Evaluation of a Polyethylene Glycol-Osteogenic Protein-1 System on Alveolar Bone Regeneration in the Mini-Pig

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Alveolar bone regeneration associated with the local release of osteogenic protein-1 (OP-1) from a polyethylene glycol (PEG) scaffold was evaluated in 14 mini-pigs. Following extraction of mandibular teeth and 26-weeks of healing time, standardized bone defects were created bilaterally in the posterior mandibles (3 sites for each hemimandible) that were randomly assigned to treatment groups. Seven treatments groups were compared: 4 different concentrations of the PEG/OP-1 test system (n = 14 for each), a positive control (collagen/OP-1, n = 14), a negative control (PEG only, n = 7) and nontreated defects (n = 7). Each animal provided all test and control groups. The animals were sacrificed after 3 weeks of healing and samples were processed for histology and histomorphometry. Three weeks after implantation, there were positive clinical responses for all test groups. Earlier bone maturation was observed in the test groups that had higher concentrations of OP-1 (0.25, 0.5, or 1 mg/mL) compared to the negative control group (PEG alone), the low concentration group (0.1 mg/mL), and the positive control group (collagen/OP-1). However, histomorphometric quantitative analyses did not reveal any statistical difference between any of the groups. No residual PEG biomaterial or inflammatory responses to the biomaterial or growth factor were observed. This study confirmed the safe local delivery of OP-1 from PEG hydrogel. Alveolar bone regeneration was not statistically different between tests groups, negative control (PEG alone) or commercial positive control (collagen/OP-1). The semi-quantitative analysis, however, showed a trend in favor of the higher concentrations of OP-1 to induce faster bone maturation.

Key Words: polyethylene glycol, osteogenic protein-1, minipig histomorphometry, alveolar bone regeneration, dose response

INTRODUCTION

The emergence of tissue engineering-based therapies for bone regeneration has opened new opportunities for the treatment of alveolar bone defects.[1] Local delivery of recombinant growth factors is of particular interest in the maxillofacial region[2] due to their commercial availability and ease of incorporation into a biomaterial carrier, which can be placed at the defect site. While a bone autograft contains useful autologous tissues and cells,[3,4] tissue engineering methods that utilize osteogenic factors eliminate morbidity related to obtaining the donor graft, often at a second surgical site. The major challenge for a wider use of recombinant growth factors in alveolar bone regeneration is the optimization of local delivery systems. Effective local delivery systems enhance efficacy by controlling the timing and dose of delivery while maximizing patient safety by reducing any potential negative effects due to an uncontrolled diffusion of the factor into the surrounding tissues.

The delivery of growth factors at the site where bone regeneration is needed may be accomplished using biomaterial scaffold made of ceramic, collagen or synthetic polymers.[5] Collagen scaffolds have been used to deliver recombinant human bone morphogenetic protein—2 (rhBMP-2) in clinical practice for sinus lift[6] or socket preservation procedures.[7] Preliminary human studies have shown the efficacy of fibrin scaffolds and synthetic polycaprolactone to deliver rhBMP-7[8] and rhBMP-2,[9] respectively. Within these systems, it is difficult to precisely tune the release of the biomolecules from the biomaterial carrier, as there is no chemical binding between the scaffold carrier and the biomolecules. Moreover, the loading and insertion of scaffold biomaterials in the surgical field may be imprecise and is technically demanding.

Bone morphogenetic protein-7 (BMP-7) also referred to as osteogenic protein-1 (OP-1) was isolated in 1990 and shown to induce ectopic bone formation.[10] rhOP-1 has been used in humans for maxillofacial bone regeneration and it has shown promising results in several case reports.[1,11–13] The approved carrier formulation for rh-OP1 in many countries is a powdered bovine bone-derived type 1 collagen (OP-1 device, Stryker, Kalamazoo, Mich) that changes into a viscous gel after rehydration. Some modifications of the type 1 collagen carrier were investigated on enhancing its mechanical properties by adding carboxymethylcellulose[14] or deproteinized bovine bone mineral.[15]

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Recently, a polyethylene glycol (PEG) hydrogel was proposed as a barrier for guided bone regeneration around dental implants and was shown to be as effective as a collagen membrane barrier.[16] In the field of alveolar bone regeneration, PEG hydrogels were also tested for the delivery of covalently bound parathyroid hormone in bone regeneration models in the dog mandible[17] and in rabbit calvaria,[18] showing significant bone repair in these models. PEG hydrogels are injectable, which make them of particular interest within the maxillofacial area for use with minimally invasive procedures. It has been observed that the handling of these injectable systems is easier when compared to the placement of particulate biomaterials. It is widely agreed that the carrier biomaterial of choice should not induce any adverse reaction and should be versatile enough to deliver different doses of the growth factor in a controlled manner. The PEG hydrogel scaffold has potential to satisfy these conditions.

