In Vivo Evaluation of Bovine Xenograft Associated With Oxygen Therapy in Alveolar Bone Repair

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To preserve alveolar bone thickness and width after extraction, clinical strategies have been adopted to reduce or eliminate the need for future surgical interventions to increase the alveolar ridge. The use of xenogeneic biomaterials has been increasing for such application. The association of bone substitutes with active oxygen-based materials, which is essential in the wound-healing process, could accelerate bone repair, optimizing the maintenance of alveolar architecture after extraction. However, the truth of this hypothesis is not clear. The present study aimed to compare the biological response to inorganic bovine bone graft Bonefill (BF), associated or not with active oxygen-based oral gel Bluem (BF+BM), in alveolar bone repair. Twenty female Wistar rats were randomly allocated. The left upper central incisor was extracted, and the dental sockets were filled with BF in the control group (n = 10) and with BF+BM in the experimental group (n = 10). The animals were euthanized at 7 and 42 days after implantation (n = 5), and the samples were processed for descriptive histological and histomorphometric evaluations. The results showed no significant difference between the groups ($P > .05$). Both groups presented a time-dependent increase in newly formed bone and biosorption biomaterial ($P = .0001$). The association between active oxygen-based gel and inorganic bovine bone graft did not interfere with or improve bone repair during the experimental periods of alveolar bone repair in rats.

Key Words: biocompatibility, rats, bone repair, biomaterial, oxygen therapy

INTRODUCTION

Clinical strategies such as guided bone regeneration, bone grafts, and dental implants, used either separately or in association, have been adopted to reduce or eliminate future surgical interventions to increase the alveolar ridge.[1,2] Intense scientific investigation has been conducted on bone substitutes with bio-absorption rate and bone regeneration capacity similar to that of autogenous bone. Hydroxyapatite is undoubtedly the most explored compound in this respect. Finding bone substitutes with bone-regeneration properties is a biomedical challenge; hence, there has been a growing interest in biomaterials that are similar to mineralized tissues, especially synthetic materials that mimic natural human bones.[3,4]

In recent years, the use of xenogeneic biomaterials in dentistry has increased to promote the maintenance of alveolar architecture after extraction. Xenograft materials are obtained from different species than the recipient and are predominantly made from the inorganic portion of bone tissue.[5,6] Bovine biomaterials present both biocompatibility and osteoconductivity, and these important biological properties allow an apposition of newly formed bone from osteoprogenitor cells located at the border of the host tissue.[7] The advantages of this material also include high predictability, high survival rate, and important preservation of the alveolar walls compared with nonalveolar preservation.[5]

The global implant biomaterials industry offers a large number of similar bone substitutes that claim to have the "best" biological properties for bone repair. However, regulators and professionals must have evidence showing the efficacy of these products through in vivo studies with standardized protocols, to reduce controversy and the use of inefficient products.[8]

Bonefill is an inorganic xenogeneic biomaterial obtained from the cortical and spongy portion of bovine bones. Its mineralized inorganic bone matrix contains a macro- and microporous structure, similar to cortical and spongy human

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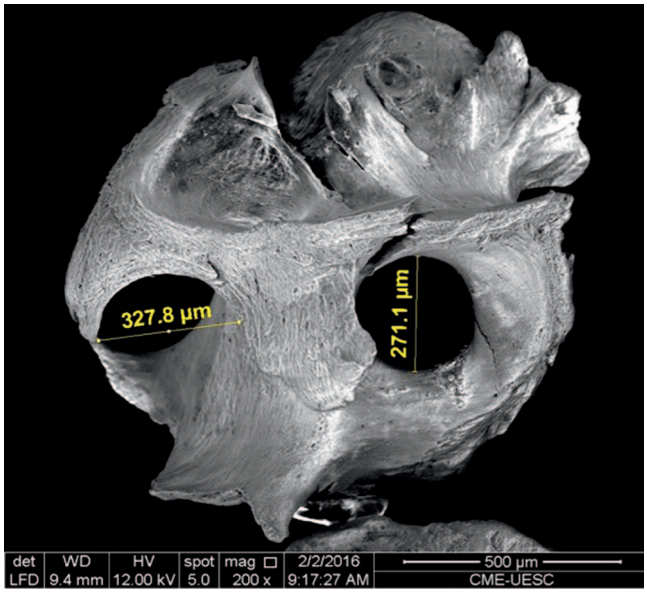


