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Comparison of amniotic membrane *versus* the induced membrane for bone regeneration in long bone segmental defects using calcium phosphate cement loaded with BMP-2

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ABSTRACT

Thanks to its biological properties, the human amniotic membrane (HAM) combined with a bone substitute could be a single-step surgical alternative to the two-step Masquelet induced membrane (IM) technique for regeneration of critical bone defects. However, no study has directly compared these two membranes.

We first designed a 3D-printed scaffold using calcium phosphate cement (CPC). We assessed its suitability *in vitro* to support human bone marrow mesenchymal stromal cells (hBMSCs) attachment and osteodifferentiation. We then performed a rat femoral critical size defect to compare the two-step IM technique with a single-step approach using the HAM. Five conditions were compared. Group 1 was left empty. Group 2 received the CPC scaffold loaded with rh-BMP2 (CPC/BMP2). Group 3 and 4 received the CPC/BMP2 scaffold covered with lyophilized or decellularized/lyophilized HAM. Group 5 underwent a two- step induced membrane procedure with insertion of a polymethylmethacrylate (PMMA) spacer followed by, after 4 weeks, its replacement with the CPC/BMP2 scaffold wrapped in the IM. Micro-CT and histomorphometric analysis were performed after six weeks.

Results showed that the CPC scaffold supported the proliferation and osteodifferentiation of hBMSCs *in vitro*. *In vivo*, the CPC/BMP2 scaffold very efficiently induced bone formation and led to satisfactory healing of the femoral defect, in a single-step, without autograft or the need for any membrane covering. In this study, there was no difference between the two-step induced membrane procedure and a single step approach. However, the results indicated that none of the tested membranes further enhanced bone healing compared to the CPC/BMP2 group.

Key words: Amniotic membrane; Masquelet induced membrane technique; Bone; 3D-printing; Tissue engineering; Bone morphogenetic protein;

1. INTRODUCTION

The Masquelet technique also called the induced membrane (IM) technique is one of the most commonly used and well-established method to manage critical-sized long bone defects [1–3]. It is a two-step surgical procedure. First, the segmental defect is debrided, the bone is usually stabilized with a plate and a polymethylmethacrylate (PMMA) cement spacer is inserted into the defect [2,3]. This spacer will induce a foreign body reaction thereby leading to the formation of a biological membrane (i.e. the IM) [1]. The second stage is performed six to eight weeks later [2,4]. After carefully removed the spacer, the cavity is filled with cancellous bone autograft from the iliac crest [2]. The IM technique has the benefit of creating an isolated compartment that will protect the bone graft from resorption and invasion by fibrous tissue. It might also be a source of mesenchymal stromal cells or blood vessels and produce osteoinductive growth factors [5–7].

However, the IM technique has some important limitations. The traditional technique involves large quantities of autograft, which can be associated with significant donor site morbidity. Furthermore, the two surgeries required create significant discomfort and risk to the patient as well as additional cost [4]. To overcome these drawbacks, alternatives to the IM technique have been suggested such as using exclusively bone substitutes (avoiding donor site morbidity) and/or using synthetic resorbable membrane (avoiding the first surgical procedure to induce membrane formation) [8–12]. Calcium phosphate-based materials are popular bone substitutes [13,14]. To enhance regeneration in large segmental defect, they usually require growth factors such as bone morphogenetic proteins (BMP). In this study, BMP-2 was chosen for its ability to induce differentiation of osteoprogenitor cells into osteoblasts and to stimulate the formation of new bone [15].

There is still no consensus in the literature regarding the osteoinductive property of the IM [16–18]. It has been indeed suggested that the IM may not have an osteoinductive effect but

only acts as a physical barrier to prevent fibrous tissue ingrowth, according to the concept of guided bone regeneration (GBR) [16,19]. The replacement of the IM by a synthetic membrane has thus been proposed to allow a single-stage procedure, which would remove the additional time needed for IM formation as well as avoid the cost and morbidity of a second surgery. Two studies reported the use of synthetic membranes (polycaprolactone and polytetrafluoroethylene) to cover segmental defects. Tarchala *et al.* observed similar results between a polytetrafluoroethylene membrane and the IM technique [19], whereas the IM promoted greater bone regeneration than a polycaprolactone membrane [9]. Apparently, these membranes clearly only acted as a physical barrier without providing any additional biological effects. Another proposed alternative to avoid an additional surgery was to use polylactic-co-glycolic acid nanofibers as a biodegradable material to replace the PMMA spacer to induce the membrane. Unfortunately, this approach was not directly compared to the IM technique [20].

Thanks to its biological properties, human amniotic membrane (HAM) has become an attractive biological tissue to act as a bioactive membrane for GBR [21–26]. HAM is a readily available biomaterial, derived from human placenta, without ethical concerns. HAM is a source of growth factors [27], and it is also known to possess low immunogenicity [28] and an anti-cancer effect [29]. HAM has been used for over a century for wound healing in medicine [30–33]. However, only a few studies have reported promising results using HAM in the field of orthopedic surgery, and especially, its potential to be used as an alternative to the IM [34–36]. Similarities between the HAM and IM have been shown: both biological membranes are a highly-organized tissue, share similar proteins components and have comparable thicknesses [35]. They both contain growth factors such as VEGF or TGF- β 1 and express anti-inflammatory proteins [5,35,37,38]. Finally, some authors reported the

osteogenic capacities of HAM [24,39–41]. Taken together, these findings suggest that HAM could replace the IM and allow a single surgical procedure.

Although the use of amniotic membrane has already been suggested as an alternative to the IM technique, these two membranes have never been directly compared. The objectives of this study were to: 1) design and fabricate a 3D custom scaffold made of osteoconductive calcium phosphate used to replace autologous bone graft in the two-stage IM method and, 2) compare the bone regeneration potential of HAM *versus* that of the IM when they cover this scaffold loaded with BMP-2.

1. Materials and methods

1.1. Materials

1.1.1. 3D Plotting of the bone substitute and post-processing

The bone scaffolds were fabricated using calcium phosphate cements (CPC) paste (InnoTERE® GmbH, Radebeul, Germany). The powder component consisted of 60 wt % α -TCP, 26 wt % calcium hydrogen phosphate, 10 wt % calcium carbonate and 4 wt % precipitated HA [42]. They were fabricated by pressure-assisted micro-extrusion using a dedicated 3D-printer (3D Discovery®, RegenHU, Switzerland). The following plotting parameters were used: needle diameter = 250 μm ; plotting speed = 4 mm.s^{-1} ; dosing pressure = 0,25 MPa.