The overall objective of the present work was to evaluate the influence of PEG/OP-1 scaffold/growth factor combination on bone regeneration in a standardized mini-pig mandibular model. The specific objectives were to: (1) Evaluate the effect of OP-1 on bone regeneration based upon dosage; (2) Evaluate the safety of this composite; and (3) Compare the effect of the PEG-OP-1 composite versus the type 1 collagen-OP1 combination.

Materials and Methods

Animals and general anesthesia

This study was conducted at the NAMSA facility (Chasse sur Rhone, France) in accordance with the OECD Good Laboratory Practice regulations, ENV/MC/CHEM (98) 17, with the European Good Laboratory Practice regulations 2004/10/EC Directive and with the United States Food and Drug Administration Good Laboratory Practice regulations, 21 CFR 58 under allowance of the local Ethical Committee. Furthermore this trial fully complies with recently published recommendations.[19]

Fourteen adult males Göttingen mini-pigs (age: 18 months; weight ranging from 26 to 42 kg) were used for this study. Food and water were given ad libitum to the animals during the entire course of the study. Prior to both surgical procedures (teeth extraction and implantation), the mini-pigs fasted overnight. The animals were weighed on the day of surgery and a preoperative injection of carprofene (Rimadyl, 4 mg/kg, subcutaneous, Pfizer, New York, NY) was administered for pain management. The animals were also premedicated with an intramuscular (IM) injection of atropine (atropinum sulfuricum, 0.05 mg/kg, Aguettant, Saint-Fons, France) and buprenorphine (Temgesic, 0.3 mg/mL, 0.05 mg/kg, Schering-Plough, Kenilworth, NJ). General anesthesia was induced with ketamine (Kétamine VIRBAC 1000, 100 mg/mL, 5 to 16 mL, IM, Virbac, Carros, France) mixed with diazepam (Valium, 5 mg/mL, 1.5 to 3 mL, IM, Roche, Basel, Switzerland). During the surgery, anesthesia was maintained when needed with additional Ketamine (Kétamine VIRBAC 1000, 100 mg/mL, 1 to 16 mL, IM, Virbac).

Materials

The synthetic scaffold implanted in the present study was a polyethylene glycol-based hydrogel, formed by reacting a 4-arm acrylate terminated PEG with a linear thiol terminated PEG in an aqueous buffer system (triethanolamine/acetic acid).[20] The PEG termini connected through a highly self-selective addition reaction, forming an elastic gel network. The PEG used here (MX-10) was designed for 10-day hydrolysis degradation in vitro.

The two PEGs were mixed stoichiometrically and dissolved in sterile aqueous 0.04% acetic acid (HAc). For activation of the gelation reaction, a 0.10 M aqueous triethanolamine solution was used (Activator). The PEGs solution and the Activator were sterile filtered and filled into sterile Eppendorf tubes under laminar air flow. OP-1 was dissolved in sterile 0.04% HAc to yield a solution with 10 mg/mL OP-1. This was further diluted to 5.0, 2.5, and 1.0 mg/mL for additional dosage groups. The 4 different OP-1 solutions were filled into sterile Eppendorf tubes and all solutions were stored frozen.

PEG hydrogel scaffolds containing 1.0, 0.5, 0.25, 0.10, or 0 mg/mL OP-1 were prepared by having the different components thawed shortly before application and 130 μl of PEGs solution were mixed with 50 μl of Activator. After 1 minute, 20 μl of the growth factor solution (10, 5.0, 2.5, or 1.0 mg/mL OP-1 in 0.04% HAc solution or 0.04% HAc solution alone as negative control) were added and the resulting solution was transferred to a sterile cylindrical steel mold (reproducing the shape of the bone defect) in which the scaffolds were left for at least 7 minutes to allow for complete gelation.

