with hematoxylin–eosin were performed.

2.8. X-ray Computed Microtomography Analysis (μ-CT)

The samples were placed in a cylindrical acrylic tube and allocated inside the SkyScan 1174v2 microtomograph (μ-CT Bruker microCT®, Kontich, Belgium), obtaining images with 13.76 μm voxel, 0.73° per sequence. Next, the two-dimensional reconstruction and realignment analyses were performed using the NRecon® 1.6.9 and DataViewer® 1.4.4.0 software, respectively. For the reconstitution of the three-dimensional images, the CTVox® 2.4.0 r870 software (Bruker microCT) was used.

2.9. Histomorphometric and Histological Analysis

Images were obtained by Olympus® BX50 light microscope (Olympus® Corporation, Tokyo, Japan) on 4×, 40× and 100× lenses at the FOB-USP Anatomy Laboratory using the DP Controller software 3.2.1.276-2001-2006 (Olympus® Corporation, Tokyo, Japan).

For the histomorphological description of the bone defect areas, 40× and 100× images were used in all specimens, considering the entire extent of the defect in order to analyze granulation tissue, inflammatory infiltrate, presence and quality (immature or mature/lamellar bone) and the degree of filling of the newly formed tissue, regarding the interaction between the HFB and PBMT with the BM used.

Quantitative analysis about the percentage of new bone volume was evaluated by 4× images using the point count planimetry method. For this, a previously established grid with 88 points [34,40] was superimposed on the histological section image of each animal, each point that overlapped the newly formed tissue was considered, and the total density was evaluated by the occupation in % of the image covering the defect in its entirety.

The grid size used was 13.2 × 9.6 cm with 1.2 cm spacing between each point marked on a transparent sheet. The measurement of the area densities of the analyzed sections was performed using the equation: $D = \Sigma PN / PT \times 100$, where PN indicates the number of overlapping points in new bone formation and PT the number of total points included in the overlapping grid. The percentage value was related to the average of all animals analyzed (Figure S1).

2.10. Statistical Analysis

Data were submitted to analysis of variance (ANOVA) to detect possible differences between groups. The ANOVA assumptions, residual normality and variance homogeneity, were verified, respectively, by the Shapiro–Wilk and Bartlett tests, both at 5% probability. Subsequently, the means were compared by the Tukey's test at 5% probability. Within each treatment, the comparison of new bone formation as a function of the treatment period (14 and 42 days) was assessed by the Student's *t*-test at 5% probability. All analyses were conducted using the R software (R Core Team®, 2019, The R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. General Evaluation

No complications were seen in the postoperative period of the animals, with normal healing and no signs of infection. Signs of pain-related behavioral changes such as decreased movement or weight loss were also not evident.

3.2. Microtomographic Evaluation

The images analyzed by μCT observed in Figure 3 showed that the biomaterial resembles the cortical bone, mainly in its radiopacity, not allowing the quantitative verification of the newly formed bone tissue.

