TITLE: ~~Effect of~~ ~~Effect of cell dosage in an~~ allogeneic ~~cell-based~~ ~~tcell-based tissue~~ ~~engineered treatments~~ ~~issue engineered~~ ~~treatment~~ in a ~~sheep osteonecrosis~~ ~~preclinical~~ model

RUNNING HEAD: Allogeneic cell-based tissue engineering

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ABSTRACT

Osteonecrosis of the femoral head (ONFH) is defined as a tissue disorder and successive subchondral bone collapse resulting from an ischemic process, which may progress to hip osteoarthritis. Cell therapy with multipotent bone marrow mesenchymal stromal cells (BM-MSC) of autologous origin appears to be safe and has shown regenerative potential in previous preclinical and clinical studies. The use of allogeneic cells is far more challenging, but may be a promising alternative to use of autologous cells. Moreover, an optimized dosage of cells from an allogeneic source is needed to obtain off-the-shelf tissue engineering products (TEP). The purpose of this study was to evaluate the efficacy of a TEP composed of undifferentiated *ex vivo* expanded BM-MSC of allogeneic origin, combined with bone matrix particles in variable doses. A comparative analysis of TEP's bone regenerative properties against its autologous counterpart was performed in an early stage ONFH preclinical model in mature sheep. Allogeneic BM-MSC groups demonstrated bone regeneration capacity in osteonecrotic lesions equivalent to autologous BM-MSC groups 6 weeks after treatment. Likewise, stimulation of bone regeneration by a low cell dose of $0.5 \cdot 10^6$ BM-MSC/cm³ was equivalent to that of a high cell dose, $5 \cdot 10^6$ BM-MSC/cm³. Neither local nor systemic immunological reactions nor tumorigenesis were reported, strengthening the safety profile of allogeneic BM-MSC therapy in this model. Our results suggest that low-dose allogeneic BM-MSC is sufficient to promote bone regeneration in femoral head osteonecrotic lesions, and should be considered in translation of new allogeneic cell-based tissue engineering products to human clinics.

IMPACT STATEMENT

Cell therapy and tissue engineering hold promise as novel regenerative therapies for musculoskeletal diseases, and particular in bone regeneration strategies. In this manuscript we report the evaluation of the efficacy of an allogeneic cell-based tissue engineering product in an early stage osteonecrosis of the femoral head preclinical model in skeletally mature sheep. Moreover, we demonstrate its bone regeneration capacity and safety in vivo and its equivalence to autologous counterparts. These findings have important implications for the translation of new allogeneic cell-based tissue engineering products to human clinics.

INTRODUCTION

Osteonecrosis of the femoral head (ONFH) is defined as the tissue disorder and successive subchondral bone collapse resulting from an ischemic process. Because of its anatomical and vascular features, the hip joint exhibits a special predisposition to development of osteonecrosis, which can progress into hip osteoarthritis and subsequent loss of function in the late stages of the disease, leading to a painful and incapacitating situation for patients and significant healthcare-related costs.¹⁻⁵ Current treatments for regeneration of damaged bone tissue in ONFH focus on stimulation of new bone formation to halt progression, and to revert early stages of the disease. Among these, core decompression (CD) has been widely used to reduce intraosseous pressure in the femoral head, restore vascular flow and alleviate pain.³ This procedure can be performed alone, or in combination with autografts, allografts, bone morphogenetic proteins (BMP), bone marrow (BM) concentrates or bone-marrow-derived multipotent mesenchymal stromal cells (BM-MSC).^{3,4} However, clinical trials have evidenced low reliability and high variability of such approaches so far⁵; therefore, development of new treatments in the