Two types of scaffolds were fabricated. Circular-shaped scaffolds were used for the *in vitro* characterization experiments. They were plotted in 60° configuration (each layer orientation varied by 60°), provided a triangular inner structure, and a size of 9 mm diameter and 1 mm thick. For the *in vivo* experiments, the scaffold was designed in order to be adjusted into a critical-size rat femoral defect [42]: they were shaped with a cylindrical outer geometry with a height of 5 mm and a diameter of 4 mm and the same triangular inner structure. One side was flattened to allow fixation onto the osteosynthesis plate [42]. Inside each layer and for both

scaffolds, porosity was set at 500 μm . After plotting, the scaffolds were incubated in water-saturated atmosphere at 37°C for 3 days for cement setting, before being sterilized by gamma radiation at 25 kGy (Gamacell® 3000 Elan, NORION MDS, Ottawa, Canada).

1.1.2. Preparation and storage of HAMs

Two treatments of HAM were assessed: lyophilized (L-HAM) and decellularized then lyophilized HAM (DL-HAM). They were prepared as previously described [43]. Two human placentas were collected after elective cesarean surgery from consenting healthy mothers (tested seronegative for HIV, Hepatitis B and C virus, and syphilis). Patients provided written informed consent as requested by the institutional review board and their placentas were anonymized. The placentas were kept in a sterile solution containing PBS 1x (Gibco®) supplemented with 1% antibiotics (penicillin/ streptomycin, Invitrogen®) and transferred to the laboratory. Residual blood clots were removed and the HAM was peeled from the chorion then rinsed with sterile distilled water. All these steps were performed under sterile conditions. To realize lyophilized HAM (L-HAM), patches were frozen at -80°C, then dried under vacuum in a freeze dryer. The decellularization of HAM was performed according to a previously established protocol [43]. Briefly, HAM was first treated with trypsin and ethylenediaminetetraacetic acid (T/EDTA, 0.125%) for two minutes at 37°C. After washing HAM with sterile PBS, HAM was transferred to a decellularization solution composed of 8 mM CHAPS, 25 mM EDTA, 0.12 M NaOH and 1 M NaCl in PBS and incubated, for 7h at room temperature under gentle agitation followed by three washes of sterile distilled water. Finally, DL-HAM was frozen at -80°C then dried in the freeze dryer. L-HAM and DL-HAM were sterilized by gamma radiation at 25 kGy and kept at room temperature.

1.1.3. Preparation of spacers

The bone cement spacers (5mm in length, 5 mm in diameter) were made of polymethyl methacrylate (PMMA) of medical grade (CMW 3, DePuy International, Blackpool, England)

and hardened in silicone moulds (Putty soft, GumarTM, Acteon®). They were sterilized by gamma radiation at 25 kGy.

1.1.4. BMP-2 solution

A commercially available kit (InductOs®, Medtronic, Biopharma B.V., Heerlen, Netherlands) was used to prepare the solution of rhBMP-2 following the manufacturer's protocol. Prior their implantation *in vivo*, the CPC scaffolds were loaded with a solution containing 10 µg of rhBMP-2: CPC/BMP2.

1.2. *In vitro* evaluation of the cytocompatibility of the scaffolds

In vitro viability and osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) cultured over the bone substitute were assessed. After getting patient written consent, the hBMSCs were isolated from patients who underwent hip surgery (experimental agreement with *CHU de Bordeaux* and *Etablissement Français du Sang*, agreement CPIS 14.14), and expanded following well-established protocols [44]. Cells were used at passage 2. Then, the circular-shaped scaffolds (9 mm diameter; 1mm height) were put in 48-well plates previously covered by agarose 2 %.

For the viability assay, hBMSCs were seeded onto the CPC scaffolds at a density of 10×10^4 cells per scaffold and they were cultured in 1 ml of two different mediums: 1) basal medium (BM= α -minimum essential medium (MEM alpha, GIBCO®), 10% fetal bovine serum (Eurobio®) or 2) osteogenic medium (OM= standard osteogenic induction medium (StemProTM, GIBCO®)). Plates were then incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. The hBMSCs cultured on tissue culture polystyrene plates served as a positive control.

A live/dead viability assay (Thermo Fisher Scientific, Invitrogen) was performed according to the manufacturer's instructions 3, 7 and 14 days after culture (n=2 per condition and per

time). The staining was visualized with a confocal microscope (Leica TCS SPE Model DMI 4000B).

Qualitative assessment of hBMSCs osteodifferentiation using ALP activity was performed after 7 and 14 days of culture (n=2 per condition and per time). The samples were fixed with 4% paraformaldehyde for 15 min, then washed twice with PBS 1x and processed using the Alkaline Phosphatase kit 86 C-1KT (Sigma-Aldrich, USA) according to the manufacturer's instructions. Briefly, cell layers were incubated with Fast Blue RR salt and Naphtol AS-MX Phosphate Alkaline solution 0.25% (Sigma-Aldrich, USA) for 30 min at room temperature in the dark before being washed in distilled water. Images were obtained using a stereo microscope (MZ10F, Leica Microsystems, Germany) coupled to a camera (Leica model DFC 450C).

1.3. *In vivo* surgical implantation

1.3.1. Animal model and implantation procedure

The present study was approved by the French Ethics Committee for animal care and experiment (agreement APAFIS n°12543-201712121537848v3). The aim was to compare bone regeneration using L-HAM or DL-HAM, to the IM technique using a femoral critical-size defect model. Fifteen 10-weeks-old male Sprague Dawley rats were used and the surgery was performed on both femurs. They were divided into the following five groups (n=6 per group) (Table 1):

- 1) Empty group: defects were left empty (**EMPTY**)
- 2) Control group: defects were filled by the rh-BMP2 loaded calcium phosphate cements (**CPC/BMP2**)
- 3) The L-HAM group: defects were filled by the CPC/BMP2, then covered by the L-HAM (**L-HAM**)

4) The DL-HAM group: defects were filled by the CPC/BMP2 and covered by the DL-HAM (**DL-HAM**)

5) The IM group: underwent a two-stage procedure with insertion of a PMMA spacer followed by its replacement with the CPC/BMP2 wrapped in the IM (**IM**)