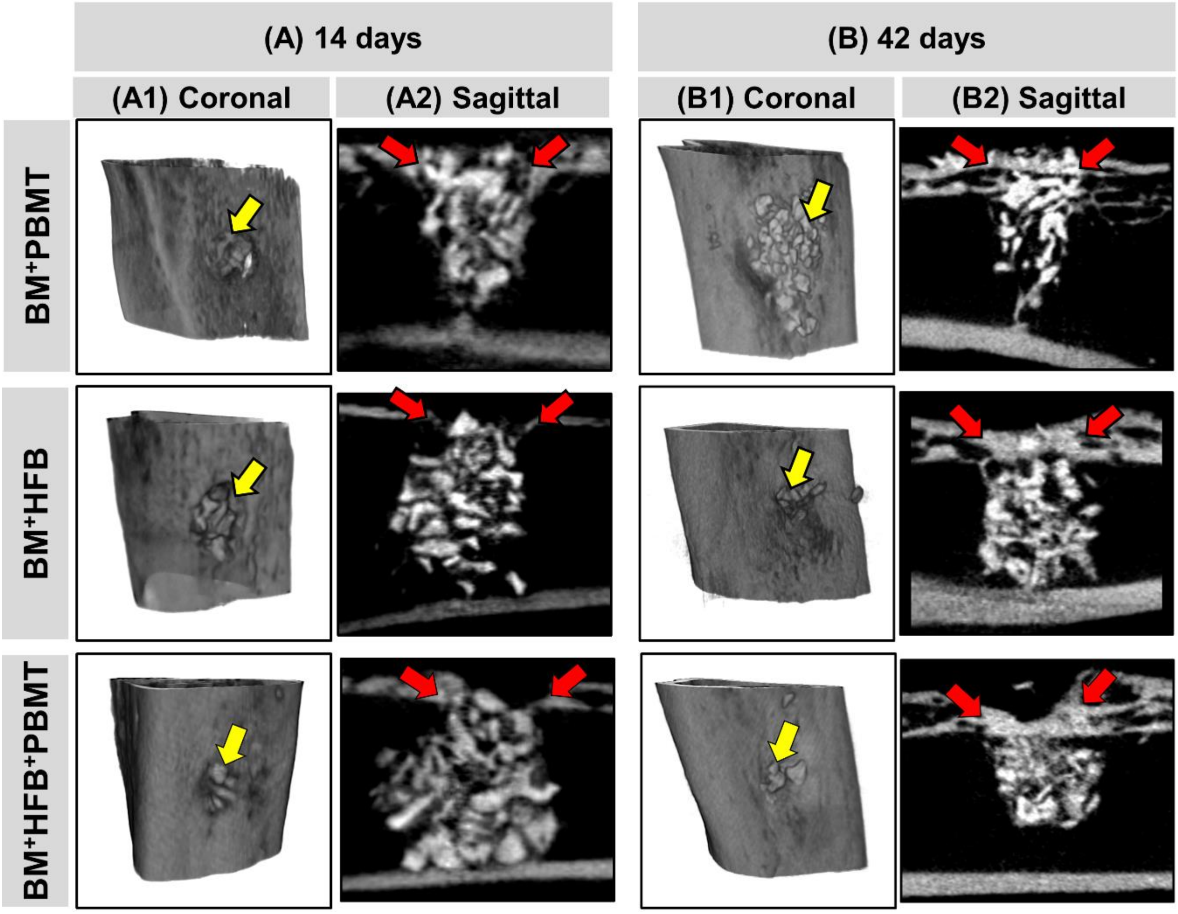


Figure 3. Representative microcomputed tomography (μCT) image of the proximal third of the tibia for each rat group: 1 (BM + PBMT) (lyophilized bovine bone matrix with photobiomodulation therapy), 2 (BM + HFB) (lyophilized bovine bone matrix plus heterologous fibrin biopolymer) and 3 (BM + HFB + PBMT) (lyophilized bovine bone matrix plus heterologous fibrin biopolymer with photobiomodulation therapy) in the periods of (**A**) 14 and (**B**) 42 days. (**A1**,**B1**) Three-dimensional coronal section shows the cortical region of the defect, biomaterial particles (yellow arrow). (**A2**,**B2**) Two-dimensional sagittal section shows the cortical and medullary region of the defect filled by BM, defect in the cortical bone (red arrows).

At 14 days (Figure 3A1), it is possible to notice the monocortical bone defect with definition of its borders and covered with biomaterial in all groups. In Group 2 (BM + HFB), the particles appear closer to each other, and in Groups 1 (BM + PBMT) and 3 (BM + HFB + PBMT) there was a tendency for bone formation in the ruptured cortical (Figure 3A2).

At 42 days (Figure 3B1), visually, there is new bone covering the cortical area, with particles of the biomaterial in the midst of this new formation with bone healing process in continuity. There was a visual difference in bone board thickness in relation to the days, being the thickest in this period. In Group 1 (BM + PBMT), some particles of the biomaterial exceeded the defect limits (Figure 3B1); in Groups 2 (BM + HFB) and 3 (BM + HFB + PBMT), the defect site appears to be thicker bone undergoing remodeling.

Table 1. Table of volume density of new bone formation (%).

	BM + PBMT	BM + HFB	BM + HFB + PBMT
14 days	22.20 ± 1.77 Aab	20.00 ± 1.87 Ab	26.40 ± 1.03 Aa
42 days	33.20 ± 2.18 Bab	31.60 ± 1.33 Bb	38.20 ± 1.59 Ba

Different uppercase letters (comparison in columns, 14 vs. 42 days) indicate a statistically significant difference. Different lowercase letters (line comparison, BM + PBMT vs. BM + HFB vs. BM + HFB + PBMT in each period, 14 or 42 days) indicate a statistically significant difference. Student's *t* and Tukey´s test, respectively, both at 5% probability.

3.3. Histological Evaluation

At 14 days, as seen in Figure 4A1, all experimental groups presented newly formed bone trabeculae in the medullary region of the defect, permeating the particles of the biomaterial and connective tissue filling the spaces adjacent to the edge of the lesion (Figure 5A1), with some integration of the biomaterial to the tissue in normal bone repair and mineralization pattern, besides the absence of inflammatory process. In Group 3 (BM + HFB + PBMT), there was a large amount of connective tissue with vascular shoots filling the medullary spaces (Figure 5A2). Overlying the injured cortical region, the groups had a thin layer of periosteum and connective tissue, slight bone neoformation from the edges of the lesion and the central cortical area filled with loose connective tissue.