field of regenerative medicine holds promise for future tissue engineering and cell therapy strategies.^{4,6} Indeed, cell therapy with BM-MSC of autologous origin appears to be safe and shows regenerative potential, as reported in previous preclinical⁷⁻⁹ and clinical studies.¹⁰⁻¹² However, cell harvesting has been associated with morbidity, risk of infection, treatment delay and high production costs.¹³ Implementing BM-MSC using cells of allogeneic origin and optimizing cell dosage could enable and support use of cell-based tissue engineering products (TEP) as off-the-shelf solutions, reduce manufacturing time and improve production sustainability.¹³ To that end, allogeneic strategies must first be validated in preclinical models, to guarantee their safety and efficacy before translation to clinical applications. Several studies have been undertaken using large-animal models, to generate experimental ONFH preclinical models in rabbits¹⁴, emus¹⁵, sheep¹⁶ and pigs¹⁷, that reproduce the clinical situation. Among these, sheep are docile animals with similar body weight, bone anatomy and mineral composition to humans, which represent a valuable model¹⁸. -and to evaluate safety, feasibility and efficacy of autologous and allogeneic BM-MSC-based TEP, with encouraging results.^{7,8,18,19} Implantation of ex vivo expanded BM-MSC has demonstrated encouraging results in early ONFH stages, as previous preclinical studies have shown bone stimulation and vascular formation in the necrotic lesions.^{8,9,19,20} Additionally, our findings indicate that a BM-MSC loaded bone matrix scaffold is capable of stimulating bone regeneration more effectively than isolated core decompression or in association with an acellular scaffold in a preclinical femoral head osteonecrosis model in sheep.⁷ Safety and feasibility of autologous and allogeneic BM-MSC-based TEP is reinforced by the absence of toxicity and adverse events in both preclinical²¹⁻²⁴ and clinical^{10,25} studies. However, dose efficacy determination requires to be evaluated in order to optimize TEP production for clinical off-the-shelf purposes. The purpose aim of the present study was to evaluate the efficacy of a tissue engineering

product composed of undifferentiated *ex vivo* expanded allogeneic BM-MSC, associated with bone matrix particles in variable cell doses. Furthermore, the bone-regenerative properties of these formulations were compared to those of their autologous counterparts in a preclinical model of early-stage ONFH in sheep.

MATERIALS & METHODS

Animals and ethics statement. All experiments were performed in 24-to 72-month-old, healthy and skeletally mature ewes (n = 27) of the Ripollesa x Lacaune (*Ovis aries*) breed, weighing 52.35 ± 6.26 kg. X-ray examination was performed to confirm tibial epiphyseal closure previous to animal inclusion. Sheep were supplied by A.M. Animalia S.L. (Girona, Spain) and recorded in the registry of breeding centers, suppliers, and users of experimental animals with reference number G9900009. Sheep were acclimatized to Vall d’Hebron Institut de Recerca (VHIR) facilities for a minimum of 7 days prior to the start of the experiments. They were housed together on a 12-hour light/dark schedule, fed a standard diet and allowed access to water *ad libitum*. All procedures described here were approved by the animal experimentation ethics committee of VHIR (protocol number 72/13) and were conducted in compliance with Spanish national and local legislation (Real Decreto 53/2013 and Decret 214/97, respectively) and the directives of the European Union for animal research (2010/63/EU).

Anesthesia, analgesia and post-surgical procedures. All surgical procedures were performed using aseptic technique and under general anesthesia, following the same protocol. Twenty-four hours prior to surgery, sheep were given pre-emptive analgesia using a transdermal fentanyl patch (2.5-5 µg/kg Fendivia®, Nycomed, Roskilde, Denmark) and underwent fasting but free access to water. On the day of surgery, animals

were sedated with an intramuscular (IM) injection of 0.5 mg/kg of midazolam (Midazolam Sala, Lab. Ramon Sala SL, Barcelona, Spain). After loss of reflexes, a venous catheter was placed in the cephalic vein and pre-oxygenation with 100 % oxygen was applied using a facemask, while the right hindquarters were aseptically washed. In the operating room, intravenous (IV) anesthesia was induced with propofol (Propofol Fresenius, Fresenius Kabi, Bad Homburg, Germany) at 4 mg/kg. Endotracheal intubation was performed immediately and anesthesia maintained with 60 % oxygen and 2 % isoflurane (Forane[®], AbbVie Farmacéutica SLU, Madrid, Spain) for the duration of the procedure. Ringer lactate (Viaflo Hartmann, Baxter SL, Valencia, Spain) was continuously infused at 2-3 ml/kg/h; an IV constant-rate infusion of 10 µg/kg/h of fentanyl (Fentanest[®], Kern Pharma SL, Barcelona, Spain) was also administered for analgesia. Anesthesia, ventilation, heart rate, oxygen saturation, end-tidal CO₂, and respiratory rate parameters were continuously monitored throughout the surgical procedure. Post-surgical analgesia included transdermal fentanyl patches for 72 h. Antibiotic prophylaxis was obtained with an IV infusion of 22 mg/kg of cephazolin (Cefazolina Sala, Lab. Ramon Sala SL, Barcelona, Spain) and postoperative IM administration of 15 mg/kg of amoxicillin (Duphamox LA, Zoetis, Madrid, Spain).