A single step surgical procedure was used for groups 1 to 4, whereas a two-steps surgical procedure was used for group 5. Surgery was carried out under aseptic conditions. Short-term anesthesia was induced by inhalation of 4% isoflurane (Air:1.5 L/min) and maintained using isoflurane 2% (Air: 0.4 L/min). Analgesia was performed by intraperitoneal injection of 0.1 mg/kg buprenorphine (Buprecare®, 0.3 mg/ ml) and rats were given a subcutaneous injection of cephalosporin (cefazoline 0.06mg/kg). The surgical site was aseptically prepared, and a longitudinal 3-cm skin incision was performed laterally across the leg. Dissection of the muscles was performed to expose the femoral shaft. The RatFix™System (RISystem AG, Davos, Switzerland) was mounted to perform standardized 5 mm defect (Fig. 1). Briefly, the osteosynthesis system consisting of a PEEK plate of 23 mm, mounted in the rat femur by six screws, facilitating the placement of the saw guide. Subsequently, two osteotomies were created using a Gigli saw (Fig 1A) and the central mid-diaphyseal bone fragment was removed (Fig 1B). A medial cerclage-wiring technique was performed to ensure enough stability over time. Group 1 defects were left empty for 6 weeks. For groups 2 to 4 the defect was either filled with CCP/BMP2 alone (Fig 1C), or the CCP/BMP-2 was covered by a HAM (Fig 1D) for 6 weeks. Group 5 received an initial PMMA spacer for 4 weeks (Fig 1E), after which the site was reopened to expose the induced membrane created (Fig 1F). After a slight incision through the IM (Fig 1G), the spacer was removed and replaced by the CPC/BMP2 scaffold (Fig 1H) for 6 weeks. The incision made through the IM was closed with absorbable sutures (Vicryl™ 5.0, Ethicon). Finally, the muscles and the superficial fascia were closed using absorbable sutures (Vicryl™ 4.0, Ethicon). The skin was closed with Michel staples and

then covered with aluminum spray (Aluspray®, Vetoquinol, Lure, France). Injection of buprenorphine and cephalosporin were performed the following day after the surgery. Rats were monitored daily for the occurrence of abnormal behavior or complications. At the final time points, the animals were euthanized using CO₂ inhalation. The femurs were dissected and fixed in 4% paraformaldehyde overnight, before being stored into 70% ethanol at 4°C.

1.3.2. Planar X-Rays

After anesthetizing the animals with isoflurane, X-Ray Radiographs were taken immediately after the surgery (Day 0) and every two weeks using Faxitron X-Ray MX20-DC2 digital imaging instrument (Faxitron Bioptics, Arizona, USA). The percentage of bone formation was scored according to a previously described system [12,45] every two weeks post-implantation. We have used a 4-points system. A score of “1” represented 0 to 25 % bone healing, “2” represented 26 to 50 % healing, “3” represented 51 to 75 % healing and “4” represented 76 to 100 % bone healing. The degree of union between the bone substitute and each edge of the defect was also assessed, according to a modified score described previously: 0 = no union, 1 = partial union, 2 = complete radiographical union [46].

1.3.3. Micro-computed tomography

The X-ray microtomographic device used in this study was a Skyscan 1276 (Bruker, Konicht, Belgium). The X-ray source was set at 100 kV and 150 µA to obtain a 15 µm resolution with an exposure time of 450 ms. After scanning, cross-sectional slices were reconstructed using NRecon® reconstruction software (Micro Photonics) and three-dimensional analysis was performed using CTAn® visualization software (Bruker, Konicht, Belgium). A volume of interest of 5 mm length and 333-slice thickness was determined for each femur and applied to all reconstructions. Bone volume fraction (bone volume/total volume [BV/TV]) was assessed.

1.3.4. Histological preparation and histomorphometric analysis

Each sample was decalcified with EDTA-based Microdec® decalcifiant for 3 weeks. The samples were dehydrated and processed for conventional embedding in paraffin. Eight- μ m-thick serial sections were prepared through the middle of the defect and stained with Masson's trichrome and HES staining. Images of the 30 samples stained with Masson's trichrome staining were obtained with an Eclipse 80i light microscope (Nikon, Japan) and captured with a DXM 1200C CCD camera (Nikon, Japan). Morphometric analysis was performed using ImageJ® software for each femoral specimen to quantify the percentage of bone formation inside the pores of each scaffold for group 2 to 5. A previously described histological quantitative scoring system [12] was also used to evaluate the tissue response around the scaffold surface and within the pores (Table 2). Finally, samples stained with HES were acquired with a slide scanner (Hamamatsu Nanosizer 2.0HT) and a quantitative analysis of blood vessels within the pores of the CPC scaffold was performed using NDPview software. Blood vessels were identified by their luminal structure and the presence of red blood cells within their boundaries. Blood vessel density (BVD) was determined by the number of new blood vessels in the scaffold divided by the entire scaffold area [47–49].

1.4. Statistical analysis

Results were expressed as mean \pm standard deviation (SD), with n indicating the number of HAM sample tested. Statistical analysis was performed using GraphPad Prism® Software (La Jolla/CA, USA). First, normality test was performed using a D'Agostino and Pearson omnibus normality test. Statistical significance for independent samples was evaluated with the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. In all cases, statistical significances are marked by stars with * indicating a two-tailed $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

2. RESULTS

2.1. *In vitro* viability and osteogenic differentiation of hBMSCs cultured on the bone substitute

We assessed the suitability of the bone substitute to be used as a scaffold upon which hBMSCs could survive and differentiate towards the osteogenic lineage. Live/dead staining revealed that hBMSCs attached to and spread over the surface of the plotted CPC scaffolds overtime (Fig 2). At day 14, qualitative observations revealed more cells in the scaffold surface when OM was used. Similar results were observed on standard tissue culture polystyrene.

At the early time-point (day 3), a slight ALP staining of hBMSCs was observed, mainly at the periphery of the scaffold, in both culture media (Fig 3). After seven days, a strong ALP staining of hBMSCs was observed on the scaffold cultured in OM. This intense staining was observed over the entire surface of the scaffold. Similarly, an increase of positive ALP staining was observed when hBMSCs were seeded on plastic in OM compared to BM. This staining appeared less intense than the one observed on the scaffold.

2.2. *In vivo* study

2.2.1. Study population

All 15 rats survived to surgery. The animals were continuously monitored and did not exhibit any signs of pain. We also observed regular feeding habits and normal ambulation from the day after surgery.