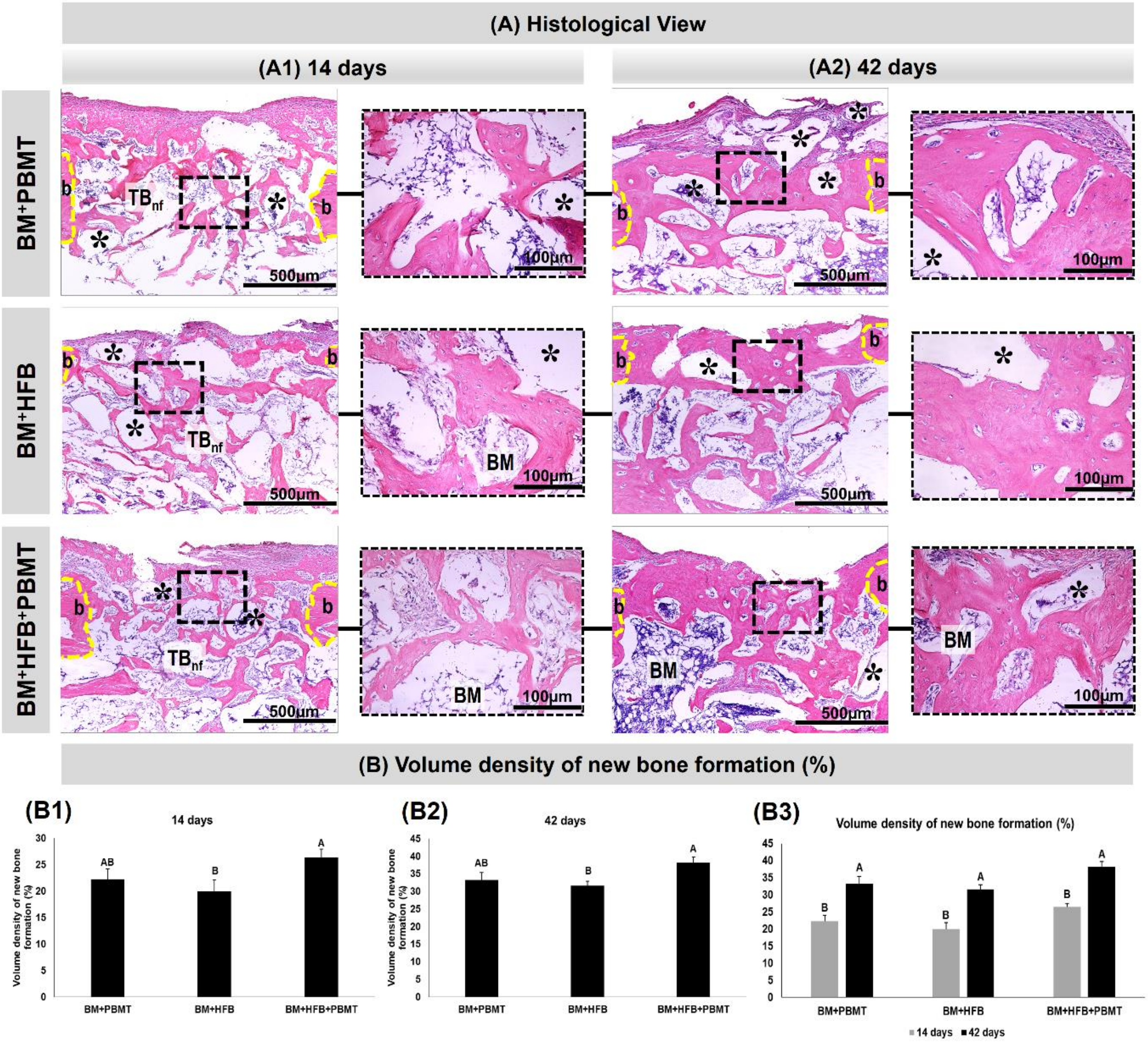


Figure 4. (**A**) Histological views at 14 and 42 days in tibia defects filled with lyophilized bovine bone matrix graft with photobiomodulation therapy (Group 1, BM + PBMT), lyophilized bovine bone matrix graft plus heterologous fibrin biopolymer (Group 2, BM + HFB) and lyophilized bovine bone matrix graft plus heterologous fibrin biopolymer with photobiomodulation therapy (Group 3, BM + HFB + PBMT). Newly formed trabecular bone (TBnf), bone graft particles (asterisk), defect border (b), bone marrow (BM). (**B**) Graphs of volume density. Graphs **B1** and **B2** demonstrate the comparisons of the volume density of the new bone formed between the groups studied in the same period of experimentation (14 or 42 days). In **B3**, the volume density of new bone formed in the same group in the two experiment periods (14 or 42 days) is compared (*n* = 5/group). Different uppercase letters (A ≠ B)

indicate a statistically significant difference ($p < 0.05$). (hematoxylin and eosin (HE); original 10× magnification, bar = 500 μm; 40× magnified images, bar = 100 μm).