ONFH induction. Animals were placed in lateral decubitus position and the right hip was aseptically prepared. A 2.5 mm diameter Kirschner wire (K-wire) (Synthes GmbH, Switzerland) was inserted through the femoral neck to define the entry point at the lateral cortex and the exact location of the center of the femoral head, under fluoroscopic control (OEC Fluorostar 7900 Compact, GE Healthcare). Subsequently, a biopsy trocar (5 mm and 2.5 mm, external and internal diameters respectively, Synthes) was inserted parallel to the guide. The K-wire was then removed and the cryoprobe (R2.4 Renal Cryoprobe, Endocare Cryocare, Health Tronics, Irvine, CA, USA) was inserted following the same

direction and orientation as the K-wire; two consecutive and alternate freezing-thawing cycles were applied, 10 minutes each, using argon-helium (Air Liquide Medicinal SLU, Barcelona, Spain). The cryoprobe was then removed and the wound was closed using **Vycril**® 2-0 sutures (Ethicon, Johnson&Johnson, Somerville, NJ, USA). The treated sheep were distributed into five experimental groups (Table 1).

Culture of BM-MSC and characterization. Three weeks after ONFH induction, BM aspirates were collected from the sternums of 8 sheep (groups A and B) under general anesthesia. These samples were subsequently used for isolation of mononuclear cells (MNC) and establishment of autologous ovine BM-MSC lines, as described elsewhere.²⁶ Cells were cultured up to passage 2 in Dulbecco's modified eagle medium (DMEM, Gibco, Paisley, UK) containing 2 mM glutamine supplemented with 10 % serum pooled from ewes used in the study. All cultures were maintained at 37 °C and 5 % CO₂ in humidified incubators. Cell number and viability were determined by the hemocytometer-based Trypan Blue dye exclusion method. Specific differentiation media formulations (StemPro®, Gibco) were used for the adipogenic, chondrogenic and osteogenic induction of undifferentiated ovine BM-MSC cultures *in vitro*. Oil Red O (Sigma-Aldrich, St. Louis, MO, USA), Safranin O (Sigma-Aldrich), alkaline phosphatase (SigmaFast™ BCIP®/NBT, Sigma) and Alizarin Red (Merck Millopore) stainings were performed to determine the outcome of the differentiation assays. Fluorescence-Activated Cell Sorting (FACS) analysis was performed to evaluate expression of cell surface markers CD31 (MCA1097F, AbD Serotec, Bio-Rad, CA, USA), CD45 (MCA2220PE, AbD Serotec, Bio-Rad), CD44 (MCA2219F, AbD Serotec, Bio-Rad), CD166 (5559263, BD Pharmingen, San Diego, CA, USA), and MHC Class II-DR Monomorphic (MCA2226F, AbD Serotec, Bio-Rad) in a FACSCalibur device (Becton Dickinson, San Jose, CA, USA).

Allogeneic treatments were prepared using an ovine BM-MSC line at passage 13, cryopreserved in liquid nitrogen prior to use. This cell line was obtained, labelled with enhanced green fluorescent protein (eGFP) and characterized in a previous study.⁸

Cells were thawed and cultured under the same conditions as our autologous BM-MSC cell lines.

TEP preparation. Different formulations of TEP were prepared and distributed in one of four experimental groups: A, B, C and D (Table 1), including high- (6×10^6) or low- (1×10^6) dose **initial inoculums of** cell suspensions of autologous or allogeneic BM-MSC, combined with 1 cm³ of cadaveric, decellularized cancellous human bone particles comprised of particle diameters between 0.05 and 1 mm, obtained from a tissue bank (Transplant Service Foundation, Banc de Sang i Teixits, Barcelona, Spain). Cells and scaffolds were incubated overnight in non-adherent T25 flasks (Nunc™ EasYFlask™ 25 cm² Nunclon™ Delta Surface, Thermo Scientific, Roskilde, Denmark) and colonization rates (%) were calculated indirectly by counting the non-seeded cells present in the supernatant and then subtracting from the number of cells present in the inoculum. Visualization of eGFP-labelled BM-MSC cells seeded onto bone scaffolds was performed under fluorescent light (L5ET filter, Leica DMIL LED, Leica Microsystems, Wetzlar, Germany).