Figure 1. Scanning electron microscopy of inorganic bovine bone graft Bonefill (BF). Note the presence of interconnected pores between 270 and 330 µm that may favor the transport of substances such as Bluem as well as the proliferation of osteogenic cells and capillary systems during endothelial proliferation.

bones. For being remodeled by the action of osteoclasts and osteoblasts, this biomaterial is considered to be a viable alternative to autologous bone when used in bone defects.[9]

Oxygen is important for intracellular processes such as biosynthesis, movement, and transport. Energy is essential for cell function and survival because it increases the rate of cell proliferation and reepithelialization. The oral gel Bluem (Bluem Care) is an active oxygen-based gel that was developed to treat periodontitis and eliminate harmful bacteria, thereby reducing and preventing gum inflammation. The oxygen-based gel increases cell metabolism and has presented interesting results in inflammation repair, accelerating the healing process in wounds and oral ulcers, relieving pain, and reducing pathogenic bacteria.[10]

We hypothesized that the association between bovine bone graft and the active oxygen-based gel, which is essential in the wound-healing process, could accelerate and improve bone repair after tooth extraction. In this study, Bluem gel was mixed with Bonefill Porous for alveolar filling.

Therefore, the present study aimed to compare the biological response to inorganic bovine bone graft Bonefill (BF), associated or not with active oxygen-based gel oral gel Bluem (BF+BM), in alveolar bone repair in rats.

Materials and Method

Biomaterials

In this study, the association between 2 different biomaterials was evaluated for alveolar bone repair in rats: bovine bone graft (Bonefill Porous, Bionnovation Biomedical; $0.10 < \varnothing < 0.60$ mm, BF group) (Figure 1), with or without active oxygen-based gel (oral gel Bluem, Bluem Care; BF+BM group). Both products are commercially available.

In vivo analysis

Ethical Aspects

This study followed the Brazilian Guidelines on Animal Use for Scientific and Educational Purposes – DBCA (National Animal Experimentation Control Board – CONCEA – 2013) and was previously approved by the Animal Ethics Committee of the Universidade Federal Fluminense (CEUA/UFF No. 949). Moreover, the procedure was carried out in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments)[11] and PREPARE guidelines (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence).[12]

The animal models were selected in accordance with the guidelines of the 3Rs Program (Reduction, Refinement, Replacement), which aims, besides reducing the number of animals during experimentation, to minimize pain and discomfort. Also, in accordance with the guidelines of the 3Rs Program, the sample size was selected using the minimum number of animals necessary to perform the normality test.[12–15] The sample size was adopted by power test analysis on the website https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html (adapted from Hassumi et al[16]). A level of significance of 5% and a power test of 80% were used, which suggested 4 animals/group. Thus, taking into consideration possible animal loss, we used 5 animals/group in each experimental period.

Animals

This study included 3-month-old, skeletally mature, female Wistar rats (*Rattus norvegicus;* $n = 20$) that weighed approximately 300 g. The animals were provided by the Laboratory of Animal Experimentation of the Laboratory Animal Center (LEANAL), Universidade Federal Fluminense (UFF), Niterói, Brazil. The animals were randomly divided by the principal investigator using an opaque envelope that contained the following group names: group 1 (BF) and group 2 (BF+BM), with $n = 5$/experimental period. Rats in group 1 were surgically treated with bovine graft fine-grained Bonefill Porous (Bionnovation Biomedical; BF). Group 2, the experimental group (BF+BM), received bovine graft fine-grained Bonefill Porous mixed with Bluem gel (Bluem Care). The rats were examined at 7 and 42 days after surgery.

Animal Welfare

The animals were kept in the same laboratory in mini-isolators containing 2 animals per box. Each mini-isolator was lined with dry wood shavings (pine wood shavings), a completely harmless, nontoxic, and inedible material that was replaced daily to provide favorable conditions for animal health and welfare as well as serving as a thermal insulator and reducing heat conduction from the body of the animals.

The animals' diet was based on a standard diet consisting of granulated feed (Nuvilab), which was changed daily to prevent fungal formation and proliferation by prolonged

exposure of the food. Water was supplied ad libitum through a glass container with a stainless-steel spout.