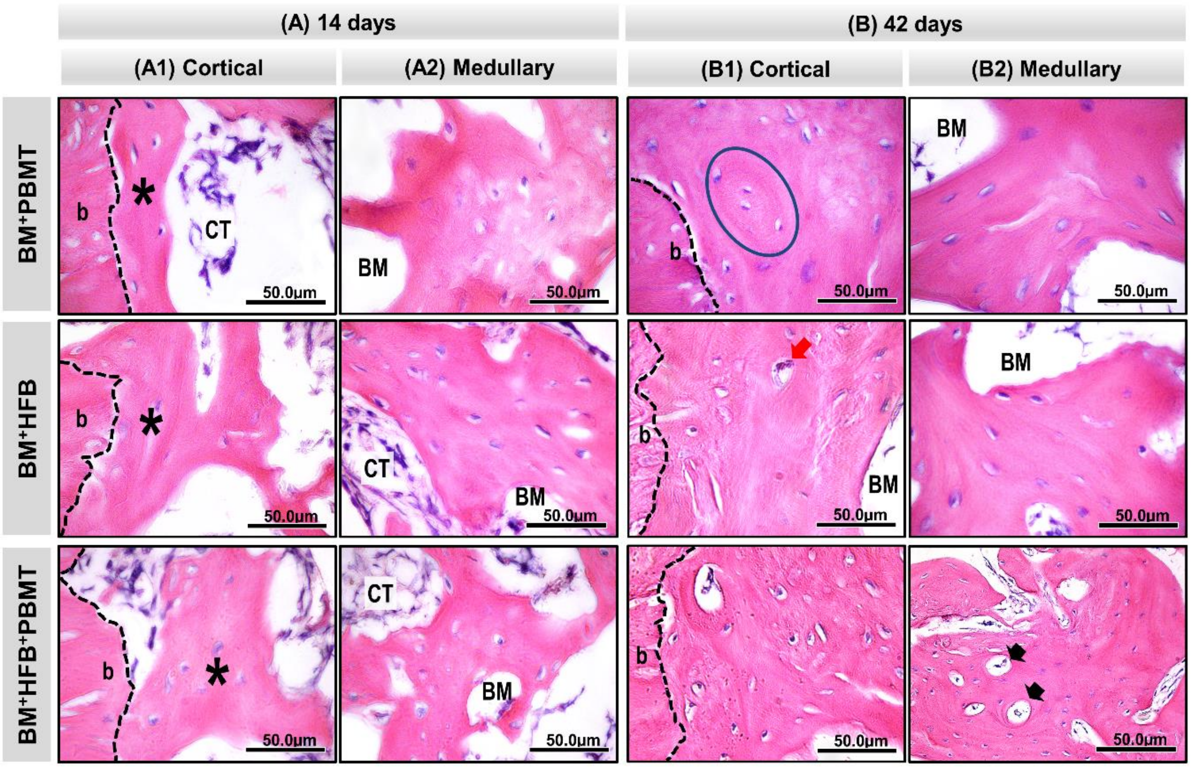


Figure 5. Time course of monocortical defect healing in rats tibia in groups: Group 1 (BM + PBMT) (defects filled with lyophilized bovine bone matrix (xenograft) with photobiomodulation therapy), Group 2 (BM + HFB) (defects filled with lyophilized bovine bone matrix plus heterologous fibrin biopolymer) and Group 3 (BM + HFB + PBMT) (defects filled with lyophilized bovine bone matrix plus heterologous fibrin biopolymer with photobiomodulation therapy) in the periods of 14 and 42 days. (**A**) At 14 days. (**A1**) In cortical area, all experimental groups show bone growth (asterisks) from the border of the defect (b) with immature trabecular conformation surrounded by connective tissue (CT). (**A2**) In the medullary area, for BM + PBMT, BM + HFB and BM + HFB + PBMT, fine bone trabeculae are noted around particles of the biomaterial (BM). (**B**) At 42 days. (**B1**) In the cortical area, increased bone formation in the bone defect border, presence of concentric laminae (inside the blue lined area) in Group 1 (BM + PBMT) and the blood vessel (red arrow) in Group 2 (BM + HFB) are observed. (**B2**) In the medullary area, the bone trabeculae are thicker and more compact with Haversian canals (black arrow) in Group 3 (BM + HFB + PBMT), in relation to the previous period with some biomaterial particles in all groups. (HE; original 100× magnification; bar = 50 μm).

At 42 days (Figure 4A2), the newly formed bone matrix in the medullary region was thicker, with compact conformation and some Havers canals in relation to the previous period, with evidence of a large amount of osteocytes in Group 3 (BM + HFB + PBMT). The collagen fibers were concentrically arranged to the remaining particles of the biomaterial and organized. At the end of the experimental period, the groups still had biomaterial in the cortical (Figure 5B1) and medullary (Figure 5B2) regions; in the latter region, this was surrounded by newly formed bone tissue. All experimental groups showed the injured cortical tending to close, thus restoring the original bone architecture. Group 3 (BM + HFB + PBMT) showed a more mature lamellar bone formation in the injured cortical region.

3.4. Histomorphometric Evaluation

In the histomorphometric evaluation of the volume density of new bone formed, it was found at 14 days that Group 3 (BM + HFB + PBMT) had a mean of 26.4% ± 1.03%, with a statistical difference compared to Group 2 (BM + HFB), which obtained the lowest values (20.0% ± 1.87%); Group 1 (BM + PBMT) presented a mean of 22.2% ± 1.77%, with no significant difference compared to the other groups (Figure 4B1; Table 1).

At 42 postoperative days, a statistical difference was observed between Groups 3 (BM + HFB + PBMT) and 2 (BM + HFB) (38.2% ± 1.59% and 31.6% ± 1.33%, respectively), with a mean of 33.2% ± 2.18% for Group 1 (BM + PBMT), the latter without statistical difference in relation to the other groups (Figure 4B2; Table 1).

Analyzing the data in the euthanasia periods (14 and 42 days), within each group, there was a statistical difference between 14 and 42 days for the three groups (Figure 4B3; Table 1).

4. Discussion

The aim of the present study was to verify the influence of PBMT on bone reconstruction of rat tibias by combining two components, heterologous fibrin biopolymer and lyophilized bovine bone matrix. We observed tissue biocompatibility of these scaffolds, in addition to greater maturation of bone tissue in the final period of the experiment. PBMT has shown positive effects on different types of tissue recovery [13,16,31,41], but there are still controversies regarding the appropriate parameters to be used.