Core decompression and treatment groups. Six weeks after ONFH induction, the animals were anaesthetized, placed in lateral decubitus position and the right hip aseptically prepared. CD procedure was performed in the same location as the ONFH had been induced. A biopsy trocar was inserted in the same direction as the original tunnel and advanced through the necrotic lesion. The trocar was then withdrawn and the bone cylinder released, fixed in 10% buffered formalin and analyzed histologically to confirm osteonecrotic changes in the bone tissue. In the control group (group E), no additional

procedures were performed; the surgical approach was closed with Vicryl® 2-0 sutures. In groups A to D, the cell-loaded bone particles were mixed with fibrinogen (Tissucol Duo, Baxter) and re-introduced into the trocar; next, an obturator was pushed through the trocar, expelling the construct within the necrotic lesion. The emptied trocar was withdrawn and the surgical approach closed as previously described. At 12 weeks – corresponding to 6 weeks after treatment surgery –the animals were sedated with an IM injection of midazolam, euthanized using an IV dose of 50-60 mg/kg of sodium thiopental (Tiobarbital Braun 1g, BBraun Medical SA, Melsungen AG, Germany) and the femoral heads harvested for further analyses.

Post-operative monitoring. All sheep underwent routine, daily clinical investigation after each surgical procedure; they were monitored closely for the 6 weeks following CD and treatment surgery, to assess for any evidence of immune reaction to the treatments. Weight change was monitored weekly throughout the study period. Blood samples were taken from all groups before ONFH induction (week 0), before CD and treatment implantation (week 6) and at the end of the experimental period (week 12), for routine blood analysis using hematological, biochemical and hemostasis systems (BC-5000 Vet, Mindray Medical España SL, Madrid, Spain; XN-2000™ Hematology Analyzer, Sysmex, Kobe, Japan). At least 50 µl of plasma was isolated from peripheral blood samples collected from each sheep before treatment and at 1, 2, 4, and 6 postoperative weeks, and stored at -80 °C to await analysis for circulating TGF-β isoforms (TGF-β1, -β2 and -β3). Briefly, samples were thawed just once and kept on ice prior to assay. All samples were centrifuged at 1000 g for 15 minutes at 4 °C. In order to remove aggregates or debris, samples were centrifuged again at 10,000 g for 10 min at 4 °C. The Bio-Plex Pro™ TGF-β Assays Kit (Bio-Rad) was used for quantification of TGF-β isoform plasmatic levels, using a Luminex 100IS analyzer (Luminex Corporation, Austin, TX,

USA) following the manufacturer's instructions. Duplicates were performed for each sample and data analysis was performed using BioPlex Manager software v6.0 (Bio-Rad).

Microcomputed tomography (micro-CT). All harvested femoral heads were scanned with a desktop micro-CT imaging system (Quantum FX micro-CT Imaging System, PerkinElmer, Waltham, MA, USA) on the day of euthanasia. Acquisition parameters were 90 kV and 160 μ A, with a field of view of 40 mm. Spherical volumes of interest (VOI) of 2 mm³ were established, cranial (n = 2), adjacent (n = 4) and distant (n = 4) to the CD tunnel in each sample, and gray scale intensity was measured using the AMIDE (Amide's a Medical Image Data Examiner) software.²⁷ Bone mineral density was measured using a phantom of known hydroxyapatite concentrations embedded in soft-tissue resin for gray scale conversion.

Biomechanical testing. Immediately after the femoral heads were scanned, they were cut into two sagittal halves using a diamond band saw (EXAKT 310, EXAKT Technologies, Inc., Oklahoma City, OK, USA). One of the halves underwent indentation testing using a servohydraulic mechanical testing machine (MTS Bionix 358, MTS Systems Corporation, Eden Prairie, MN, USA) equipped with a cylindrical indenter of 4 mm diameter. Each specimen was positioned horizontally on a flat steel cylinder and two indentations were performed, localized in two established regions of interest (ROI) – cranial (n = 1) and distant (n = 1) to the CD tunnel – at a constant speed of 2 mm/min, to a displacement of 1.5 mm below the surface. Load-displacement data were acquired using TestWorks software (MTS Systems Corporation) and compressive strength was calculated. Treated samples were compared to untreated contralateral femoral heads.

Histological analysis. Harvested sagittal halves of femoral heads intended for histology were sliced using a diamond band saw (EXAKT 310) and immediately fixed in 10% buffered formalin. After fixation, a dehydration process was performed by immersion in

a series of aqueous solutions with increasing ethanol concentrations. Subsequently, the sections were embedded in polymethylmethacrylate (PMMA) resin (Technovit 7200VLC®, Heraeus Kulzer, Hanau, Germany), polished to a thickness of 50-100 µm; toluidine blue staining was then performed. The prepared histologic sections were observed under a light microscope (Leica DMD108, Leica DMIL LED, Leica Microsystems) and four images were obtained of the three established ROI (cranial, distal and distant to the CD tunnel). Images were examined by three independent observers and evaluated by a semiquantitative strategy adapted from histopathological scoring parameters described by Udehiya *et al.*²² and Simank *et al.*²⁸ (Supplemental Table 1). The averages of the defined parameters were calculated and studied independently. Briefly, it was considered that a greater stimulation and bone regeneration had occurred in that or those groups with the highest scores. In addition, the presence of acute or chronic inflammatory signs and immunological reactions was evaluated.