The temperature of the room was maintained between 20 and 22°C, which was ideal for the growth of the animals, with a controlled light cycle (12-hour light and 12-hour dark) to provide the correct metabolic cycle. An experienced veterinarian provided nutritional recommendations and oversaw the care and pre- and postoperative fasting of the animals. To minimize the effects of subjective bias when allocating animals to treatment, they were randomly assigned to the groups using a system of opaque envelopes.

Surgical procedure

The animals were anesthetized with an intramuscular administration of 75 mg/kg ketamine hydrochloride (Ketalar, Veltbrands, São Paulo, SP, Brazil) and 1.5 mL/kg xylazine hydrochloride (Rompun, Veltbrands, São Paulo, SP, Brazil). They were placed on the operating table in ventral decubitus and had their perioral region disinfected with chlorhexidine solution 2% (Riohex 2%); their oral mucosa was then disinfected with chlorhexidine oral solution 0.12% (Riohex 0.12%). The animals then underwent syndesmotomy and extraction of the left upper central incisor using a dental explorer No. 5 (Duflex) and pediatric forceps number 151 (Duflex), respectively. The surgical procedure was conducted carefully to cause minimal damage and to preserve the alveolar walls. Subsequently, 0.2 mg of the biomaterials was implanted in the alveolar cavity according to each group's protocol, followed by simple suture for primary wound closure (Ethicon, Johnson & Johnson) and final cleaning with 10 volumes of hydrogen peroxide (Farmax).

After surgery, the animals received subcutaneous meloxicam 1 mg/kg (Duprat) for postoperative analgesia, which was repeated every 24 hours for 3 days. They were examined daily to evaluate and record any postoperative complications.

Sample Collection

At 7 or 42 days after the surgery procedures, 20 animals ($n = 5$ per group) were euthanatized by anesthesia overdose. After that, the samples containing the biomaterials were collected for histological processing.

Histological processing

Histological processing followed the criteria established by the Laboratory of Applied Biotechnology (LABA-UFF) and Associate Laboratory for Clinical Dental Research (LPCO), both at Universidade Federal Fluminense (UFF). After euthanasia, the blocks containing the alveolar socket with implanted biomaterials were fixed in a 3.7% buffered formaldehyde for 24 hours. Due to the irregular alveolar socket morphology, the region of interest was obtained. The socket was sectioned in the transverse direction into 3 parts, and the middle third was used for evaluation. The EXAKT cutting system (Exakt 310 CP series; Exakt Apparatebau) was used for the above procedure. Sectioned samples were treated using the Allkimia decalcifying solution for 7 days. After decalcification, the samples were histologically processed via embedding in paraffin, cut into 5-μm-thick sections, and stained with Masson's trichrome protocol for light microscopy assessment.

Histological Evaluation

A light microscope (OLYMPUS BX43) was used for descriptive histological analysis of the slides. Selected images were captured using a camera coupled to the microscope (OLYMPUS® SC100), associated with high-resolution software (CELLSENS 1.9 Digital Image). A 4× objective lens was used for a broader view of the area. A 40× magnification lens was used for better cellular and histological visualization. The reaction of the cells around the biomaterials was observed, focusing on the intensity and nature of the inflammatory response, the presence of connective tissue, newly formed bone in direct contact with the graft, and remaining biomaterial. In addition, the histological evaluation was conducted by an experienced pathologist who was blinded through coded slides.

Histomorphometric Evaluation

Five nonsuperimposing photomicrographs were captured from each slide, at 40× magnification, corresponding to the regions of dental socket surrounding the implanted biomaterials. The histomorphometric evaluation was conducted using Image-Pro Plus 6.0 software (Media Cybernetics) to generate a grid of 250 points, which allowed quantification of the volume density of newly formed bone, the residual biomaterial, and connective tissue.

Statistical Evaluation

The obtained data were considered normal by Shapiro-Wilk normality test. A quantitative description of the volume density of newly formed bone, biomaterial, and connective tissue was conducted by parametric description, with a P value of $<.05$ considered significant. A Student t test was applied to analyze the differences between the 2 treatments and experimental periods. The calculations were performed using Prism Graph Pad 8.0 software. The statistical evaluation of this study was reviewed by an independent statistician.