For the positive control samples, 100 mg of the collagen-OP-1 powder (Stryker) were mixed with 350 μl of sterile saline before use, according to the instructions of the manufacturer. This represents a concentration of 1 mg/mL of OP-1 in the positive control groups.

Surgical procedures

Local anesthesia was performed preoperatively for both extraction and implantation procedures using local injection of lidocaïne (Lidocaïne 2%, Aguettant, 2.5 to 8 mL).

The extraction procedure consisted of flap elevation and removal of the three mandibular premolars and the first molar in each hemi-mandible. The surgical wounds were closed with an absorbable suture (Vicryl 3-0 or 4-0, Ethicon, Somerville, NJ).

Twenty-six weeks after tooth extraction, 6 cylindrical defects of 8-mm diameter and 6-mm depth were made in the edentulous alveolar ridge (3 defects per hemimandible): The recipient sites were exposed by elevation of mucoperiosteal flaps and the alveolar crest was flattened to allow precise preparation of the recipient sites. The recipient cylindrical defects were prepared using spiral drills of increasing diameter under constant irrigation with sterile physiological saline. To help the localization of the defects during histological processing, metallic pins were placed in the alveolar bone, between the cylindrical defects, at the top of the alveolar crest (2 pins per mandible) prior to filling the defects with the different biomaterials. Three devices were inserted in each side of the mandible and the position of each device was randomized in each mini-pig. Primary wound closure was achieved with an absorbable suture material (Vicryl, Ethicon).

Antibiotic therapy (amoxicillin, 15 mg/kg IM, Duphamox LA, Fort Dodge Santé Animale, France) was administered preoperatively for extraction and implantation procedures and every 2 days for 6 days postoperatively to prevent infection at the

TABLE

Design of the study*

Name	Scaffold	Growth Factor	N =
Test 0.1	PEG	OP-1 (0.1 mg/mL)	14
Test 0.25	PEG	OP-1 (0.25 mg/mL)	14
Test 0.5	PEG	OP-1 (0.5 mg/mL)	14
Test 1	PEG	OP-1 (1 mg/mL)	14
Positive control	Collagen	OP-1 (1mg/mL)	14
Negative control A	PEG	-	7
Negative control B	-	-	7

*PEG indicates polyethylene glycol; OP-1, osteogenic protein-1.

surgical site. Carprofene (Rimadyl, Pfizer Santé Animale, approximately 4 mg/kg SC) was administered once preoperatively and 2 days postoperatively to manage the pain related to both surgical procedures.

Study design

The tests samples received the PEG hydrogel infused with 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, or 1 mg/mL OP-1 for the groups Test 0.1, Test 0.25, Test 0.5, and Test 1.0, respectively (n = 14 per group). The negative control samples received the PEG hydrogel alone (n = 7) or were left empty (n = 7). The positive control samples (n = 14) were grafted with the collagen/OP-1 composite (1 mg/mL; Table).

Sample retrieval

All the animals were sacrificed 3 weeks following surgical implantation by lethal injection of barbiturate (Dolethal, Vetoquinol, Lure, France) under general anesthesia (Zoletil 100: tiletamine-zolazepam, 100 mg/mL, Virbac, approximately 10 mg/kg).

The mandibles were resected, and the implant sites were observed macroscopically. Local complications (inflammation, necrosis, hemorrhage or other lesions) were graded and recorded using a numerical scale: (0: absent; 1: slight; 2: moderate; 3: marked; 4: severe). Conventional X-rays have been done and then the implanted sites including bone and soft tissues have been fixed in 10% buffered formalin during 1 week for histology.

Qualitative and quantitative histology

Histological Preparation

After complete fixation, the samples were dehydrated in alcohol solutions of increasing concentration, cleared in xylene, and embedded in polymethylmethacrylate resin. After embedding, the locations of the experimental sites were retrieved and central bucco-lingual (frontal) sections at the position of the implant were obtained.[21] Finally, the histological sections were stained using Basic Fuchsin–Toluidine Blue stain.

Histopathological Interpretation

Histopathological qualitative analysis, involving evaluation of inflammation, signs of infection, necrosis, foreign body reaction, bone remodeling process, and material debris were performed.