Immunohistochemistry for eGFP-labelled BM-MSC. Bone sections obtained from femoral heads from groups C and D were fixed, decalcified with 5 % formic acid (Decalcifier I, Surgipath Canada Inc., Winnipeg, Canada) and embedded in paraffin so that 4 µm thick sections could be obtained. Samples were stained with hematoxylin and eosin, or underwent immunohistochemical staining using polyclonal antibodies against the eGFP reporter protein (GFP-1010, Aves Labs Inc., Tigard, OR, USA). Briefly, tissue sections were deparaffinized, hydrated and treated with 3 % hydrogen peroxide (Sigma-Aldrich) for 35 minutes at room temperature. Non-specific antibody-binding sites were blocked with 30 % rabbit serum in 0.1 % Tween 20 in phosphate-buffered saline (PBS-T, Sigma) for 1 hour. Sections were then incubated with the primary antibody (dilution 1:500) overnight at 4°C. Biotinylated secondary rabbit anti-chicken IgY antibodies (ab6752; Abcam, Cambridge, UK) were incubated (dilution 1:200) for 1 hour at room

temperature, and antibody binding was visualized using a peroxidase staining kit (Thermo Fisher Scientific, Madrid, Spain) in combination with EnVision System HRP Rabbit DAB+ (K4011, Dako, Agilent, Palo Alto, CA, USA), according to the manufacturer's instructions, and then counterstained with hematoxylin.

Statistical analysis. All experimental data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using the SPSS statistical software for Windows (Version 20, SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA was conducted for analysis of variance with Bonferroni post-tests to compare among groups. Statistical significance was set at $p < 0.05$.

RESULTS

Isolation, culture and identification of BM-MSC and bone particle colonization. No donor site morbidity was observed in any sheep in the autologous groups (groups A and B) after BM collection; all animals recovered immediately following the procedure. The mean volume of BM aspirated was 29.63 ± 6.30 mL, yielding $4.87 \cdot 10^6 \pm 4.18 \cdot 10^6$ MNC/mL_{BM} with 89.90 ± 4.63 % cell viability. Ovine BM-MSC were morphologically large adherent cells and showed fibroblastic morphology (Figure 1A) and expressed CD44 and CD166 surface markers, while CD31, CD45 and MHC Class II-DR markers remained negative (Figure 1B). They demonstrated trilineage potency *in vitro* (Figure 1C). Autologous ovine BM-MSC growth profiles in culture were similar to those of allogeneic eGFP-labelled BM-MSC (doubling time was 2.57 ± 0.59 and 1.99 ± 0.42 days; expansion factor was 27.39 ± 6.06 and 39.99 ± 7.52 , respectively). Colonization rates of BM-MSC onto the bone particles were similar between cellular origin groups (autologous

74.29 ± 22.79 % vs allogeneic 82.59 ± 13.43 %) but statistically different between cell doses (high-dose 87.87 ± 4.55% vs low-dose 68.35 ± 22.66 %, p = 0.046). Final cell doses obtained were $5.27 \cdot 10^6 \pm 2.73 \cdot 10^5$ and $0.67 \cdot 10^6 \pm 2.20 \cdot 10^5$ BM-MSCs/cm³ of bone particles in high-dose and low-dose, respectively. Attachment of eGFP-labelled BM-MSCs was confirmed in cell-seeded bone particles by fluorescence microscopy (Figure 1D).

Clinical results. Twenty animals completed the entire experimental study distributed in the experimental groups with four animals each. Two sheep died in the operating room due to cardiorespiratory arrest; three sheep died due to complications derived from anesthetic procedures immediately following ONFH induction. Two sheep presented subtrochanteric fractures and had to be euthanized. All animals that completed the entire experimental study showed mild protected weight bearing in the initial 48 h following each hip surgical procedure. Afterwards, all sheep walked normally without evidence of limp. Weight gain pattern was similar among the different groups. Neither significant changes in body temperature nor signs of infection or inflammation near the implantation areas were found 6 weeks after treatment surgery. Blood parameters fell within the physiological range for all sheep during the study time. Total white blood cell counts in the allogeneic groups C and D showed no differences preoperatively or after 12 weeks, and were similar to those of autologous groups A and B and control group E (data not shown).