The choice of long bones such as the tibia for bone repair is related to its ease of manipulation and access and its similarity to the clinical application in humans, regarding remodeling, repair in the physiology of muscle strength and tension. When dealing with critical defects, larger animals should be used, such as sheep and pigs [31,33,34,36,42]. In addition, biomaterials are used in orthopedic medical surgeries performed to correct bone defects of dimensions that do not spontaneously repair, as well as in patients with osteoporosis or cancer [42,43]. Tissue engineering states that the periosteum of the long bones as an auxiliary element in reconstructive process research, and another factor is the size of defects for biomaterial analysis, with the highest absorption being cited for noncritical defects [44].

The distribution of animals in their respective groups in the present study, as well as the number of specimens (*n*), was based on the principle of the 3 R's, in which there is a commitment by the world scientific community to follow the Russell–Burch Principles (1959) "reduction, replacement and refinement" in the use of animals that, increasingly, remain active in scientific and academic circles. Therefore, it was decided to not perform groups with defects filled only by clot, autogenous bone [3,17] or Bio-Oss® [5,7,28,45], widely previously published in the literature, including the same methodology used in the present experiment and also from the same research group [39,46–48], focusing on only in the originality and aims of the research.

Following the same methodology used in this study, including similar analyzes, Song et al. [49] evaluated a hydrogel based on carboxymethylcellulose (CMC) randomly separating the rats into three groups: CMC/BioC (biphasic calcium phosphate), CMC/BioC/BMP-2 0.1 mg (bone morphogenetic protein-2) and CMC/BioC/BMP-2 0.5 mg, concluding that the hybrid material CMC/BioC/BMP-2 induced greater bone formation than the other tested materials. Likewise, focused only on the tested biomaterials, Kido et al. [36] randomly separated the rats into two groups: Biosilicate group (BG) and poly PLGA Biosilicate group (BG/PLGA), reaching concluding that BG/PLGA showed a faster degradation of the material, accompanied by greater bone formation when compared with BG, after 21 days of implantation.

As observed by the μCT, in this study, all groups tended towards bone neoformation and there was presence of biomaterial particles until the last experimental period. Studies differ between resorption [50] and non-resorption [51] of biomaterial after PBMT, and no study using HFB cited this relationship. It was not possible to perform a quantitative distinction of the newly formed tissue since the xenograft has great similarity with cortical bone, mainly in its radiopacity [52,53], so its application in this analysis has been challenging. In agreement with the literature, we identified in the present study the presence of newly formed bone tissue in the midst of the biomaterial particles, demonstrating active repair progress, and the close relationship between the μCT and histomorphometric analysis as complementary in bone evaluation [53,54].

The groups that used the HFB showed greater visual proximity of particles and integration of components. In addition, its use facilitated the agglutination of the biomaterial particles for insertion in the bone defect, as well as a rapid decrease in bleeding caused by the injury. Initially called as heterologous fibrin sealant (HFS), this bioproduct, derived from snake venom produced by the CEVAP, was used in the recovery of venous ulcers [10,55,56] and as a glue for injured nerves [57]. Next, new experiments showed more advantages of the sealant, such as the ability to act as a scaffold for stem cells [30] or biomaterials [15–17,50,58] and as a new medication administration system [9]. Considering that the use of this bioproduct goes beyond its adhesive capabilities, its nomenclature has been reconsidered and has recently been called "fibrin biopolymer", but there are still not many studies on the osteogenic potential of HFB.

In the present study, the results of the histological analysis showed that the physiological inflammatory process of bone repair was already completed at 14 days in all groups due to the absence of reaction tissue, demonstrating that the association of biomaterial with both HFB and PBMT and both at the same time present biocompatibility characteristics [59,60], corroborating studies associating HFB with autogenous bone and PBMT (830 nm) [17]. Research with 830 nm [18,61] and 808 nm [62] lasers identified the modulatory action of the laser relating to mature bone neoformation with increased osteoblast factor proteins and genes, and the xenograft tested here is widely used in experiments in the literature with applications in animals and humans, with bovine bone being elected with biocompatible properties and good acceptance.

Moreover, in the first analysis period of the study (14 days), the forming bone tissue increased in a centripetal way from the edges of the defect and was characterized by thin and disorganized fibers. However, at the end of the experiment (42 days), newly formed bone tissue was thicker, mature and organized with a physiological process of repair, similar to studies with 830 nm laser [60,63,64] that identified such bone maturation over the analyzed periods. These studies report that especially after 30 days, there is more organization and tissue repair maturation, besides the presence of denser collagen fibers in long bones.

Between Groups 2 (BM + HFB) and 3 (BM + HFB + PBMT), there was a statistical difference in relation to bone percentage in both periods, relating findings compatible with the literature when observing higher bone density [61,65], blood vessels, Havers canal development, maturation and organization of bone tissue, following the principles that the tissue will only respond to biostimulation if the energy is adequate, reaching the minimum limit, and when the energy is excessive, there may be tissue inhibition [22,66].

