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RESEARCH ARTICLE

Evaluation of the Osteogenic Potential of a Three-Dimensional Bioimplant for the Treatment of Bone Defects: Innovations in bone regeneration

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ABSTRACT

Introduction: Massive bone defects (MBD) represent a significant clinical challenge due to the difficulty in achieving effective bone consolidation. This study evaluates the osteogenic potential of a three-dimensional bioimplant composed of demineralized bone matrix (DBM), collagen, hydroxyapatite (HAp), and bone marrow nucleated cells in animal models.

Methods: An experimental study was performed in 45 lambs (Ovis aries) with bone defects in the proximal tibia. Three groups were compared: (1) three-dimensional bioimplant, (2) bioimplant with autologous bone marrow nucleated cells, and (3) bone allograft. Bone regeneration was assessed by radiological, histological, histochemical, and immunohistochemical analysis.

Results: The group treated with the bioimplant, and nucleated cells showed greater osteoid formation and osteoblastic activity compared to the other groups. PAS stain positivity and the presence of osteoblasts (PTHR1+) were significantly higher in this group ($p<0.001$).

Conclusion: The combination of a three-dimensional bioimplant with nucleated bone marrow cells significantly improves bone regeneration compared to the use of a cell-free bioimplant or an allograft, suggesting a promising alternative for the repair of massive bone defects.

1. Introduction

Massive bone defects (MBD) represent a significant clinical challenge due to the complications associated with their treatment and the limited capacity of conventional methods to efficiently restore bone function. A MBD is defined as a substantial loss of bone tissue greater than 25 mm, which is frequently accompanied by the main complication: the lack of bone consolidation or union at the affected site, which prevents a complete recovery of the patient [3, 5]. This condition considerably affects the quality of life and functionality of the patient, generating a growing demand for more effective therapeutic options.

There are various therapeutic approaches to treat DOM, including autografts, isografts, allografts, xenografts, specialized prostheses, and combinations of these methods [18, 19, 20, 21, 22, 23, 24, 26, 27]. Each of these alternatives has advantages and limitations, which have been widely documented in the scientific literature [18, 19, 20, 21, 30]. Although autografts remain the treatment of choice due to their osteogenic, osteoinductive, and osteoconductive capacity, options such as allografts and xenografts continue to be considered in specific situations due to their availability and compatibility characteristics. In recent years, tissue bioengineering has emerged as a promising discipline, offering new possibilities for the treatment of DOM [25]. This interdisciplinary area focuses on the design of bioactive implants that promote bone regeneration by replicating the physiological microenvironment. The fundamental principles of tissue bioengineering include: (1) the adequacy to the biological requirements of the implant, (2) the correct bioactive composition, and (3) the structural properties that favor the integration and functionality of the implant [6].

In 2017, we conducted a study in which we designed a three-dimensional bioimplant composed of demineralized bone matrix (DBM), collagen, hydroxyapatite (HAp), and bone marrow-derived cells. This bioimplant showed promising results by promoting osteoconduction, osteoinduction, and osteogenesis in animal models, demonstrating its ability to effectively integrate and regenerate bone in experimental bone defects [9].

The present study aims to evaluate whether the use of a three-dimensional bioimplant, based on the above-mentioned components, improves the speed and quality of bone integration compared to allografts, in the treatment of DOM in animal models. The bioimplant in question was developed from bioactive materials specific to bone tissue, with the purpose of more accurately replicating the microenvironment necessary for bone regeneration. In addition, we seek to compare the effectiveness of this implant in terms of acceleration and quality of integration in the recipient bone, establishing its potential as an advanced alternative to traditional methods. This study also expands the scope of our previous research, incorporating a greater number of animal models for a more robust evaluation of the results obtained.

2. Material and methods.

STUDY DESIGN.

This is an experimental, longitudinal, prospective, comparative study, with cases and controls.

SAMPLE SELECTION.