TGF-β1 and -β2 plasma concentrations in groups A and C showed maximum concentrations at weeks 7 and 12 (one and six weeks after treatment, respectively). However, groups B and D showed minor changes throughout the post-treatment weeks, and group E showed a decline at week 7 and a peak at week 10 (one and four weeks after treatment, respectively). TGF-β3 showed less variable plasma concentrations in all cases,

except for groups A and B, which presented maximum concentrations at week 8 (two weeks after treatment). There were no statistically significant differences among groups or time periods (all cases, $p>0.05$) (Figure 2).

Micro-CT evaluation. Bone mineral density values distant to the CD tunnel showed homogeneity between groups, whereas values adjacent to the CD tunnel displayed higher variability and no significant differences between groups (all cases, $p>0.05$). VOIs cranial to the CD tunnel were slightly more mineralized in groups A, B, C and D in comparison to group E, although not statistically significant (all cases, $p>0.05$) and were close among groups, independent of cellular origin or cell dose (Figure 3A). None of the hips progressed to collapse or articular incongruence (Figure 3B).

Biomechanical assessment. Values of compressive strength were similar among groups from the same ROI, showing no statistical differences (all cases, $p>0.05$). Values were significantly superior in distant ROIs to the CD tunnel in comparison to cranial ROIs in groups A and C ($p = 0.036$, $p = 0.011$, respectively) (Figure 4).

Histological assessment of osteogenesis. Histologic analysis revealed osteoclast lacunae with osteoclast-like cells at the border of host pre-existing trabeculae devoid of osteocytes. At 6 weeks after treatment surgery, newly formed bone tissue was visible, containing osteocytes and osteoblasts in a linear pattern and separated by cement lines from the pre-existing lamellar bone trabeculae. These findings indicate a regulated process of bone regeneration with osteoclastic resorption followed by osteoblastic apposition, being more intense cranial to the CD tunnel. There was no adverse tissue reaction or inflammatory response in any of the sections examined (Figure 5B-C). Semiquantitative histopathological evaluation showed that osteogenesis scores in ROIs cranial and adjacent to CD tunnel in groups A, B, C and D were significantly superior to those in group E (all cases, $p<0.001$), but similar among allogeneic and autologous

treatment groups as well as high- and low-cell-dose groups. Trabecular bone resorption and bone marrow scores were similar between groups and ROIs (Figure 5A).

Immunohistochemical detection of eGFP-labelled BM-MSC. Six weeks after treatment surgery for ONFH, we found no eGFP-positive cells in the immunohistochemical study of bone tissue sections from allogeneic C and D groups (Figure 6).

DISCUSSION

ONFH is an incapacitating disease with an unfavorable clinical scenario and low quality of life due to degeneration of a joint involved in body load, movement and ambulation. Current surgical and non-surgical treatments to halt bone tissue destruction and stimulate bone regeneration have shown low reliability and high variability. In this scenario, novel regenerative therapy approaches with cell-based products hold promise for stimulating new bone formation and preventing disease progression, as ONFH is thought to have a cellular etiology.^{29,30} Interestingly, implantation of ex vivo expanded BM-MSC has demonstrated encouraging results in early ONFH stages, in both preclinical^{7-9,26,27} and clinical studies,²⁸⁻³⁰ so efforts are currently being directed toward developing new strategies in this field. Interestingly, implantation of ex vivo expanded BM-MSC has demonstrated encouraging results in early ONFH stages, in both preclinical^{7-9,26,27} and clinical studies,²⁸⁻³⁰ so efforts are currently being directed toward developing new strategies in this field. However, clinical use of autologous BM-MSC delays the start of treatment and can cause donor morbidity, while allogeneic BM-MSC from young, healthy donors could be well characterized in advance and readily banked as off-the-shelf products for immediate use when needed.¹³

Preclinical animal models allow evaluation for safety and efficacy of a new TEP before its translation to clinical settings. In this study, we successfully established an early-stage pre-collapse ONFH large-animal model in sheep, based on our previous studies,^{7,16} and using an argon-helium cryotherapy system similar to that described by Wang and collaborators.¹⁴ This system provides high reliability, good repeatability and a precisely controlled necrotic region. Importantly, core decompression procedures and the administration route employed in this study are in current use, and are associated with low morbidity,³¹ thus favoring their translation to the clinical setting.