The PBMT protocol used in the present experiment is due to previous studies (in vivo) on tissue regeneration [8,12,13,16], plus a literature review on the action of PBMT specifically on bone tissue, in which the length of 830 nm was cited as the most used (40.79%) and with positive effects in 98.68% [67]; in another review, satisfactory results were observed between the PBMT and xenograft in 17 of the 18 articles included [23]. The literature points to laser-modulated bone formation with increased bone growth factors in differentiated cells by stimulating matrix secretion, cell proliferation and reduced inflammation [65,68].

The results presented by our study corroborate the results of Iatecola et al. [17], who used autogenous bone graft (ABG) associated with HFB and PBMT. PBMT also showed satisfactory results in helping to reconstruct long bones when associated with biomaterials such as synthetic hydroxyapatite [41], biosilicate® [31], and Celecoxib® [69], in addition to an increase bone mineral density with salmon calcitonin [70].

The biological action of the laser depends on the penetration, propagation, absorption and length of light. The infrared laser is capable of reaching deeper tissues, penetrating about 2 mm into the tissue before losing 37% of energy, so when compared to the red light-length laser (which has 0.5–1 mm triggered energy) [71], it penetrates more with less energy loss, being indicated for bone lesions [19,71,72], affecting mitochondrial stimulation, formation of new blood vessels and proinflammatory and regenerative cytokines [33,73].

PBMT has been shown to be an ally in the process of bone reconstruction; however, the consensus about parameters for the purpose of bone regeneration is still uncertain in the literature, since studies show varieties of protocols, thus obtaining different results [23,74,75] such as differentiation, density and osteogenic proliferation [67,74,76].

5. Limitations

The implementation of functional analyses such as biomechanics [33] and immunomarkers [77] for bone cells help to better interpret the process of tissue maturation and may be considered as a limitation of the present research. In addition, a greater number of groups and specimens studied, provided they are ethically approved, can also be considered a limiting factor. We list as future studies the analysis and quantification of collagen fibers [78] and comparison between different concentrations of biopolymer in order to verify its osteogenic characteristics.

6. Conclusions

It was concluded that PBMT, through the use of low-level laser, associated with the biocomplex formed by the heterologous fibrin biopolymer (HFB) added to the lyophilized bovine bone matrix (BM), has the potential to aid in the reconstruction process of bone defects in the tibia of rats.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Representative image of the methodology used to quantify the area of newly formed bone.

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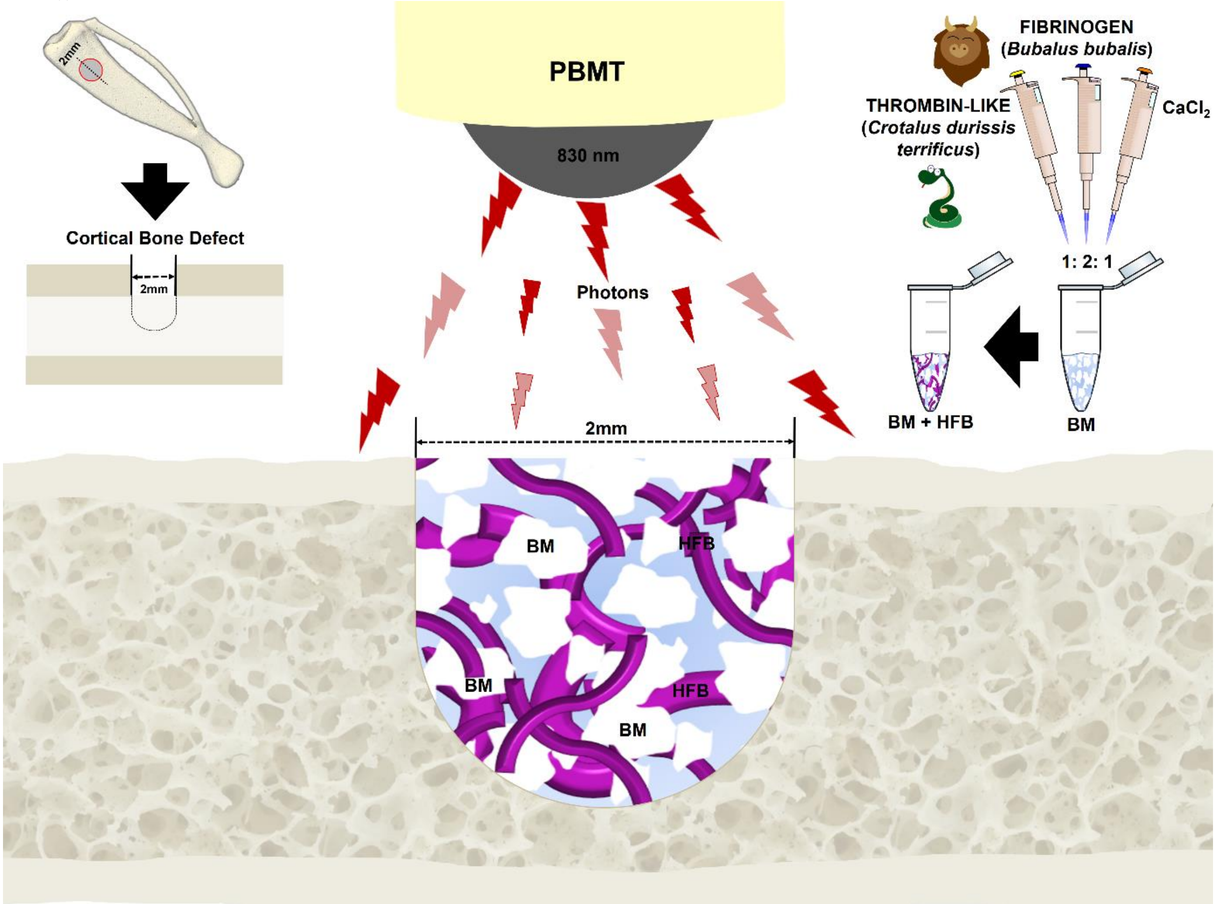
Graphical Abstract