Histomorphometric Analysis

Histomorphometric analysis was conducted by digitalizing and examining slides with a Zeiss Axioscope microscope equipped with a color images analyzing system (Samba, version 4.27, Samba Technologies, France). A quantitative analysis was performed to assess the percentage of the initial defect filled by new bone (bone surface), the new bone height in the center of the defect (bone height) and the osteoid density in the center of the defect (osteoid density). The region of interest was delimited in the dimensions of the initial surgical defect and was used for the percentage calculations. The osteoid density was expressed in mm/mm^2 and was measured on a central standardized area.

Statistical analysis

Statistical analysis of the histomorphometrical data was performed using a 1-way ANOVA test (Graph Pad Prism): Kruskall-Wallis test followed by a Dunn's multiple comparison test, with a risk established at 5%. The different groups were compared with ANOVA for bone surface (%), bone height (%) and osteoid density ($\mu m/mm^2$).

RESULTS

Surgical observations

During the implantation procedures, only minor abnormalities were observed at the 84 sites treated. Four sites showed remaining roots, which were removed before implantation. In 7 sites, a breakdown of the buccal/lingual wall or communication with the mandibular canal was observed.

Macroscopic observations

No sign of any negative outcome was observed macroscopically at all study sites. Remaining bone fragments from the implantation surgeries were recorded in 8 out of 84 sites and were associated with a slight inflammation. Neither necrosis nor hemorrhage was noticed at the sites.

Histopathological analysis

After histopathological examination, 4 out of the 84 sites were discarded from histomorphometric analysis. This was due to signs of infection (n = 2), presence of a pseudocyst (n = 1), and difficulties to localize the defect area (n = 1). Consequently, there were 13 samples for the groups PEG-OP-1 0.25 mg/mL, PEG-OP-1 0.5 mg/mL, PEG-OP-1 1 mg/mL and collagen-OP-1. There were 14 samples in the PEG-OP-1 0.1 mg/mL group and 7 samples in the empty and PEG only groups.

In the nontreated defect group, the bone regeneration was seen from the defect margins as immature cancellous bone with thin trabeculation. Osteoblasts and osteoid rims were abundant. No sign of bone remodeling was observed. Restoration of the defect was not achieved and the remaining portion of the defects was filled with a vascularized fibroconnective tissue.

In the groups PEG (MX-10) and PEG (MX-10)-OP-1 (all concentrations), no residue of PEG matrix were detected. The

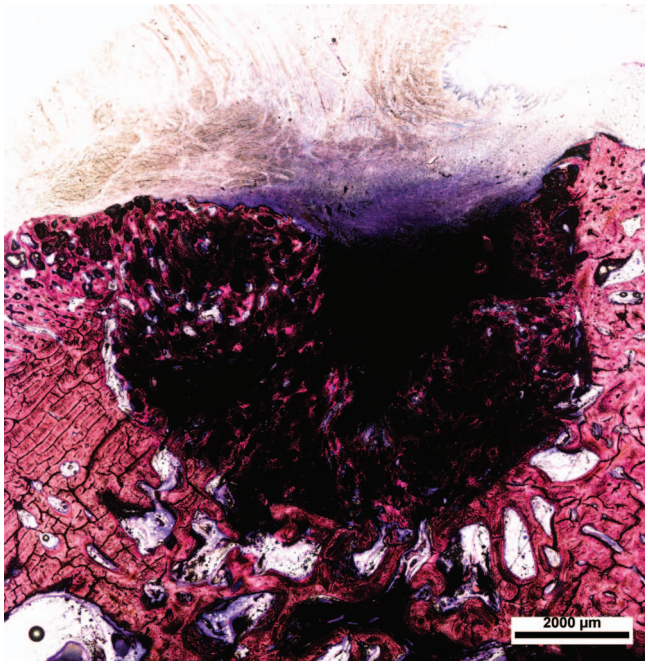


Figure 1. Histological section of a sample from the PEG (MX-10–0.25 mg/mL) group (Goldner Trichrome 2X). Bone regeneration merged from the defect margins as a young spongious bone with generally thin interconnected trabecula. The portion of the defect not filled with new bone is occupied with a vascularized fibroconnective tissue.

bone regeneration was also seen from the defect margins as immature cancellous bone with generally thin interconnected trabecular spaces (Figure 1). Osteoblasts and osteoid rims were abundant. Almost no sign of bone remodeling was observed. When the defect was not fully filled with new bone, the remaining portion of the defects was occupied with a vascularized fibroconnective tissue.