2.2.2. Planar X-Rays

Figure 4A shows representative x-ray radiographs for the five groups: 1) defect left empty (EMPTY), 2) filled by the rh-BMP2 loaded calcium phosphate cements (CPC/BMP2), 3) filled by CPC/BMP2 then covered by the lyophilized HAM (L-HAM), 4) filled by CPC/BMP2 then covered by the decellularized/lyophilized HAM (DL-HAM), 5) filled by CPC/BMP2 wrapped in the IM. In all cases, bone formation substantially increased between two and four weeks after the surgery. Six weeks after the surgery, complete or nearly complete healing (defined as a score greater than 3) was observed in groups 2 to 5 (Fig 4B). No ectopic bone formation was observed in surrounding subcutaneous tissues or muscles. The scores resulting from the degree of union showed that bridging between scaffold and bone increased overtime for all conditions (Fig 4C).

2.2.3. Micro-CT analysis

Figure 5A shows representative 3D-reconstruction of the region of interest. Bone volume fraction (BV/TV) within the defect was evaluated quantitatively at 6 weeks (Fig 5B). BV/TV was significantly higher for the following three groups CPC/BMP2, IM and DL-HAM compared to the defect left empty, but neither of these two membranes promoted bone healing compared to the CPC/BMP2 group. Covering the BMP2 loaded bone substitute using L-HAM significantly reduced the BV/TV compared to the DL-HAM group.

2.2.4. Histomorphological analysis

No fibrous encapsulation was observed in response to the scaffold (Fig 6A). Tissues at various stages of bone healing were found in the defect sites, with extensive regeneration of mineralized bone in all groups except for the empty group. Newly formed bone and marrow tissues were found throughout the scaffolds both in direct contact with the scaffold and within the pores.

Histomorphological analysis was performed to quantify bone regeneration inside the pores of the scaffold for groups 2 to 5 (Fig 6B). The nature of the tissue formed inside the pores was mostly bone, and few dense fibrous as well as marrow tissues were also observed. The highest level of bone formation inside pores was observed when the bone substitute was covered by the IM, without significant difference.

To further investigate the tissue response at the interface between the bone and the scaffold and within the pores, a histological tissue response scoring was performed for groups 2 to 5 (Fig 6 C-E). Fig 6C shows tissue response scores at the rod interface. The majority of the samples showed a score greater than or equal to 3 demonstrating a direct bone contact with the scaffold without fibrous tissue encapsulation. Tissue within the pores was mostly bone associated with mature or immature fibrous tissue (Fig 6D). Only the L-HAM group showed some samples where connective tissue was mostly observed inside pores, as demonstrated by a significantly lower scoring of the hard tissue response within the pores compared to the IM group. Fig 6E showed the total tissue response score. The L-HAM group showed the lowest score without statistical significance.

Finally, the vascularization within the pores of the CPC scaffold was assessed from histological sections stained with HES (Fig 7A and B). Newly formed blood vessels were observed within the pores of the scaffold similarly in these conditions (Fig 7C).

DISCUSSION

The aim of this study was to compare, for the first time, the well-established IM method to a single step procedure using DL-HAM or L-HAM for bone regeneration. We also aimed to investigate the ability of the 3D-printed CPC scaffold loaded with rh-BMP2 to promote bone regeneration without autograft harvest in a critical size femoral defect in rats.

One major limitation of the IM technique is the need for a high quantity of autologous bone graft. However, only a few studies have investigated bone substitutes in a Masquelet-inspired animal model using traditional PMMA [9,19]. The layer-by-layer construction of 3D structures allows precise control over the inner architecture and the shape of the scaffold in order to fit a specific defect [42]. The porosity of a scaffold plays a key role for cell migration, vascularization and new bone ingrowth. We choose a calcium phosphate-based biomaterial because these biomaterials are known to increase osteoprogenitors cells and capillaries ingrowth in large bone defects [13,14,50]. We produced inner pores with a triangular cross-section because it affects positively scaffold vascular colonization and its osteointegration [51,52]. *In vitro* results showed that the CPC scaffold allows hBMSCs proliferation when seeded over the scaffold. Furthermore, hBMSCs osteodifferentiation was observed through the entire surface of the scaffold.

The CPC scaffold is an osteoconductive biomaterial which lacks growth factors and living cells found in autologous grafts. We thus loaded the CPC scaffold with 10 µg of rh-BMP-2 to overcome its lack of osteoinductive properties. This dosage is consistent with previous studies of critical-sized femoral defect of 3 to 6 mm in rats [53,54,54–56]. However, further studies would be necessary to assess the release kinetics and the dose-response of BMP-2 on the CPC to improve the system. In our study, nearly complete healing was observed with the CPC scaffold loaded with BMP-2. Histological analysis showed a direct bone contact between the scaffold and the adjacent host bone without fibrous tissue encapsulation. Newly formed bone was also observed within the scaffold pores. In addition, as vascularization of a bone substitute in large bone defect is often challenging [57], we investigated the angiogenesis inside the CPC scaffold. Interestingly, results showed blood vessels formation within the CPC pores, which is necessary to allow bone regeneration. The lack of a BMP-2 control group and of a conventional Masquelet control group are two limitations of this study. Conventional

Masquelet technique required cancellous bone autograft to fill up the cavity. However, it was not possible to harvest such an amount of cancellous bone using this small animal model. In future research, an alternative would be to perform a study in a larger animal model [58–60]. Finally, we did not perform a BMP-2 control group, as well as a CPC control group, as the goal of this study was to compare osseous healing between membrane groups, not to study the BMP-2 or the CPC biomaterial effects.

In clinical practice, PMMA is used as a cement spacer to fill up the bone cavity to induce membrane formation and did not require prior sterilization. In our model, it was not possible to cast the spacer into the defect during surgery. We thus premade PMMA spacers which required to use gamma-radiation to sterilize them prior to surgery as previously described [61]. The lack of information regarding influence of gamma-radiation on the membrane properties as well as on degradation products release is another limitation of this study.