3 Discussion

3 DISCUSSION

Biostimulation demonstrates wide applicability with positive tissue effects, however the literature emphasizes the lack of standardization in the protocols used in the research, in addition to its interaction with various scaffold models. With this view, the main focus of this experiment was to verify the influence of photobiomodulation therapy in the reconstruction of long bones using a graft with lyophilized bovine bone matrix (BM), already used in clinical practice, associating or not with the heterologous fibrin biopolymer (HFB), which were unprecedentedly mixed and analyzed simultaneously. The purpose of the HFB in this study was to act as a scaffold to the BM, and the PBMT as an aid to this reconstructive process.

The functional changes that a bone lesion can cause in an individual's quality of life leads to the need for auxiliary methods in the tissue reconstructive process. The progress in the use of biomaterials of bovine origin stands out mainly in histological and clinical characteristics, being an alternative to autologous bone grafting, due to the setbacks caused by the second surgical area in tissue collections. With the improvement of methods that help in rehabilitation, PBMT has positive photochemical and photobiological effects, however there are still gaps to be studied in relation to its association with grafts.

In search of the facts already published by scientific research, we conducted a literature review (article 1) on PBMT associated with bovine bone, listed as the most frequent type of graft used (29–31). When observing the need for means that anchor bone grafts, such as scaffolds, the heterologous fibrin biopolymer appears as an alternative to conventional homologous sealants, and of high cost. Thus, the application of PBMT in the reconstruction of long bones with the use of lyophilized bovine matrix was evaluated, associating it to the heterologous fibrin biopolymer (article 2).

Research that investigates the effect of PBMT on bone repair using bovine bone occurs mostly in long bones of rats, with the choice of bovine bone in its inorganic phase being the most common, as observed in the 18 articles of the systematic review (article 1). As for the biostimulation protocols, a wide range of parameters was observed, however the wavelength in the infrared spectrum was undoubtedly the most chosen, with 830nm as the most cited (article 1). Due to this basis and the existing questioning in the literature about various mixed components (BMP, homologous sealant, PRF) to bovine bone in order to assist bone reconstruction, such as scaffolds or cellular stimulating means, the experimental design was conducted in the innovative expectation of verify the results of HFB as scaffold for lyophilized bovine bone matrix (article 2).

During the survey of the scientific literature for article 1, some research was excluded because it did not cite important data about its scientific methodology, such as wavelength (nm), output power (mW), energy density (J / cm^2) and amount of radiation. These parameters assist in the evaluation of the results (32,33) and in the reproduction of such protocols. A concern for providing as much data as possible should be a key point for future research, in order to support its findings. This report led to care in the development and experimental design applied in article 2 with all the details of the parameters.

The microtomographic evaluation of article 2 was based on qualitative parameters with visualization of the permanence of the biomaterial particles, a controversial relationship in the literature (article 1), since partial absorption of bovine bone particles was found in studies associated with PBMT with infrared irradiation (31,34). The presence of neoformed tissue was noticed around the graft granules in all groups, identifying an active bone repair process. In the groups with HFB, the granules of the biomaterial were closer with greater integration of components, however it is necessary to use more accurate analyzes about their potential osteo-helper.

It was not possible to have a quantitative assessment of the newly formed bone due to the difficulty of automatic delimitation of the area together with the global limitation of resolution (35), which can be explained according to Waarsing et al. (36), the density of the new bone being smaller and more variable, associated with the technical limitations of µCT where tissue depreciation or overvaluation may occur, since it presents low specificity when compared to lyophilized bone matrix and high sensitivity for quantification only when there is high resolution.

The histological results did not show evidence of an inflammatory process in the first period (14 days) evaluated in all groups, which corroborate previous research regarding the effects of anti-inflammatory drugs already confirmed by the laser. The PBMT optical window between 600 and 1100nm reaches the cell chromophores, where the energy photons are absorbed, and a primary photographic signal amplified by transduction occurs. The biomolecular mechanisms associated with cellular changes due to biomodulation, have the participation of free radicals, nitrogen species and reactive oxygen. (37,38). The same conclusion of the inflammatory process associating HFB biocompatibility was observed in femurs and rat calvaria. (39,40).

The progressive evolution of the bone remodeling process demonstrated over the periods (from 14 to 42 days) with the approximation of the defect edges is cited in the literature as an indicator of favorable results for the use of the tested components. (39,41). In addition to the formation of new bone around the granulated material, the greater organization, thickness and maturation of the bone trabeculae at the end of the experiment are indicators of positivity in bone repair, as observed in studies with 830nm laser in long bones. (42–44).

