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# Osteogenesis induced by a three-dimensional bioimplant composed of demineralised bone matrix, collagen, hydroxyapatite, and bone marrow-derived cells in massive bone defects: An experimental study

Carlos E. Cuervo-Lozano[a], Adolfo Soto-Domínguez[b], Odila Saucedo-Cárdenas[b,c], Roberto Montes-de-Oca-Luna[b], Sergio Alonso-Romero[d], María del Consuelo Mancías-Guerra[e], Eduardo Álvarez-Lozano[a,*]

[a] Servicio de Traumatología y Ortopedia, Hospital Universitario "Dr. José Eleuterio González", Universidad Autónoma de Nuevo León. Av. Madero y Gonzalitos, Monterrey, N.L., A. P. 1563, Mexico
[b] Departamento de Histología, Facultad de Medicina, Universidad Autónoma de Nuevo León. Av. Madero y E. Aguirre-Pequeño, Monterrey, N.L., A. P. 1563, Mexico
[c] Centro de Investigación Biomédica del Noreste (CIBIN), IMSS. San Luis y 2 de Abril. Col. Independencia. Monterrey, N.L., C.P. 64720, Mexico
[d] Departamento de Investigación, CIATEC. León, Gto., Mexico
[e] Servicio de Hematología, Hospital Universitario "Dr. José Eleuterio González", Universidad Autónoma de Nuevo León. Av. Madero y Gonzalitos, Monterrey, N.L., A. P. 1563, Mexico

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ABSTRACT

Treatment of massive bone defects is one of the most difficult problems to solve in orthopedics. At present, there is no consensus on the best way to resolve these problems. The aim of our study was to evaluate the effect of a three-dimensional bioimplant over massive bone defects, and to analyse if it improves the speed and quality of integration in recipient bone compared to allograft treatment.

Fifteen female lambs with massive bone defects, surgically created in their tibias, were randomly divided into three groups of five lambs each: Group I – treated with the bioimplant; Group 2 – treated with the bioimplant plus nucleated cells of autologous bone marrow; Group 3 – treated with a frozen allograft. Radiographs were taken post-treatment at weeks 1, 6, and 12. Animals were euthanized to obtain the studied bone segment for morphological analyses.

Treatment: with bioimplants *vs.* bioimplant plus bone marrow nucleated cells (BMNCs) showed a notorious osteogenic effect, but with greater osteoid synthesis and cellularity in the latter. These results suggest that combined treatment with bioimplants and BMNCs have an additive effect on massive bone defects in lambs. These experimental results could be applied to repair damaged human bone.

## 1. Introduction

Since the beginning of the 1970s, when the era of limb salvage began (Eilber et al., 1980), different forms of reconstruction have been used to treat massive bone defects, ranging from the use of an autograft, allografts (Aponte-Tinao et al., 2014, 2015, 2016; Houdek et al., 2016; Rabitsch et al., 2013), bone substitutes, special prostheses (Gkavardina and Tsagozis, 2014; Tiwari et al., 2014), or combinations of these reconstruction methods (Antoci et al., 2009; Arslan et al., 2015; Qu et al., 2015; Sevelda et al., 2015; Venkatramani et al., 2015; Vlad et al., 2013). Autologous bone remains the gold standard for bone grafts; however, it has the enormous disadvantage of being limited in quantity, shape, and size. Additionally, the patient undergoes a second surgical procedure, thus increasing morbidity by 10% (Younger and Chapman, 1989). The use of allografts has increased in the last 20 years, but these are also far from ideal because of the lack of complete integration, especially for large defects (Bus et al., 2014); this problem leads to its main complications, such as fractures, infections, and resorptions (Ayvaz et al., 2014; Delloye et al., 2014; San-Julian and Canadell, 1998). The recent development of "tissue engineering" offers new options for repairing bone defects (Langer and Vacanti, 1993) and one of the corresponding fields is the creation of three-dimensional bioimplants that provide a suitable environmental medium for tissue growth (Cunniffe et al., 2010; Hansen et al., 2005). Demineralised bone matrix

* Corresponding author at: Servicio de Traumatología y Ortopedia, Hospital Universitario "Dr. José Eleuterio González", Universidad Autónoma de Nuevo León, Av. Madero y Gonzalitos S/N Colonia Mitras Centro, Monterrey, N.L., México. A. P.1563.
*E-mail address:* dedaloz@gmail.com (E. Álvarez-Lozano).