While it is preferable to maintain a two-stage technique in case of infection and septic bone non-union [62], the repair of bone defects following trauma or tumor resection may be performed in a single step. To this end, it has been suggested to replace the induced membrane with a readily available biological membrane to shorten therapy [63]. Similar osseous healing has been observed when using a human acellular dermis, in a one-stage surgery, or the induce membrane for the treatment of non-infectious large bone defects in rats [63]. Here, we decided to evaluate the effect of HAM which is a biological membrane showing similar properties to the induce membrane. It has been shown that F-HAM displays a strong osteogenic potential [41,64] and has a “periosteum effect” when used to cover a segmental long bone defect in rabbits [40]. However, to avoid the risk of infectious disease transmission and to allow long-term storage, HAM cannot be used as a fresh tissue. Cryopreservation, lyophilization or decellularization/lyophilization combined with gamma sterilization, are the most commonly used protocols for HAM preservation [24,65]. We

previously characterized these three membranes and we already demonstrated our decellularization protocol efficiency [43]. We reported that L-HAM degraded faster than DL-HAM, but that they both showed a slower resorption rate than cryopreserved HAM when implanted subcutaneously [43]. We also previously observed that both L- and DL-HAM displayed a higher ability to act as a barrier membrane for GBR in a non-critical size femoral defect in mice than cryopreserved HAM [26]. We thus decided to compare the IM to the L-HAM and DL-HAM. We hypothesized that the presence of the membrane would limit dispersion of the BMP-2 and maintains the growth factor inside the bone defect. However, no additional effect on bone regeneration was achieved by covering the CPC/BMP2 scaffold with the IM or the DL-HAM. Despite the low dose of BMP-2, the barrier membrane effect appears to have been masked by the effect of the growth factor. The association of BMP-2 and a bone substitute to fill a rat segmental defect using a two-step surgical approach has only been investigated in one study [53]. Interestingly, they observed a significant higher level of bone formation when they performed a debridement of the IM before filling the defect, therefore suggesting an inhibition of the effect of BMP-2 by the IM. One preclinical study investigated bone regeneration using a calcium phosphate scaffold loaded with BMP-2 covered or not with three different barrier membranes in a rabbit calvarial model. They also observed no beneficial effect of barrier membranes on bone regeneration [13]. This would suggest an inhibitory activity between the added rhBMP-2 and growth factors contained in both the IM and the HAM such as TGF- β 1 [5,35]. Indeed, the opposite effects between TGF- β 1 and BMP-2 on osteoblast differentiation and maturation have been previously reported [66]. This hypothesis would be consistent with our previous study, in which we observed no additional benefit when using cryopreserved HAM to cover a calvarial defect filled by a bone substitute and BMP-2 in mice [21]. Finally, Masquelet *et al.* also reported no additional effect of the IM when associated with BMP-7, which is another osteoinductive growth factor [2].

After removing the spacer during the second surgery, they filled the IM space with bone autograft mixed with rhBMP-7 in eleven patients. The authors concluded that the results of this case-series were not improved compared with the conventional technique. It could thus be interesting to perform the same study without BMP-2 so we might observe a membrane effect.

To our knowledge, the decellularized HAM had never been evaluated in a segmental bone defect model. In this study, results highlighted a negative effect of L-HAM on total bone volume formation and on bone formation within the pores of the CPC scaffold (Fig 5 B and fig 6 D) thereby suggesting that the DL-HAM is the most suitable preserved HAM to be compared with the IM. This could be explained by the higher ability of DL-HAM to promote hBMSCs proliferation and to support their osteodifferentiation compared to L-HAM [26]. Moreover, bone healing required several weeks and we previously reported that DL-HAM had a superior persistence *in vivo* [43], thereby preventing the invasion of fibrous tissue over a longer period. Few studies have previously reported promising results achieved with a decellularized HAM for non-critical bone regeneration [24,67].

CONCLUSION

In conclusion, the CPC/BMP2 scaffold very efficiently induced bone formation and led to satisfactory healing of the femoral defect at 6 weeks, in a single-step, without autograft or the need for any membrane covering. In this study, there was no difference between the two-step Masquelet approach and a single step approach. However, the results showed that none of the tested membranes further enhanced bone healing compared to the CPC/BMP2 group.

FIGURE LEGENDS

Figure 1. Surgical protocol. (A) Two osteotomies were created using a Gigli saw and the saw guide, (B) the central mid-diaphyseal bone fragment was removed and (C) replaced with the CPC scaffold, (D) before being covered by the HAM. The two steps-protocol consisted of (E) the surgical implantation of the PMMA spacer in the bone defect, followed by the subsequent (F) formation of the induced membrane four weeks later (G) which was incised to remove the spacer and (H) replace it with the CPC scaffold wrapped in the induced membrane. Black arrowhead : CPC scaffold ;White arrow: HAM; Blue asterix: PMMA spacer; Yellow arrow: Induced membrane. (I) Bone fragment and CPC scaffold.

Figure 2. *In vitro* cell viability assays. Fluorescence micrographs of hBMSCs cultured on 3D-printed CPC based bone substitute or standard culture polystyrene (control). Living cells (green) attached and proliferated well on the synthetic scaffold. Dead cells (red) were rarely observed. BM: Basal medium; OM: Osteogenic medium. Scale bar: 100 μ m.

Figure 3. *In vitro* osteodifferentiation. Alkaline phosphatase staining (blue) was performed to assess the ability of hBMSCs to osteodifferentiate when seeded on the CPC scaffold or on tissue culture plates (control).

Figure 4. Longitudinal X-Rays follow-up. (A) Representative 2D-radiographs showing the segmental defect immediately after the surgery as well as 2, 4 and 6 weeks after the surgery. Radiographic scoring was performed to quantify (B) bone formation in the 5 groups and (C) the degree of union between the CPC scaffold and the bone. Data are presented as means \pm SD; n=6 per condition and per time; * $p < 0.05$, ** $p < 0.01$.

Figure 5. Micro-CT analysis. (A) Representative 3D rendering showing the region of interest at six weeks. (B) Quantitative analysis was performed for bone regeneration (BV/TV(%)). Data are presented as means \pm SD; n=6 per condition; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6. Histomorphological analysis of bone regeneration. (A) Representative histological sections stained with Masson trichrome staining at six weeks to visualize mineralized bone (blue). Yellow arrow: newly formed bone; BM: bone marrow; Red line represents the edges of the CPC scaffold inside which bone formation was quantified (B) Quantitative analysis was performed to assess the percentage of bone formation within pores. (C-E) A histological tissue response scoring was performed for groups 2 to 5. Data are presented as means \pm SD; n=6 per condition; * p<0.05.

Figure 7. Histomorphological analysis of angiogenesis in decalcified femoral defects. (A) Representative histological sections (HES staining) of explanted femoral defect six weeks after the surgery. Scale bar: 1 mm. (B) Higher magnification image of the blue rectangle. Erythrocytes were stained pink and densely organized collagen fibers in orange-pink. NB: new bone; BM: bone marrow; Red line represents the edges of the CPC scaffold inside which the vessels were quantified; Black arrow: new blood vessels; Red arrow: newly formed bone. Asterix: surrounding native bone. Scale bar: 250 μ m. (C) New blood vessels density. Data are presented as means \pm SD, n=6 per condition.