When observing statistical difference for bone percentage in both periods in groups 2 (BM + HFB) and 3 (BM + HFB + PBMT), infrared biomodulation (IR) seems to be the key factor that contributes to the tissue reconstructive process. The literature mentions a variability of protocols when comparing radiation with red and infrared wavelength (IR) in long bones, with IR being associated with faster bone repair, being one of the factors, the lower surface energy absorption and consequent greater penetration reaching bone tissue (45) and greater bone density, especially after 7 days, associating the satisfactory effects of laser therapy with time and wavelength (46). The 830nm laser applied to the tibiae for bone reconstruction seems to intensify osteogenic activity with the formation of new bone permeated by great vascularization in intermediate (13 days) and final (25 days) stages (47).

The long bones have physiological characteristics that can help research for bone reconstruction processes. Tension forces applied by the body weight are mentioned, the presence of a good periosteum assisting the local protection process avoiding excess penetration of the nearby tissue, in addition to the ease of making the defect and, at the same time, less harmful to health compared to cranial defects. Osteotomies of 2 to 3.5 mm evaluated in periods of 15 to 30 days seem to be suitable for observing the biological behavior of drugs and materials, since the complete thickness of the defect area has not been completely repaired when using only a clot (48).

New analyzes with biomarkers and biomechanical functionality could deepen the findings of histomorphometry, as mentioned in article 2, and can be considered as limiting factors of this research. As perspectives, future studies with different concentrations of HFB and specific analysis of collagen fibers may improve the evaluation of the osteogenic capacity of the association of the biopolymer.

4 Conclusion

4 CONCLUSION

The literature consulted on PBMT associated with bovine bone for the treatment of bone reconstructions scores positive results, however there is a diversity of parameters, in addition to the absence of important data that apparently can interfere with the reproducibility of research and consequently on its effects.

The interaction between PBMT and the biocomplex composed of the heterologous fibrin biopolymer mixed with lyophilized bovine bone matrix is potentially helpful in the reconstruction of rat tíbia.

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APPENDIXES

APPENDIX A – Declaration of exclusive use of the article in thesis signed by the authors of the article 1: **“Photobiomodulation Therapy (PBMT) Applied in Bone Reconstructive Surgery Using Bovine Bone Grafts: A Systematic Review”.**

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article **“Photobiomodulation Therapy (PBMT) Applied in Bone Reconstructive Surgery Using Bovine Bone Grafts: A Systematic Review”** will be included in Thesis of the student Marcelie Priscila de Oliveira Rosso 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 28th, 2020.

Marcelie Priscila de Oliveira Rosso

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APPENDIX B – Declaration of exclusive use of the article in thesis signed by the authors of the article 2: **“Photobiomodulation Therapy Associated with Heterologous Fibrin Biopolymer and Bovine Bone Matrix Helps to Reconstruct Long Bones”**

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article **“Photobiomodulation Therapy Associated with Heterologous Fibrin Biopolymer and Bovine Bone Matrix Helps to Reconstruct Long Bones”** will be included in Thesis of the student Marcelie Priscila de Oliveira Rosso 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 28th, 2020.

Marcelie Priscila de Oliveira Rosso
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Sandra Maria Barbalho

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Murilo Priori Alcalde

Author | Signature

Marco Antonio Hungaro Duarte

Author | Signature

Jesus Carlos Andreo

Author | Signature

Rogério Leone Buchaim

Author | Signature

Annexes

ANNEX

Annex 1: Authorization of the publisher when article accepted for publication

Journal Materials and Journal Biomolecules, both are part of MDPI group.

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

USP

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

Authorization for availability in repository

Fancy.Zhai <fancy.zhai@mdpi.com> 18 de março de 2020 22:37
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Annex 2: Approval of Animal Ethical Committee

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Uso de Animais

CEUA-Proc. Nº 006/2019

Bauru, 25 de setembro de 2019.

Senhor Professor,

Informamos que a proposta de pesquisa intitulada ***"A influência da terapia por fotobiomodulação em defeito ósseo preenchido com matriz óssea bovina associada ou não ao selante heterólogo de fibrina"***, registrada sob *CEUA-Proc. Nº 006/2019*, tendo Vossa Senhoria como Pesquisador Responsável, foi analisada e considerada APROVADA em reunião da Comissão de Ética no Uso de Animais (CEUA), realizada no dia 20 de setembro de 2019.

Finalidade	*( ) Ensino (x) Pesquisa Científica*
Vigência da autorização:	*01/10/2019 a 30/03/2020*
Espécie/Linhagem:	*Rattus Norvegicus / Wistar (blocos de parafina com espécimes oriundos do Prot. CEEPA 011/2016*
Nº de animais:	*Não se aplica (utilização de peças incluidas em parafina)*
Peso/Idade	*250g/90 dias*
Sexo:	*Macho*
Origem:	*Amostras obtidas no Prot. CEEPA 011/2016*

Esta CEUA 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 Uso de Animais

Prof. Dr. Rogério Leone Buchaim
Docente do Departamento de Ciências Biológicas

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