45 female lambs (Ovis aries) aged 4 to 5 months and weighing 20 to 30 kg were used. The inclusion criterion was that there were no lambs of ages or weights outside the range of characteristics described at the time of acquisition. The sample size was calculated by estimating a certain parameter with a desired confidence interval in relation to our previous study by Cuervo-Lozano et al. (2017) [9, 16]

PREPARATION OF THE THREE-DIMENSIONAL BIOIMPLANT.

Three different compounds were used to create the three-dimensional bioimplant: MOD, collagen and HAp. The first material used for the bioimplant was MOD, which was obtained by demineralizing the diaphysis of the long bones of the hind limbs of three donor lambs, following the method previously described by Rivera et al. (2003).

The second component was collagen. Collagen was used to provide rigidity to the implant and maintain its desired shape, as well as making it insoluble when it encounters body fluids. The collagen was extracted from the skin of pig fetuses through a process that consisted of the bioimplant being prepared by mechanically grinding the skin in a meat grinder, followed by the addition of a 0.5% hydrochloric acid (HCl) solution in distilled water.

The third component for the bioimplant was HAp. Since bone is a tissue with a nanostructure, HAp nanoparticles were selected to achieve a porosity of less than 100 nm. HAp is the main inorganic component of bone and, in the form of nanoparticles, improves the microenvironment and increases the surface available for cell growth. HAp nanoparticles were processed from discarded eggshells using a hydrothermal synthesis process, following the methodology described by Elizondo-Villarreal et al. (2012).

The proportion in which these materials were mixed was 30% MOD, 60% collagen and 10% HAp. This mixture was placed in prefabricated 5 cc molds with specific shape and size, then dried and sterilized in doses of 20–22 kg.

DESCRIPTION OF THE SURGICAL TECHNIQUE.

The surgical technique was based on our previous study by Cuervo-Lozano et al. (2017) [9, 16]. Forty-five female lambs (Ovis aries) aged 4 to 5 months and weighing 20 to 30 kg were used. A bone defect was surgically created in the proximal diaphysis of the left tibia of each lamb, using an oscillating saw and a metal guide specifically designed for this procedure. The bone defect spanned 75% of the cortical circumference and was 5 cm long. The defects were treated in three different ways; animals were randomly assigned to one of three groups, each consisting of fifteen lambs. The first group was treated exclusively with the three-dimensional bioimplant. In the second group, the three-dimensional

bioimplant was used in combination with autologous nucleated cells; Before placement, the bioimplant was immersed for 5 minutes in a container with cells obtained by aspiration of the iliac crest, which were processed by a special centrifugation process performed a few hours before surgery. In the third group, the defect was treated with a frozen allograft. In all cases, internal fixation was applied with a special plate and screws to secure the allograft in position.

The lambs remained in the vivarium for 5 days for wound monitoring and administration of antibiotics (20 mlU of procaine benzylpenicillin), without restriction in limb support, and with unlimited access to water and food. From the sixth day onwards, the animals were transferred to a common habitat.

The entire framework of the protocol was carried out with prior authorization from the Bioethics Committee of the Faculty of Medicine of the Autonomous University of Nuevo León in Monterrey, Nuevo León, Mexico. The protocol was approved in October 2023 and the actions of the protocol were carried out in the period October 2023 - September 2024, from the drafting of the protocol, its authorization and the final writing of this study.

The experiments were carried out in accordance with the International Guidelines for the Appropriate Use of Experimental Animals and the Mexican Standard NOM-062-ZOO-1999 on Technical Specifications for the Production, Care and Use of Laboratory Animals (SAGARPA, 1999).

RADIOLOGICAL ANALYSIS.
In radiological analysis, anteroposterior and lateral radiographs of the limb were taken at 1-, 6-, and 12-weeks post-treatment, and the appearance of massive bone defects in the tibiae of the study groups was evaluated.