Results

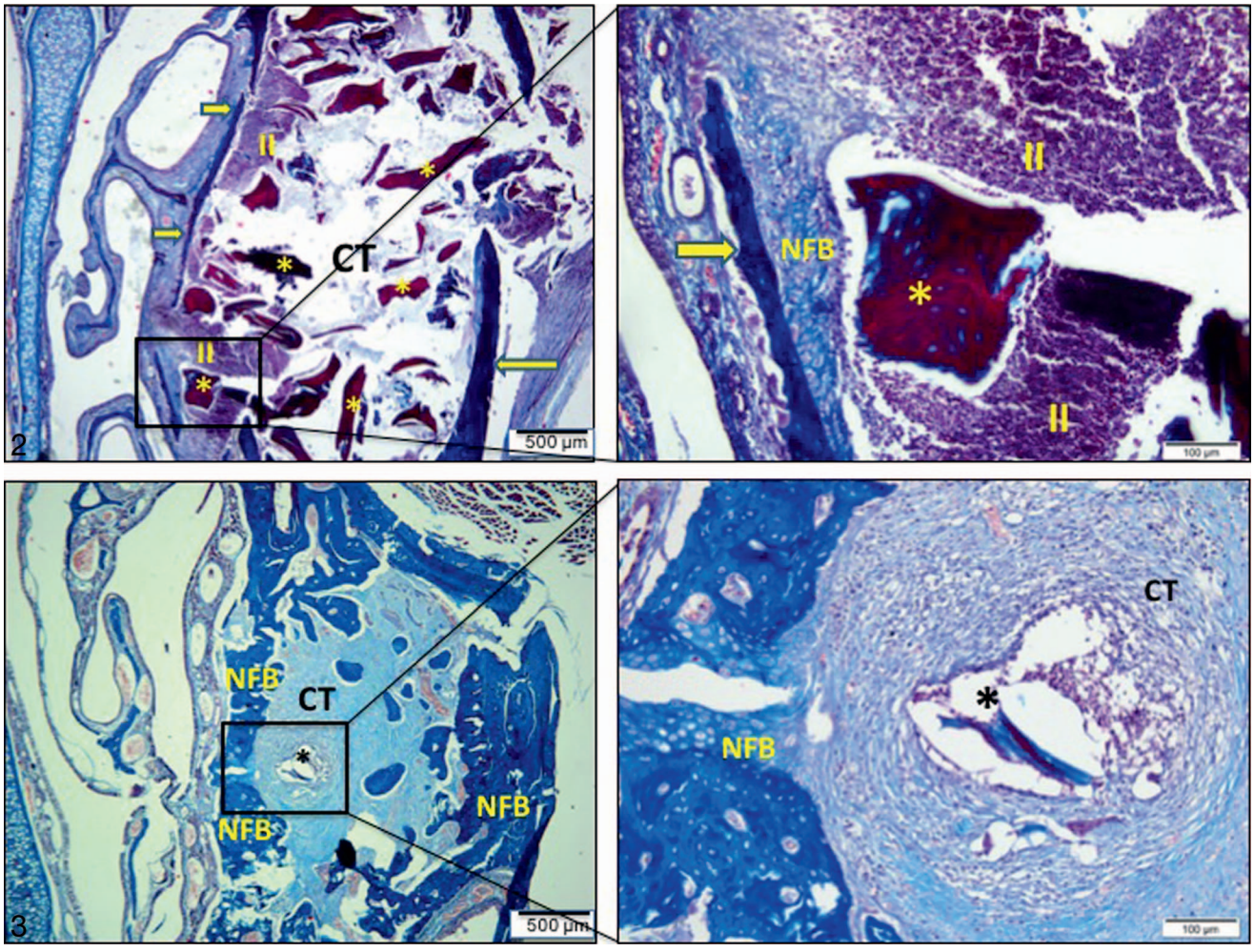
The experimental periods were uneventful. All animals survived the experimental period and remained in good health during the healing period, and there was no sign of inflammatory response that could be associated with the alveolitis process. The surgical procedures and follow-ups presented no complications regarding technical conditions, postoperative infection, or other clinical concerns. No animals were excluded from the study.