In the group collagen-OP1, very limited residue of collagen matrix, associated with phagocytic cells was detected. These residues were osteointegrated with signs of ongoing ossification. The bone regeneration merged from the defect margins as a young spongious bone, displaying thin to thick interconnected trabecules. Osteoblasts and osteoid rims were abundant. Slight signs of bone remodeling were observed. Restoration of the defect was not achieved and the remaining portion of the defects was filled with a vascularized fibroconnective tissue.

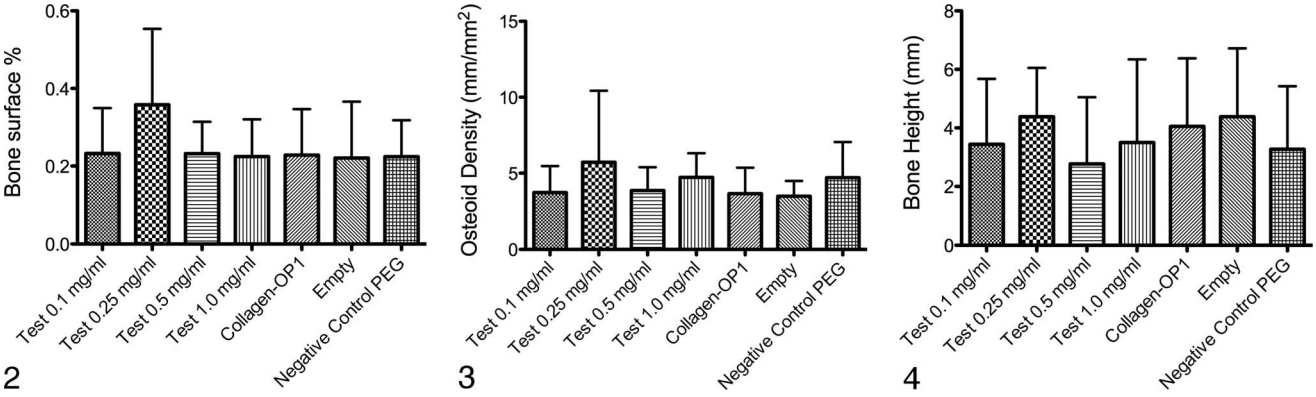
Histomorphometric analysis

The presence of signs of infection and or inflammatory reaction impairing bone growth resulted in the exclusion of 4 of 84 sites from descriptive statistics or biostatistical analysis. The quantitative analysis displayed no statistical differences between the studied groups when expressed in percentage. The percentages of bone area (Figure 2) as well as the osteoid density (Figure 3) were slightly higher in the group PEG (MX-10)–OP-1 (0.25 mg/mL), but not significantly different. The bone height in the center of the defects was not statistically different between the groups (Figure 4).

Discussion

This study describes the effect of the delivery of various doses of OP-1 in standardized intrabony alveolar defects in the minipig, using a novel polyethylene glycol (PEG) hydrogel carrier. We observed similar bone regeneration in all test and control groups, without specific adverse reactions due to rh-OP-1. PEG hydrogel loaded with different doses of OP-1 has a similar positive effect on alveolar bone regeneration as compared to a type-1 collagen matrix loaded with OP-1. Additionally, bone regeneration was similar in the test and negative control sites (empty defects and PEG alone defects). No specific adverse effects were reported in any of the experimental groups.

In the present study, we have obtained substantial bone regeneration after 3 weeks at sites where biomaterial was not placed. This could partly explain why we have observed small differences between the groups. As scaffolds from various treatment groups (with different OP-1 dosage) and controls were implanted relatively closely in the same hemimandibles, we cannot exclude an interaction between these sites after



Figures 2–4. Figure 2. The percentage of bone fill relative to the initial defect (new Bone Area) is slightly higher in the group PEG (MX-10)–OP-1 (0.25 mg/mL), but not significant. **Figure 3.** The percentage of osteoid density is slightly higher in the group PEG (MX-10)–OP-1 (0.25 mg/mL), but not significant. **Figure 4.** The bone height was not statistically different between the groups. For all groups, the bone height in the center of the defect was lower as compared to the maximum bone height measured in the defects.

implantation. In this case, the best groups would have a positive influence in the worst groups and that would reduce the observed differences at the end of the experiment. We have observed few complications (infections), which have been excluded from the study.