HISTOLOGICAL AND HISTOCHEMICAL ANALYSIS.
The animals were sacrificed with sodium pentobarbital (90–210 mg/kg of weight, intravenous administration) at 12 weeks to obtain the studied bone segment for morphological analyses. After sample collection, they were fixed in a 4% paraformaldehyde solution in 1X phosphate-buffered saline [PBS], pH 7.2–7.4 for 24 h. Subsequently, a 1 cm thick segment was collected and treated with the decalcification technique with 10% HCl for 21 days, changing the solution every three days, observing that the bone showed a soft consistency. Then, the samples were processed by conventional histological techniques until their inclusion in paraffin blocks.

The general cellular characteristics, the presence of bone trabeculae, and the orientation and organization of the collagen fibers were evaluated in histological sections (4 µm thick) stained with hematoxylin and eosin [H&E] and Mallory-Azan tricolor [M-AT] for histological analysis.

Additionally, the periodic acid-Schiff [PAS] staining method was used to identify the osteoid components. The samples were evaluated by bright field microscopy.

IMMUNOHISTOCHEMICAL ANALYSIS.
To identify the presence of osteoblasts in the samples of interest, immunohistochemical analysis was performed on 4-µm-thick histological sections immunolabeled with anti-Parathyroid Hormone Receptor R1 [PTHR/PTHR1] polyclonal antibody (aa388-406, LS-C313515 (1:200), LifeSpan Biosciences, Inc., Seattle, WA, USA). An Abcam anti-mouse and rabbit HRP/DAB detection kit (ab64264 Cambridge, MA, USA) was used as the detection system. Positivity was visualized with 3,3′-diaminobenzidine [DAB] and nuclei were identified using Gill's hematoxylin.

MORPHOMETRIC ANALYSIS.
To quantify the percentage of osteoid, the samples were grouped according to the type of treatment without specifying the treatment; 40 consecutive fields in each group were photo documented with a powerful dry objective (40×) in the PAS-positive samples (8 fields/section, 1 section/lamb and 5 lambs/group). High-resolution digital images were obtained with a Nikon Eclipse 50i microscope and a Digital Sight dDS-2Mu image analysis system with NIS-Elements software. The images were then analyzed with ImageJ version 1.49 (National Institutes of Health); the color, tone distribution, saturation and illumination were the same for all images. This morphometric analysis allowed the percentage of osteoid to be determined in the study groups.

Subsequently, in these same images, the intensity [IntDent] of the positivity in the osteoid of each sample was quantified by a microdensitometer. The values obtained were expressed as optical density. The mean value and standard deviation [SD] of all morphometric analysis values were obtained for statistical analysis and for comparison between study groups.
In addition, morphometric analysis of PTHR/PTHR1-positive cells was performed, in which positive cells/field were quantified in 40 fields for each group using the same methodology described previously. Morphological analysis of the samples was performed by two morphology specialists, who were blinded to the treatment used in each sample and blinded to each other.

STATISTICAL ANALYSIS.
Measures of central tendency (mean) and dispersion (standard deviation) were used to report the results of the quantitative variables, while qualitative variables were expressed as frequencies and percentages. Since the samples did not follow a normal distribution, as indicated by the Kolmogorov-Smirnov test, the nonparametric Kruskal-Wallis test was applied to compare the differences between the three groups. In addition, a post-hoc analysis was performed to identify significant differences between the groups. The statistical package IBM SPSS Statistics version 25 (Armonk, NY; IBM Corp) was used for data analysis and a p value < 0.05 was considered statistically significant.

3. Results.

3.1. BIOIMPLANT CHARACTERIZATION

Before implantation, the bioimplant was characterized; we observed collagen fiber bundles with acidophilic staining and cells with basophilic nuclei. These data were confirmed by M-AT staining, showing blue collagen fibers and cells with red cytoplasm. In addition, small

semitransparent granular structures corresponding to DBM and HAp were observed in the bioimplant samples.