Descriptive histological evaluation

The histological results of the middle third of the alveolar socket at 7 and 42 days after surgery are shown in Figures 2 through 5.

BF Group

At 7 days after implantation, the area of defect was filled with connective tissue, showing fragmented biomaterial particles, hemorrhagic exudate, and scarce inflammatory infiltrate. Along the margins of the defect, some immature trabecular



Figures 2 and 3. Figure 2. Representative photomicrographs of the biological response to treatment placed in the alveolar socket after 7 days in the inorganic bovine bone graft Bonefill® (BF) group. The small square is displayed at 20-fold magnification adjacent to the figure with lower magnification (4×). Preexisting bone (yellow arrow); connective tissue (CT); newly formed bone (NFB); inflammatory infiltrate (ii); remaining biomaterial (*). Histological section stained with Masson's trichrome. **Figure 3.** Representative photomicrographs of the biological response to treatment placed in the alveolar socket after 42 days in the inorganic bovine bone graft Bonefill (BF) group. The small square is displayed at 20-fold magnification adjacent to the figure with lower magnification (4×). Connective tissue (CT); newly formed bone (NFB); remaining biomaterial (*). Histological section stained with Masson's trichrome.

bone was seen proceeding to the center of the defect (Figure 2).

At 42 days, small particles of biomaterial (and in a smaller amount than observed at 7 days) were noted. Fibrocellular connective tissue filled the center of the defect, showing some islands of bone trabeculae. Along the margins of the defect, newly formed bone tissue was seen, proceeding toward the center of the defect (Figure 3). An increase in newly formed bone was observed compared with 7 days after implantation.