TABLE LEGENDS

Table 1. Description of the five experimental conditions used for the in vivo implantation (n=6 per group).

Table 2. Histological quantitative scoring system.

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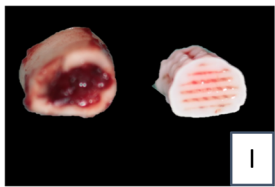
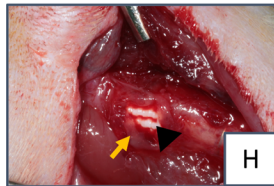
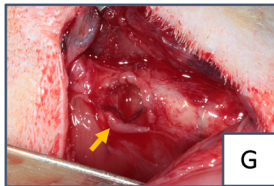
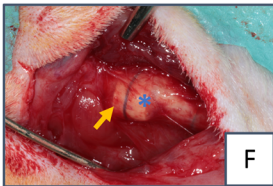
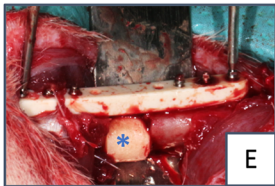
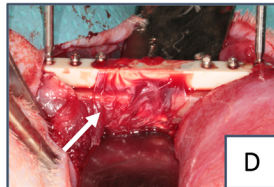
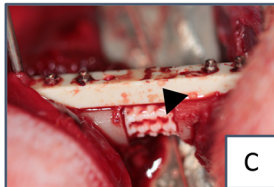
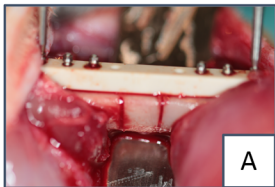
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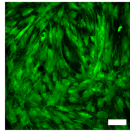
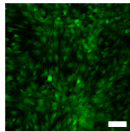
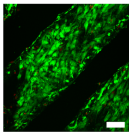
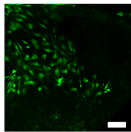
CPC scaffold
BM

CPC scaffold
OM

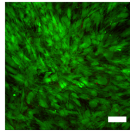
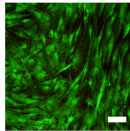
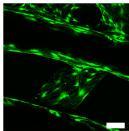
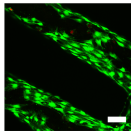
Control
BM

Control
OM

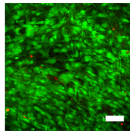
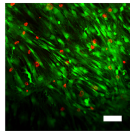
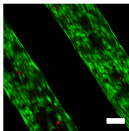
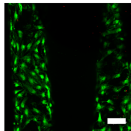
DAY 3



DAY 7



DAY 14



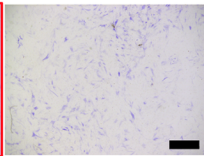
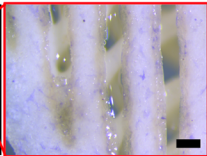
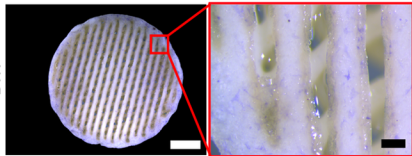
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DAY 3

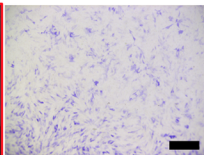
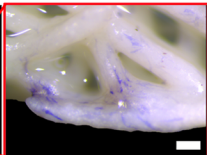
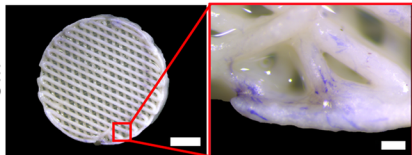
CPC SCAFFOLD

CONTROL

BM



OM



Scale bar: 2mm

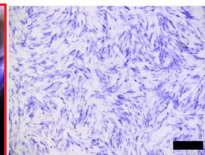
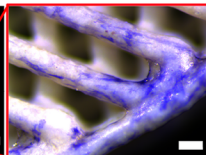
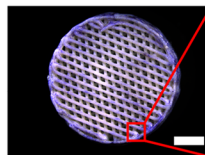
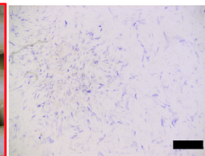
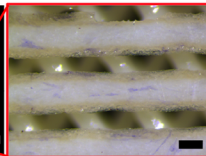
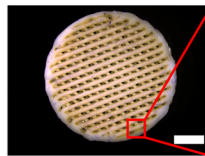
Scale bar: 200 μ m