3.2. RADIOLOGICAL ANALYSIS

Anteroposterior and lateral radiographs of the limbs were taken at 1 (control), 6-, and 12-weeks post-treatment for radiological evaluation. The radiographs were evaluated by three different orthopedic physicians. They awarded 1 point if the X-ray showed no ossification or integration, 2 points if the ossification or integration was less than 50%, and 3 points if they found more than 50% ossification or integration. The anteroposterior X-ray of the first postoperative week, with the bioimplant and nucleated cells from the autologous bone marrow, showed the bone defect. At week 12, the same limbs showed 100% ossification. No statistical differences were found between the groups.

3.3. HISTOLOGICAL ANALYSIS

In the histological analysis of the implantation site of Group 1 (bioimplant), areas with different organization of collagen fibers and cellular distribution were observed, classified into three zones according to cellular and tissue organization.

• Zone 1: Irregularly organized fibers, with cells between and at the periphery of the bundles, with spaces like small blood vessels between the collagen fibers.

• Zone 2: Wider organization of fibers and cells, with spaces of greater diameter.

• Zone 3: Clear characteristics of an immature bone tissue, with bundles of collagen fibers organized around a cavity with cells inside, like the endosteum of Haversian canals. Acidophilic material representing osteoid was observed.

In Group 2 (bioimplant + nucleated bone marrow cells), the same areas previously described were observed, but with greater cellularity and greater organization of osteon structures. In addition, a greater amount of osteoid was observed compared to Group 1.

In Group 3 (allograft), clear divisions between the laminae of bone tissue were observed, with a distinct organization of the osteons. In this group, endosteal remnants were observed in the Haversian canals, but no cells were found in the spaces of the concentric laminae.

The results were corroborated with M-AT staining, where pale red lines indicative of osteoid were observed at the periphery of the developing bone trabeculae or in areas containing cells in Groups 1 and 2, being more pronounced in Group 2. In Group 3, few areas with the described characteristics of osteoid were observed.

3.4. HISTOCHEMICAL ANALYSIS (PAS POSITIVITY IN OSTEOID)

Histochemical analysis allowed the identification of positivity in cells from both groups 1 and 2.

• Group 1: 6.152% ± 2.145% positivity.
• Group 2: 15.978% ± 3.321% positivity.
• Group 3: 1.487% ± 0.745% positivity.

In the density intensity analysis using ImageJ, the following values were observed:

• Group 1: 4.612×107 ± 3.215×107
• Group 2: 1.489×108 ± 4.015×107
• Group 3: 4.657×106 ± 2.357×106

3.5. IMMUNOHISTOCHEMICAL ANALYSIS (PTHR1 FOR OSTEOBLASTS)

Immunolabeling with anti-PTHR/PTHR1 antibodies showed areas with positive cells, indicating the presence of osteoblasts, observed mainly in Groups 1 and 2.

• Group 1: Average of 8.3±0.98.3 ± 0.98.3±0.9 positive cells per field.
• Group 2: Average of 11.7±0.611.7 ± 0.611.7±0.6 positive cells per field.
• Group 3: Average of 1.4±0.11.4 ± 0.11.4±0.1 positive cells per field.

A higher cell density was observed in developing trabeculae in Group 2, followed by Group 1. Group 3 showed minimal cellular activity.

3.6. STATISTICAL ANALYSIS

The Kolmogorov-Smirnov test showed that the samples did not follow a normal distribution. The Kruskal-Wallis test was used to compare the groups, with a post-hoc analysis revealing significant differences: • Group 1 vs Group 2: $p<0.05$ • Group 1 vs Group 3: $p<0.01$ • Group 2 vs Group 3: $p<0.001$.

Discussion.

The aim of this study was to evaluate the effectiveness of a three-dimensional bioimplant composed of demineralized bone matrix (DBM), collagen, hydroxyapatite (HAp) and nucleated autologous bone marrow cells in the regeneration of massive bone defects, comparing its performance with that of a bone allograft. For this purpose, an animal model was used with 45 subjects distributed in three experimental groups: bioimplant alone, bioimplant with nucleated bone marrow cells and allograft.