The BM-MSC obtained from ovine sternum complies with the three minimum criteria established by the International Society of Cell and Gene Therapy,³² showing expected morphology, phenotype and multipotency to mesenchymal lineages *in vitro*, comparable to previous studies in sheep.^{8,33–36} eGFP labelling allowed us to observe ovine BM-MSC adhered to the surface of bone particles showing fibroblastic morphology, resembling human and canine BM-MSC seeded onto different types of scaffolds.^{37,38} We noticed that seeding efficiency was influenced by cell dose: the higher the number of inocula, the higher the colonization rate.⁸ We also observed that cryopreservation of allogeneic BM-MSC did not affect their adherence capability, as their rates were comparable to those of BM-MSC obtained from autologous aspirates – thus providing evidence for the feasibility of formulating TEPs with banked cells.

Clinically, no local or systemic manifestations of acute or chronic toxic reactions were observed during or after either allogeneic or autologous BM-MSC administration in our ovine ONFH model. Blood-marker levels were normal in all sheep, and allogeneic BM-MSC did not alter subject leukocyte counts at 6 weeks after CD and TEP implantation. Our results are in line with the observations of previous reported preclinical studies,

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3 which noted the absence of cell rejection after allogeneic treatment with BM-MSC^{21,23,39–}
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5 ⁴² and could be related to the immunoprivileged status of MSC.⁴³
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7 Bone mineral densities obtained from micro-CT analysis in ROIs adjacent and cranial to
8 the CD tunnel in all TEP groups were slightly increased with respect to the control group,
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10 although these differences were not statistically significant. These micro-CT results
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12 suggest a moderate effect in bone regeneration, confined to the proximities of the
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14 implanted treatments in the CD tunnel, being more intense cranial to it, and similar for all
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16 cellular-origin and cell-dose samples. Other authors have reported similar findings, where
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18 allogeneic and autologous BM-MSC demonstrate equivalent effect in bone
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20 regeneration.^{23,24,42} Newly formed bone cranial to the CD tunnel showed less tissue
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22 strength than the trabecular structure distant from the treatments, which could be due to
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24 the plexiform or primary structure of new bone formed in proximity to the treatments,
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26 mechanically weaker in comparison to vital lamellar mature-bone trabeculae found
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28 distant from the CD tunnel.⁴⁴
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34 Remarkably, TEP formulated with allogeneic BM-MSC promoted osteogenesis at 6
35 weeks after treatment surgery, according to our semiquantitative histopathological
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37 assessment. Osteogenesis scores were superior to those of CD procedure alone, and
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39 similar to autologous BM-MSC treatments. This comparable effect in bone repair from
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41 allogeneic and autologous groups is in concordance with observations made in other
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43 preclinical studies.^{21–24,41} Furthermore, low- and high-cell-dose groups demonstrated
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45 similar scores in osteogenesis, in line with results reported by Coathup *et al.*⁴⁵.
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47 Histological findings indicate an early regenerative ossification process, with new bone
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49 formation appositional to pre-existent trabeculae with empty lacunae, accompanied by
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51 resorption areas and incipient signs of bone marrow cellularity and vascularization. The
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53 literature provides evidence of direct contribution by BM-MSC to bone repair, whether
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of autologous^{8,19} or allogeneic origin.⁴⁶ In our study, stimulation of bone regeneration is most likely related to a paracrine effect rather than a direct cell differentiation process, as we did not find positive eGFP-labelled BM-MSC engrafted cells in new appositional bone tissue in the allogeneic high-or low-cell-dose groups. Therefore, our results are in accordance with other publications, supporting the ability of BM-MSC to secrete cytokines, growth and trophic factors which provide a regenerative microenvironment and promote tissue repair by means of a local, tissue-resident progenitor population.^{8,13,47} As with all animal studies, our study has some limitations that should be acknowledged. First, we used healthy animals, with neither high corticoid intake nor comorbidities such as obesity, diabetes, or the concomitant medical treatments often seen in ONFH patients. Although very similar anatomically, ovine and human femurs present some variations to be taken into account.⁴⁸ Finally, both the 6-week postoperative period after treatment and the sample size may have been too brief/small to evince significant differences among experimental groups, though these parameters are in line with other published studies.^{7,21–}

²⁴ **Future studies would thus require the prolongation of the postoperative period.**

In summary, our study demonstrates that allogeneic BM-MSC tissue engineering products stimulate bone regeneration in osteonecrotic lesions 6 weeks after implantation in an ovine ONFH model, equal to that found with autologous counterparts. Likewise, bone regeneration stimulation by means of a low cell dose is equivalent to that obtained with a high cell dose. In addition, this study further supports the safety profile of allogeneic BM-MSC administration. These results should be considered in the design of new tissue engineering strategies for human application.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that no competing financial interests exist.