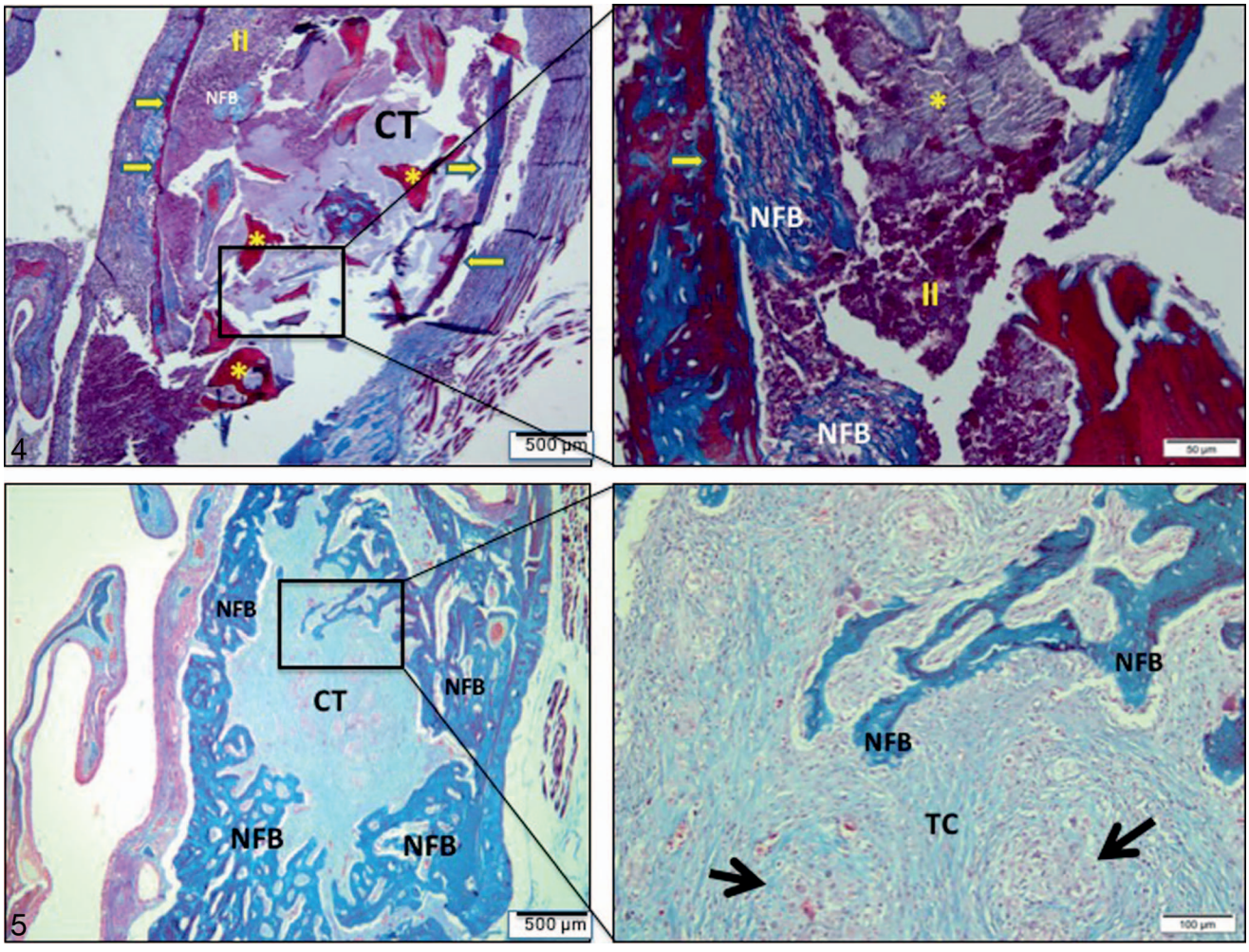
BF+BM Group

At 7 days after the surgical procedures, granules of the biomaterial were observed interspersed with the connective tissue, showing areas of moderate inflammatory infiltrate with hemorrhagic exudate and amorphous basophilic material, suggesting a gel. The presence of newly formed bone was observed in the periphery of the defect (Figure 4).

At 42 days, the area of the defect was filled with fibrocellular connective tissue in the middle part, and there were small islands of granulation reaction, in addition to a scarcity of mononuclear inflammatory infiltrates. There was an important reduction in the volume of biomaterial compared with the previous period. Higher areas of mature bone trabecular were observed compared with 7 days following healing from the periphery to the interior of the defect (Figure 5). Bone repair at 42 days appeared to be similar between the groups.

Histomorphometric results

Through the qualitative analysis of the histomorphometric results, it was possible to compare the progress of different



Figures 4 and 5. Figure 4. Representative photomicrographs of the biological response to treatment placed in the alveolar socket after 7 days in the active oxygen-based gel oral gel Bluem (BF+BM) group. The small square is displayed at 20-fold magnification adjacent to the figure with lower magnification (4×). Preexisting bone (yellow arrow); connective tissue (CT); newly formed bone (NFB); inflammatory infiltrate (ii); remaining biomaterial (*). Histological section stained with Masson's trichrome. **Figure 5.** Representative photomicrographs of the biological response to treatment placed in the alveolar socket after 42 days in the active oxygen-based gel oral gel Bluem (BF+BM) group. The small square is displayed at 20-fold magnification adjacent to the figure with lower magnification (4×). Connective tissue (CT); newly formed bone (NFB); inflammatory infiltrate (black arrow); remaining biomaterial (*). Histological section stained with Masson's trichrome.