Scale bar: 1 mm

DAY 7

CPC SCAFFOLD

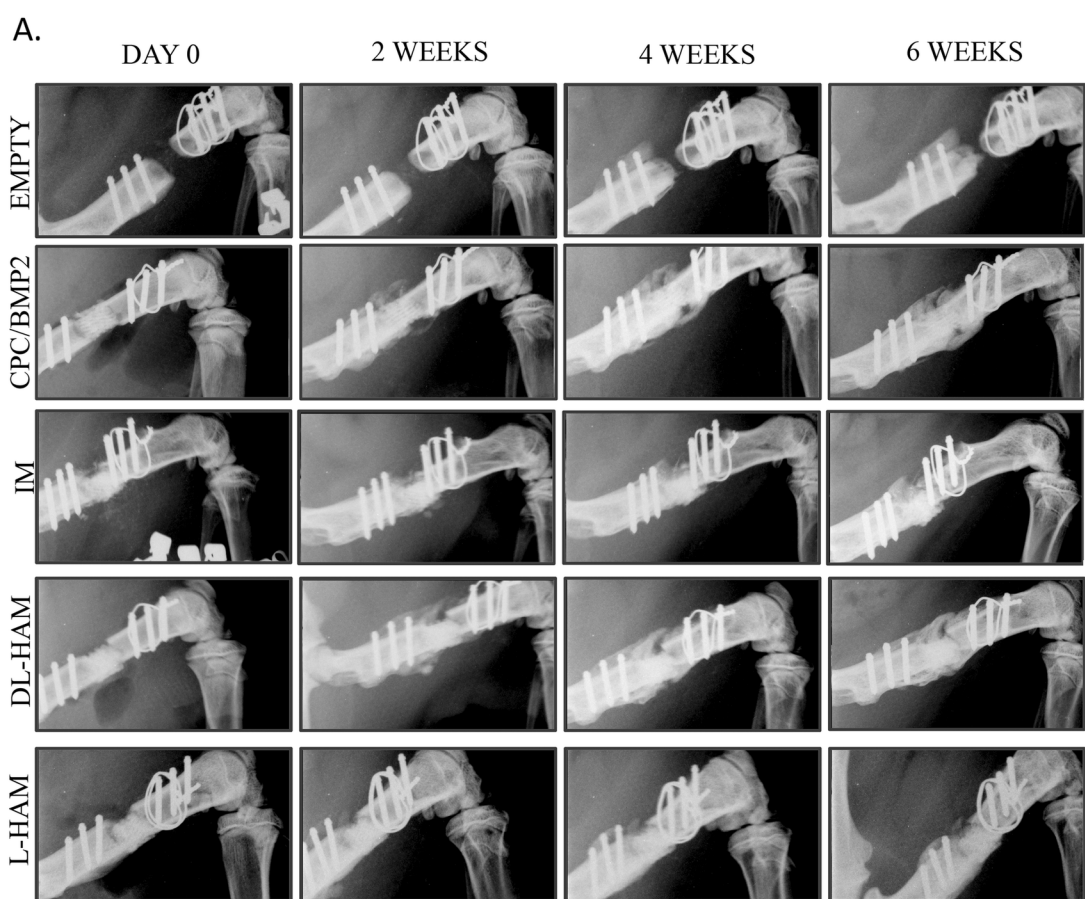
CONTROL



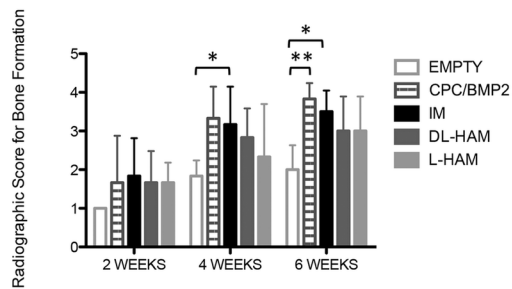
Scale bar: 2mm

Scale bar: 200 μ m

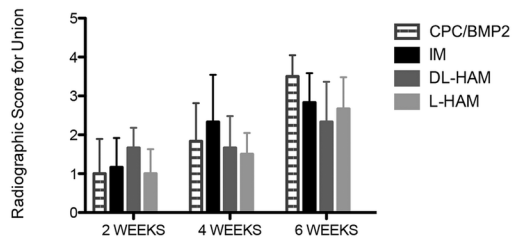
Scale bar: 1 mm



B.



C.



A.

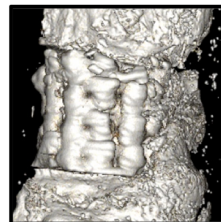
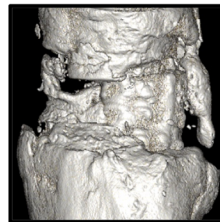
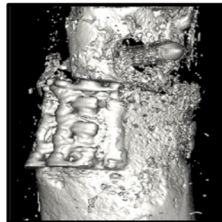
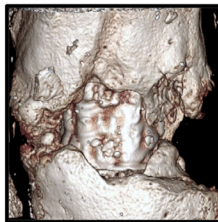
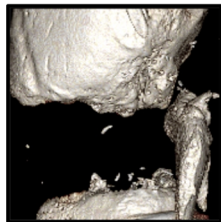
EMPTY

CPC/BMP2

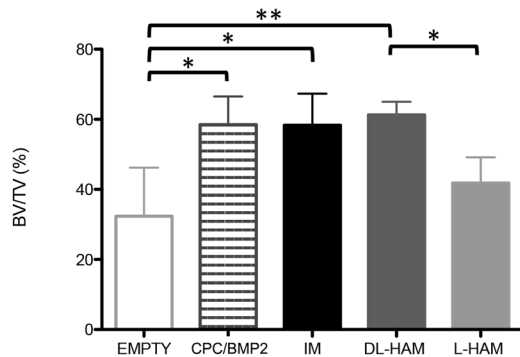
IM

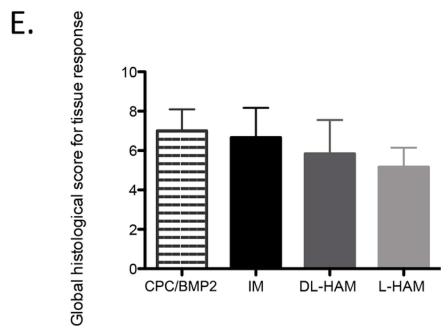
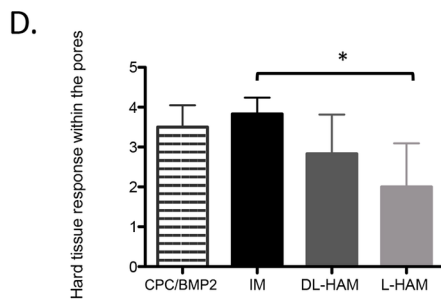
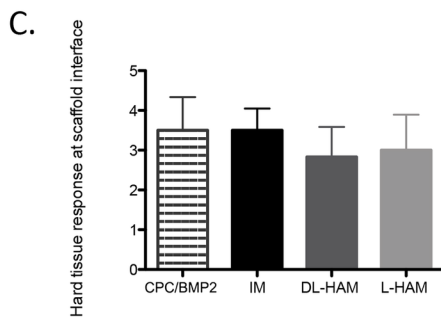
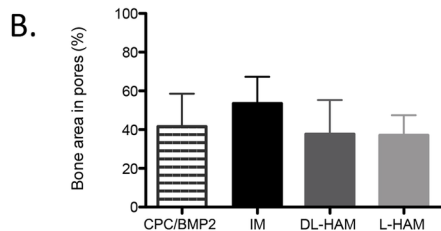
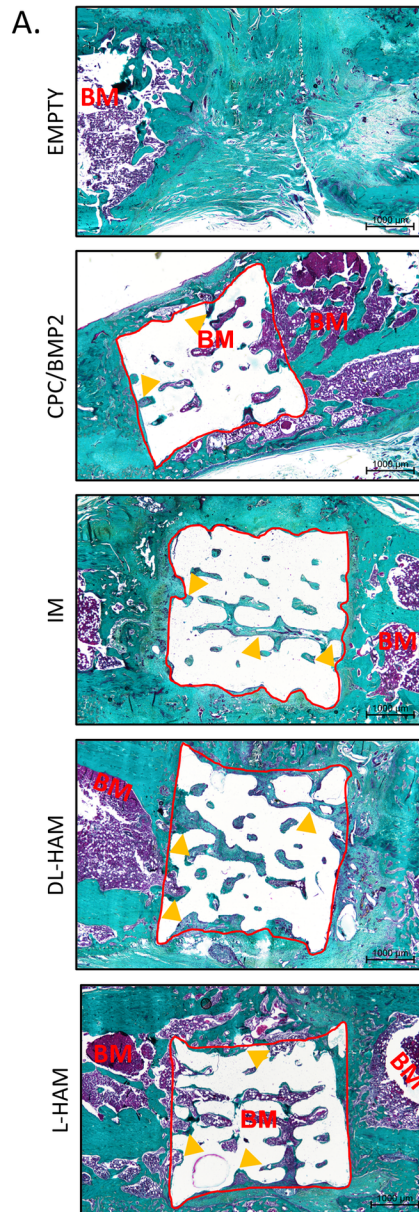
DL-HAM