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FIGURE LEGENDS

Figure 1 A-D. Characterization of ovine BM-MSC and colonization of bony scaffolds. (A) Image of ovine BM-MSC isolated from sternum aspirates at day 1 and 9. (B) Immunophenotype characteristic of ovine BM-MSC of a representative population for markers characteristic of MSC and hematopoietic cells that react with sheep. Plots correspond to a representative experiment. (C) Trilineage potential of ovine BM-MSC was demonstrated upon induction into the osteogenic (Alkaline Phosphatase (ALP) and Alizarin Red (AR)), chondrogenic (Safranin O) and adipogenic (Oil Red O) lineages. (D) Before and after BM-MSC seeding onto bone particles. Scale bar 100 μ m.

Figure 2. TGF- β 1, - β 2 and - β 3 plasma concentrations are expressed as absolute values and normalized to initial concentration at week 6, 7, 8, 10 and 12 of the study period (weeks 0, 1, 2, 4 and 6 after treatment, respectively).

Figure 3 A-B. Micro-computed tomography analysis. (A) Bone mineral density (mgHA/cm³) values obtained from VOIs distant, adjacent and cranial to CD tunnel from micro-CT analysis. (B) Representative sagittal and coronal micro-CT images at week 12.

Figure 4. Compressive strength at distant and cranial ROIs to the CD tunnel (mean MPa $\pm$ SD) *p<0.05.

Figure 5 A-C. Outcomes of different TEP 6 weeks after treatment. (A) Semiquantitative histopathological scores. (B) Representative histological images from Group A. (C) Representative histological images from Group D. Arrow-heads show bone lining cells

on the surface of new bone tissue (*). Arrow shows osteoclast lacuna with osteoclast-like cells. LB = lamellar bone. Scale bars 5 mm and 200 μ m.

Figure 6. Immunohistochemical staining of GFP-labelled BM-MSC seeded onto bone particles. Arrow-heads show non-positive staining bone lining cells on the surface of newly formed bone tissue (*). Arrows show cement lines between newly formed bone tissue and pre-existing lamellar bone trabeculae. LB = lamellar bone. Scale bar 100 μ m.

SUPPLEMENTAL FIGURES AND TABLES

Supplemental Table 1. Histopathological scoring system followed for the assessment of bone formation in the ovine model of ONFH (adapted from Udehiya *et al.* [40] and Simank *et al.* [39]).

Figure 1

Figure 2

200x207mm (600 x 600 DPI)

Figure 3

1000x1000mm (72 x 72 DPI)

Figure 4

193x107mm (600 x 600 DPI)

Figure 5

Figure 6

Table 1. Description of the experimental groups and treatments.	
Group	Treatment
A	CD + autologous ovine BM-MSC high dose ($5 \cdot 10^6$ cells) + bone particles (1 cm^3)
B	CD + autologous ovine BM-MSC low dose ($0,5 \cdot 10^6$ cells) + bone particles (1 cm^3)
C	CD + allogeneic ovine BM-MSC high dose ($5 \cdot 10^6$ cells) + bone particles (1 cm^3)
D	CD + allogeneic ovine BM-MSC low dose ($0,5 \cdot 10^6$ cells) + bone particles (1 cm^3)
E	CD (control)

CD, core decompression; BM-MSC, bone marrow-derived multipotent mesenchymal stromal cells.

Supplemental Table 1. Histopatological scoring system followed for the assessment of bone formation in the ovine model of ONFH (adapted from Udehiya *et al.* [40] and Simank *et al.* [39]).

Supplemental Table 1. Histopathological scoring		
1	Osteogenesis	
	No osteogenesis	0
	Weak osteogenesis	1
	Medium osteogenesis	2
	Good osteogenesis	3
	> 75% tissue is bone neoformation	4
2	Trabecular bone	
	Non osseous cellular activity	0
	Early apposition of new bone	1
	Active apposition of new bone	2
	Reorganizing cancellous bone	3
	Completely reorganization of cancellous bone	4
3	Resorption of trabeculae	
	Marked resorption	0
	Mild resorption	1
	Absent resorption	2
4	Bone marrow	
	None is resected area	0
	Beginning to appear	1

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	Present in more than half of the defect	2
	Complete colonization by red marrow	3
	Mature fatty marrow	4