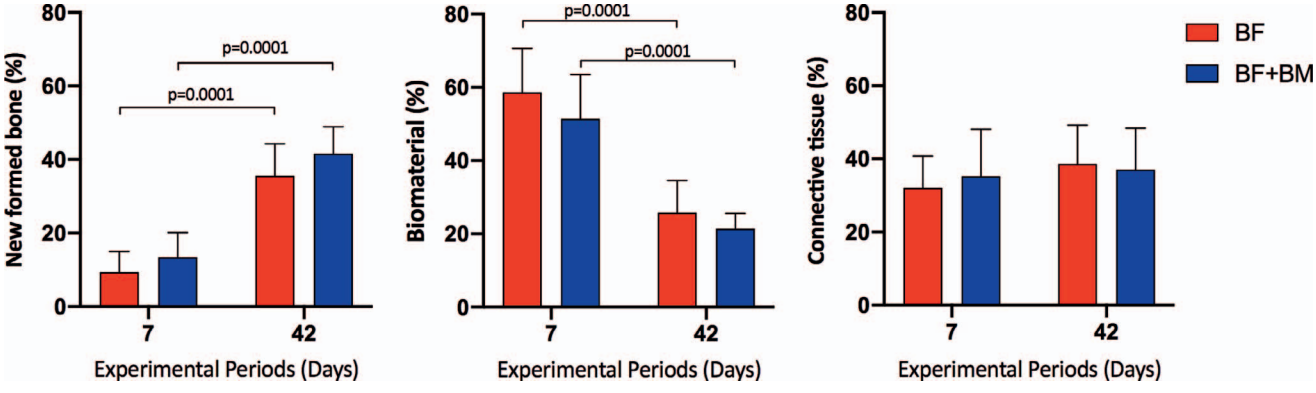


Figure 6. Volume of newly formed bone (NFB), biomaterial (BM), and connective tissue (CT) (%) of the inorganic bovine bone graft Bonefill (BF) and active oxygen-based gel oral gel Bluem (BF+BM) groups (n = 5) after 7 and 42 days. The results are presented as mean ± confidence interval. Horizontal bars represent the statistical differences between the same group at different experimental periods and its respective *P* values. There were no differences between treatments at same experimental period. Student *t* test, $P < .05$.

parameters studied: newly formed bone, biomaterial, and connective tissue at 7 and 42 days after implantation (Figure 6).

The dental sockets of the BF and BF+BM groups 7 days after surgery presented low levels of newly formed bone, and there were no statistically significant differences between the groups (9.4% ± 4.56 and 13.4 ± 5.45, respectively). During this experimental period, there were also no differences between biomaterial (BF, 58.6 ± 9.71; BF+BM, 51.4 ± 9.76) and connective tissue (BF, 32 ± 7.03; BF+MB, 35 ± 10.37).

At 42 days after implantation, we observed a time-dependent increase in newly formed bone in the BF (35.6 ± 6.98) and BF+BM (41.6 ± 5.89) groups as compared with the previous period (7 days; $P = .0001$). The reaction was inversely proportional in the biomaterial parameter. There was a time-dependent reduction in the volume of biomaterial in the BF (25.8 ± 7.08) and BF + BM (21.4 ± 3.43) groups at 42 days as compared with at 7 days ($P = .0001$). However, there were no differences between groups in the same experimental period. For the connective tissue parameter, no statistically differences were observed between groups (BF, 38.6 ± 8.53; BF+BM, 37 ± 9.16) and when compared with the previous period.

Discussion

The present preclinical study compared the biological response to a commercially available bovine bone graft, used with active oxygen-based gel, or used alone, at 7 and 42 days after implantation in alveolar bone repair in rats.

To understand the biocompatibility (safety) and the influence of Bluem oral gel on bone repair, the authors used the preclinical model in the rats' dental sockets. Considering the concept of translational research and according to the results, a phase 1 clinical study could be conducted to evaluate the safety and efficacy of these materials in humans. The animal model used in the present study can be regarded as a significant limitation. There are known disadvantages to using animal models for validating the results to humans externally. However, the authors did not intend to extrapolate the results of this study to humans but rather to understand how the materials could behave after implantation. Thus, the presented evaluation aimed to evaluate the materials' local effect on the rats' dental sockets with 4wall in an infection-free model using the commercially available active oxygen-based oral gel Bluem. Nevertheless, this work presents scarce scientific evidence on the healing process and specifically in bone repair.

Alveolar bone healing after upper incisor extraction in rats is considered a classical model of preclinical studies for the evaluation of the biocompatibility of biomaterials.[16] In addition, this model is small and inexpensive and facilitates experimentation. The use of animals with more significant physiological, anatomical, and organic similarities is also vital for producing reliable results that can be applicable to humans in the future.[16] We chose the alveolar socket model because, according to the ISO 10993-6/Annex D, equivalent anatomical sites should be used according to the end use of the material.

The healing periods evaluated in this study were selected because they show the beginning of the cicatricial response (7 days) and a later stage of bone repair. Therefore, these intervals have been used in previously reported studies.[12,13,17,18] The 7-day postimplantation represents coagulum formation and cell proliferation from the connective tissue, whereas the 42-day period represents the period with significant new-formed bone.[19]

Bone-regeneration procedures to preserve, increase, or reconstruct the height, thickness, and quality of the alveolar ridge, immediately after dental extraction, seem to be essential. This reduces the need for later grafting by simplifying and optimizing in situ rehabilitation.[20] The presents study evaluated the bone repair of dental sockets after different treatments conducted through 2-dimensional (2D) analysis: histology and histomorphometry. The 2D evaluation may be considered a limitation of the study, as computed microtomography analysis may be associated with investigating a 3-dimensional parameter of bone healing.