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[DBM] (Urist, 1965; Vaccaro et al., 2002) is a very useful material because it has bone morphogenetic proteins [BMPs] (Urist et al., 1970; Urist and States, 1971), which act as osteoinductive mediators; however, an osteoinductive agent without a carrier will be lost or degraded rapidly without producing any osteogenic effect (Duflo et al., 2006; Hotz and Herr, 1994; Liu et al., 2006). Because bone is a nanostructured tissue (Balasundaram and Webster, 2007), this carrier must have certain chemical and structural characteristics (Seref-Ferlengez et al., 2015; Song et al., 2015; Terauchi et al., 2015). "Stem cells" (Nowakowski et al., 2015) have recently aroused a growing interest in their therapeutic potential in diseases that until now lacked an effective treatment. These cells are found in the quiescent state in the body, mainly in bone marrow and adipose tissue, as multipotent stromal cells (Maumus et al., 2011; O'Brien et al., 2007). The structure, cellularity, function, and biological mechanisms of bone tissue make it difficult to find a material, biological or otherwise, that replaces bone and replicates all its characteristics. It is important to provide a biological and structural aid to the body to facilitate the repair of massive bone defects through fast, high-quality integration. The aim of this study was to evaluate if a three-dimensional bioimplant improves the speed and quality of integration in the recipient bones, compared to allograft treatment, of massive bone defects. This bioimplant was generated from the combination of the components of bone tissue: DBM with BMPs, collagen, hydroxyapatite [HAp] $Ca_{10}(PO_4)_6(OH)_2$, and osteoprogenitor cells.

## 2. Materials and methods

### 2.1. Study design and setting

This is an experimental, longitudinal, comparative, blind and controlled study.

### 2.2. Preparation of bioimplant

The first component used for the bioimplant was DBM, which was generated via the demineralisation of the diaphysis of the long bones of 3 hind limbs of donor lambs using a technique previously described by Rivera et al. (2003). The DBM has biological characteristics that favour osteoinduction (Urist et al., 1970; Urist and Strates, 1971; Vaccaro et al., 2002).

The second component was collagen because collagen is the most abundant structural protein in bone; collagen was used to provide rigidity and maintain the desired shape of the bioimplant while making the implant insoluble when contacting body fluids. Collagen was obtained from the skin of foetal pigs by a process consisting of mechanically macerating the skin in a meat grinder and dripping a 0.5% by weight solution of hydrochloric acid [HCl] with constant stirring in distilled water.

Bone is a nanostructured tissue, and therefore to achieve a porosity of less than 100 nm, HAp nanoparticles were selected as the third component. HAp is the main inorganic component of bone and its use in nanoparticles improves the microenvironment and increases the useful surface area for cell growth.

The HAp nanoparticles were processed from waste egg shells by a hydrothermal synthesis process, as described in the technique published by Elizondo-Villarreal et al. (2012).

The three components were mixed in a proportion of 30% DBM, 60% collagen, and 10% HAp. The mixture was placed in prefabricated 5-cc moulds of a specific shape and size, dried, and subsequently sterilised in doses of 20–22 kg.

### 2.3. Description of experiment, treatment, or surgery

We employed 15 female lambs (*Ovis aries*), 4–5 months of age and weighing 20–30 kg. A bone defect was surgically created in the proximal diaphysis of the left tibia of the lambs using an oscillating saw and a metallic guide specifically fabricated for this purpose. The bone defect comprised 75% of the cortical circumference and 5 cm of the length. The bone defects were treated in three different manners; animals were randomly selected and divided into 3 groups of five lambs each. Group one lambs were treated only with the bioimplant. Group two lambs were treated with the bioimplant plus autologous nucleated cells; prior to its placement, the bioimplant was immersed for 5 min in a container with the cells obtained from an iliac crest aspirate by a special centrifugation process a few hours before surgery. In group three, the defect was treated with a frozen allograft. In all cases, internal fixation with a special plate and screws was used to keep the allograft in place. The lambs remained in the bioterium for 5 days for wound monitoring and administration of antibiotic (20 mIU procaine benzylpenicillin) without restriction of limb support and with *ad libitum* access to water and food. From the 6th day onwards, the animals were transferred to a common habitat.

The experiments were performed in accordance with the International Guidelines on the Appropriate Use of Experimental Animals and according to the Mexican Norm NOM-062-ZOO-1999 on the Technical Specifications for the Production, Care and Use of Laboratory Animals (SAGARPA, 1999). The protocol was approved by the Bioethical Committee of the Faculty of Medicine, UANL in Monterrey, Nuevo León, México.

### 2.4. Radiological analysis

In the radiological analysis, antero-posterior and lateral radiographs of the limb were taken at weeks 1 (control), 6, and 12 post-treatment and the appearance of the massive bone defects in the tibias of the study groups were evaluated.

### 2.5. Histological and histochemical analysis

Animals euthanized with sodium pentobarbital (90–210 mg/kg weight, intravenous administration) at 12 weeks to obtain the studied bone segment for morphological analyses. After the samples were collected, they were fixed in 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 10% HCl decalcification technique for 21 days by changing the solution every third day, observing that the bone showed a soft consistency. Afterward, samples were processed by conventional histological techniques until their inclusion in paraffin blocks.

The general cellular characteristics, presence of bone trabeculae, and orientation and organisation of the collagen fibres were evaluated in histological sections (4 μm thick) stained with haematoxylin and eosin [H&E] and Mallory-Azán Trichrome [M-AT] for histological analysis.

Additionally, the histochemical periodic acid-Schiff [PAS] staining method was used to identify the components of the osteoid. Samples were evaluated using light field microscopy.

### 2.6. Immunohistochemical analysis

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