The histological results showed significant differences in bone regeneration between the groups. In the group that received only the bioimplant (Group 1), the organization of the collagen fibers was irregular, and the presence of vascular spaces in the tissue was observed, typical characteristics of early bone formation (zone 1). However, bone regeneration in this group was limited, with areas of immature tissue that did not show complete organization. In contrast, the group that received the bioimplant together with nucleated bone marrow cells (Group 2) showed greater organization of collagen fibers, with a structure like that of mature bone tissue in more advanced areas of regeneration (zone 3). This indicates that the addition of nucleated bone marrow cells favors osteogenesis, promoting more efficient and rapid integration of the implant. This finding is consistent with the literature, which has shown that the combination of bioactive materials and autologous stem cells can significantly enhance bone formation in massive defects [14, 17, 28, 29], given that bone marrow stem cells have the potential to differentiate into osteoblasts and contribute to bone regeneration [28, 29]. In this sense, the presence of osteoid, observed in zones 2 and 3 of group 2, suggests a superior osteogenic activity compared to group 1, corroborating the hypothesis that autologous stem cells can stimulate bone formation at the injury site.

The allograft group (Group 3) showed a more classical organization of the bones with a clear delimitation of the bone laminae and osteon structures, indicating a bone regeneration that follows a slower integration process. This result agrees with previous studies indicating that, although allografts are effective in bone regeneration, tissue integration may be delayed and depend on graft revascularization[4,7,10]. Furthermore, the lack of cells in the spaces between the concentric laminae suggests a lower cellular activity compared to the other two groups.

Immunohistochemical analysis showed a higher number of positive osteoblasts in the groups treated with bioimplant (Group 1 and 2) compared to the allograft group. The presence of osteoblasts in the peri trabecular spaces in groups 1 and 2, particularly in group 2, is indicative of an active process of osteogenesis. Immunolocalization of the PTH receptor protein (PTHR1), which is a specific marker of osteoblasts, allowed us to identify the highest concentration of these cells in the group that received the bioimplant with nucleated cells. This finding reinforces the idea that the addition of autologous stem cells improves osteoblastic activity and, therefore, favors bone regeneration [4].

On the other hand, in group 3, the number of osteoblasts observed was significantly lower, suggesting a lower cellular response in the bone regeneration process. This is consistent with previous studies reporting that allografts may not promote osteoblast proliferation as effectively as implants containing autologous stem cells [4,12, 28, 29] which may explain the lower bone regeneration observed in this group.

In terms of PAS positivity, which indicates the presence of osteoid, the results showed that Group 2 (bioimplant with nucleated cells) presented a higher positivity compared to the other two groups. This confirms that bone marrow nucleated cells not only promote the organization and differentiation of cells towards an osteoblastic lineage, but also contribute to the production of extracellular matrix, which is essential for bone formation. The increased PAS positivity in Group 2 suggests a higher osteogenic activity, which corroborates the histological and immunohistochemical results.

Statistical analyses, using the Kruskal-Wallis test and post-hoc tests, showed significant differences between all groups. The comparison between Group 1 and Group 2 demonstrated a substantial improvement in bone regeneration when bone marrow nucleated cells were added, underlining the importance of this cellular approach for the improvement of bone regeneration outcomes. The results of Group 3, although showing bone regeneration, were significantly lower compared to the other two groups, confirming that the bioimplant with stem cells is a superior option compared to the allograft.

Conclusion.

The results of this study confirm that the combination of a three-dimensional bioimplant with nucleated cells from autologous bone marrow significantly improves bone regeneration in massive defects compared to the use of a bioimplant without nucleated cells or the use of an allograft. These findings offer a solid basis for the development of innovative therapies in bone tissue engineering and open new possibilities for the treatment of complex fractures and bone defects in humans.

Conflict of interest.

The authors declare that there are no conflicts of interest related to this study. They have not received financial, material or other support from entities that could have influenced the results or interpretation of the data. In addition, none of the authors have personal, professional or financial ties with companies or products that may have affected the objectivity of this work. This declaration ensures the integrity and transparency of the study.

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