Despite this limitation, through histological evaluation, we were able to evaluate the response to the material with and without active oxygen-based gel (Bluem oral gel) on bone repair. To increase the strength of the results, we conducted histomorphometric analysis, with a protocol already described in the literature,[21–23] to obtain dynamic reactional tissue up to 7 and 42 days after biomaterials implantation and presented its analytical performance. The results of the presented study show a time-dependent increase in newly formed bone in the dental socket in both experimental groups; however, the clinical and histological evaluation did not show differences between the tested groups.

The inorganic bovine bone used in this study is a highly purified mineral structure produced from natural bone through a multiphase process, which complies with the safety regulations advocated by the control agencies. The fresh bone is ground by receiving a sequence of baths that solubilize the organic structures, such as remaining cells, fibers, and proteins. Only the mineral portion remains, avoiding the induction of possible immunogenic processes into the organism.[9]

A previous study[9] suggested that the biomaterial used in this evaluation is partially remodeled through the action of osteoclasts and osteoblasts and is a viable alternative to autologous bone in defects, for which its use is indicated. The presence of biomaterial particles in a blood clot helps to anchor the fibrin network, preventing the clot from retraction. In the granulated form, this biomaterial acts in an osteoconductive mechanism, favoring bone growth and regeneration. Its mineralized inorganic bone matrix has a porous macro- and microstructure, similar to human cortical and spongy bones (Figure 1). The literature suggests that the presence of macropores in a biomaterial structure favors bone cell and vascular insertion, adhesion, proliferation, and differentiation,[24] supporting our finding of osteoconduction presented by the biomaterial after 42 days of implantation in both tested groups, probably due to the large volume of interconnecting pores.

The active oxygen-based oral gel Bluem is a commercially available active oxygen-based gel that proposes to increase antibacterial activity, whereas low concentrations of sodium perborate and glucose oxidase, an enzyme found in honey, increases active oxygen in wounds, thereby promoting faster healing. Its formula contains low concentrations of hydrogen peroxide, consequently increasing the effective elimination of pathogenic bacteria, as has already been described in the

literature. The active oxygen-based gel delivers active oxygen (H_2O_2) directly to the surgical site. Once in contact with saline solution, sodium perborate is converted into sodium borate at low concentrations of 0.003%–0.015%. In this form, hydrogen peroxide has a disinfectant action, a chemostatic effect on leukocytes, and an antibacterial action on normal wound fluid during the neutrophil respiratory burst.[10] From this context, the active oxygen-based gel may reduce both infection during surgery and postoperative risk factors, accelerating the healing process.

In the current study, no abscess, tissue necrosis, gingival scarring, or exacerbated inflammatory reaction was observed in the postoperative periods in either group, and the presence of the active oxygen-based gel did not interfere with bone repair. From the results presented in this study, the biocompatibility of the active oxygen-based oral gel Bluem was confirmed. In addition, use of the gel did not interfere with alveolar bone repair in a preclinical model of rats. However, because there are only a few studies on the use of Bluem gel, future studies should be conducted to provide new data to corroborate its effectiveness in clinical use. Our findings may support a study with infected sites in preclinical studies or phase 1 clinical research.

According to the results presented in this study, it is possible to define the active oxygen-based oral gel Bluem as a biocompatible biomaterial in rats' dental sockets with 4 walls in an infection-free model. From these findings, promising perspectives can be associated with active oxygen-based gel associated with a mineralized bovine bone to preserve alveolar ridge after extraction. Meanwhile, future research is required to overcome the limitations of the current study.

Conclusion

Based on the obtained results, it is possible to conclude that the association between an active oxygen-based gel and inorganic bovine bone graft did not interfere with or improve bone repair during the experimental periods of bone healing in the ratsdental sockets. Future studies should be conducted using other research models to validate the results to humans externally.

Abbreviations

BF: inorganic bovine bone graft Bonefill

BF+BM: active oxygen-based gel oral gel Bluem

Acknowledgments

Jose Mauro Granjeiro, PhD, senior researcher, INMETRO, served as an independent statistician for this study.

Note

There are no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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