L-HAM

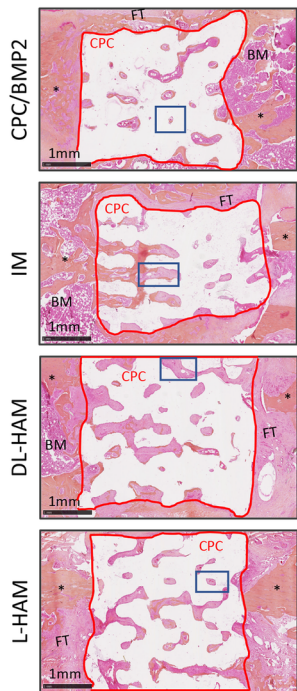


B.

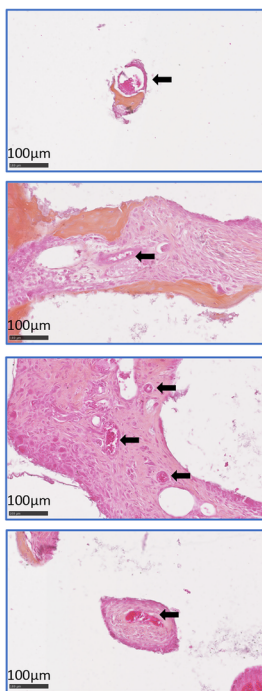




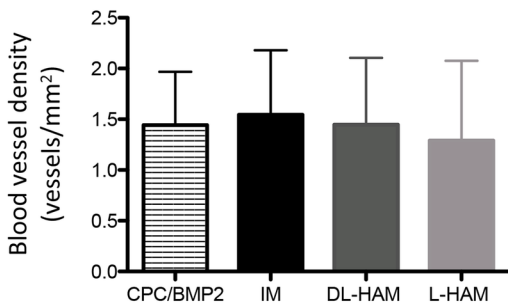
A.



B.



C.

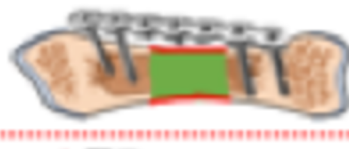
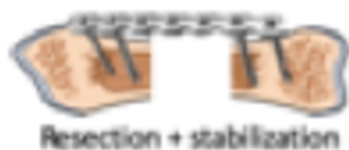


Group	Name	Defect Fillings
1	EMPTY	Left empty
2	CCP/BMP2	CPC scaffold + rhBMP-2
3	L-HAM	CPC scaffold + rhBMP-2 covered by L-HAM
4	DL-HAM	CPC scaffold + rhBMP-2 covered by DL-HAM
5	IM	CPC scaffold + rhBMP-2 covered by the induced membrane

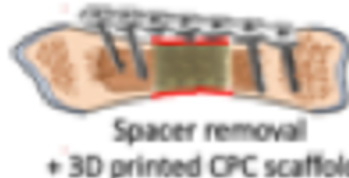
Hard tissue response at scaffold interface	Score
Direct bone to implant contact without soft interlayer	4
Remodeling lacuna with osteoblasts and/or osteoclasts at surface	3
Majority of implant is surrounded by fibrous tissue capsule	2
Unorganized fibrous tissue (majority of tissue is not arranged as a capsule)	1
Inflammation marked by an abundance of inflammatory cells and poorly organized tissue	0
Hard tissue response within the pores	Score
Tissue in pores is mostly bone	4
Tissue in pores consists of some bone within mature, dense fibrous tissue and/or few inflammatory response elements	3
Tissue in pores is mostly immature fibrous tissue (with or without bone) with blood vessels and young fibroblasts invading the space and few macrophagespresent	2
Tissue in pores consists mostly of inflammatory cells and connective tissue components in between (with or without bone) or the majority of the pores areempty or filled with fluid	1
Tissue in pores is dense and exclusively of inflammatory type (no bone present)	0
Presence of cartilage tissue formation	Score
Yes	1
No	0

INDUCED MEMBRANE

1st surgery

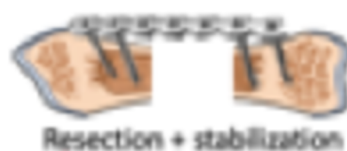


2nd surgery



AMNIOTIC MEMBRANE

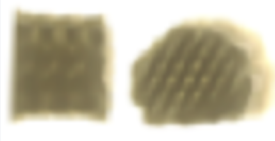
1 surgery



L- HAM
or DL- HAM



3D-PRINTED
CPC SCAFFOLD

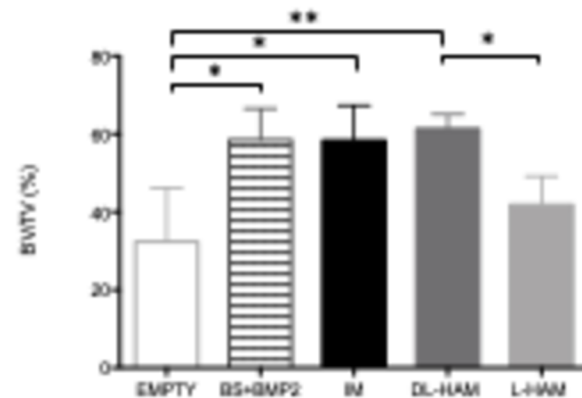


BONE REGENERATION ?

LONGITUDINAL PLANAR X-RAYS



MICRO-CT ANALYSIS



HISTOLOGICAL ANALYSIS