For the development of a longer-term study, it would probably be necessary to create larger defects in the mini-pig mandible to insure spontaneous healing, but the standardized defects described here are judged sufficient for this short-term study.[22] An extra-oral approach to create a bone defect in the lateral aspect of the mandible in the mini-pig has also been described to evaluate the osteoconductive properties of biomaterials and the role of barrier membranes.[23,24] The extra-oral approach is localized in the angle of the mandible and provides some advantages related to the large amount of bone available However, as the defects are localized in basal bone, the translation of study results to the intra-oral alveolar environment could be uncertain. An intra-oral alveolar bone model system provides a realistic opportunity for the evaluation of scaffold biomaterials and relevant translation from a preclinical test to a clinical outcome.

PEG hydrogels are currently used for several biomedical applications due to their similarities with extracellular matrix structure and properties, ease of surgical insertion and ease of synthesis and surface modification (grafting of biomolecules). Their degradation kinetics and interactions with the environment can be tuned by modifying hydrogel properties.[25] It has been shown in vitro that it is possible to modify this PEG hydrogel by grafting RGD peptides, and this modification had no deleterious effect on biocompatibility with human gingival fibroblasts.[26] A PEG hydrogel similar to the one tested within the present study was used before in vivo in preclinical experiments delivering covalently bound rhPTH (1-34)[17,18] and rhBMP-2.[27] In these studies, the authors reported on a good tolerance of the implanted constructs without any adverse reaction observed on histological sections. Moreover, a significant increase in bone area and mineralized bone was observed. Our results confirmed that the adjunction of rh-OP-1 to a PEG hydrogel had no negative influence on bone regeneration and that the results are similar when using the commercial carrier (carboxymethylcellulose) and rh-OP-1 (positive control group). The main clinical advantage of using PEG hydrogel is the easy handling of this injectable formulation.

In a previous study testing the ability of a PEG membrane to prevent soft tissue ingrowth in bone defects, Thoma et al[28] used a bone defect model similar to the present study in the minipig (8-mm diameter and 8-mm depth). They reported that this model provided favorable conditions for bone regeneration, due to the presence of 5 bony walls, the proximity of bone marrow cell progenitors and its relatively small size.[28] The same group successfully used PEG hydrogels at different pH alone or in combination with HA-TCP stimulate bone regeneration. In these localized alveolar defects, it has been shown that a PEG with pH = 8.7 was favorable and that the addition of the particulate ceramic bone substitute provided no benefits in terms of bone regeneration in acute and chronic alveolar bone defects.[29]

Bone morphogenetic proteins are currently used for bone regeneration in many clinical situations in orthopedics and oral and maxillofacial surgery. The U.S. Food and Drug Administration and several regulatory administrations worldwide approve BMP-2 and BMP-7 (OP-1) for specific applications.[30] In several preclinical studies OP-1 has demonstrated a positive effect on bone formation for different models of oral and maxillofacial bone regeneration. In the mini-pig, OP-1 has been used successfully to prefabricate and transplant a vascularized bone graft[15,31,32] and it has been used to enhance the bone formation and the osseointegration of titanium implants after sinus graft procedures as compared to platelet rich plasma.[33] Moreover, a preclinical study showed that the placement of BMP-7 on titanium (dental implant) surfaces regenerated bone in a vertical direction around dental implants partially inserted into bone.[34] The characteristics of the scaffold delivery system used for local growth factor release are of critical importance. The delivery system will likely have substantial effect on the local tissue response largely due to the speed and timing of drug release.[35] Several drug release systems used to enhance bone regeneration are currently being developed. These include hydrogels, injectable cements or other natural or synthetic polymers to release peptides, growth factors or antibiotics, or other bioactive agents.[36]

The safe local and efficient local delivery of OP-1 by a PEG system was demonstrated in this study. Future studies are needed to confirm the dose-response effect of this scaffold/growth factor combination in a model where site proximity does not introduce a confounding variable. Given continued positive outcomes, time-course experiments will also be needed to evaluate the time needed to obtain mature bone regeneration using PEG-OP-1.

Abbreviations

HAc: acetic acid
IM: intra-muscular
OP-1: osteogenic protein
PEG: polyethylene glycol
rhBMP: recombinant human bone morphogenetic protein

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