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Enhanced bone regeneration with a synthetic cell-binding peptide—in vivo results

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Abstract

This in vivo study compared the regenerative processes within defined defects of the porcine skull after delivery of a porous algae-derived hydroxyapatite (adHA), a similar, experimental adHA carrying the cell binding peptide P-15, used solely and in combination with 25% autogenous bone (AB). Particulated AB served as a control group. During an observation period of 26 weeks, microradiography and histology were performed at four specific times. Significantly higher mineralization rates ($p = 0.008$) were found 4 weeks after application of the bioactive material in combination with AB. At 12 weeks there was a significantly higher mineralization ($p = 0.036$) following the application of the bioactive form alone. This study showed significantly higher mineralization after use of a P-15 bioactivated material at early stages. Thus, it can be concluded that the application of the P-15 sequence to an hydroxyapatite accelerates the process of early bone formation, whereas no long-term effect was traced.

Keywords: Bone regeneration; Algae-derived hydroxyapatite; P-15 peptide; Bone graft; Collagen

A variety of bone replacement materials have been tested in the last decades [1]. However, an ideal material has not yet been found [2]. Current materials do not contain cellular components required for osteogenesis. They only form a base for new bone growth and possess only osteoconductive properties [3]. In order to obtain osteoinduction and to improve the bone quality of the augmented region, the combined application of such materials together with autogenous bone is recommended by some authors [4–7].

Bone consists of an organic and an inorganic component. Approximately 25% of the bone is organic and is mainly composed of type 1 collagen, three closely entwined strands of amino acids. The sequence (766–780) of an α 1 chain is responsible for cell bonding and the initiation of a cascade of events (migration, proliferation, and differentiation) that results in the formation of new bone. Of all 1300 amino acids only this specific sequence, referred to as P-15 peptide, is involved in the bone formation [8].

It has been demonstrated in vitro that anorganic bone matrix carrying the P-15 sequence enhances the attachment of cells and provides an environment that is permissive for cell migration, cell differentiation, and morphogenesis [8–10]. The P-15 peptide may modulate cell number and tissue structure by the enhancement of viable cell attachment and via the regulation of apoptosis [11]. Results of a recent study suggest that anorganic bone matrix carrying the P-15 complex can be used as an injectable biomimetic matrix to facilitate bone repair [10].

Until now the P-15 peptide has been primarily under investigation in in vitro studies. Possible applications in the repair of large bony defects in a prospective in vivo

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setting have not been explored. Aim of this study was to compare the regenerative potential of a routinely utilized bone graft material to the capacity of the identical material after bioactivation by the P-15 peptide. An established porcine model was chosen to guarantee the transferability of the obtained results to clinical practice [12,13]. Application of the material was performed in the forehead area, which closely resembles the conditions of the maxillofacial region because this bone is of desmal origin and does not depend on central blood supply.

Materials and methods

Bone graft materials. A porous algae-derived hydroxyapatite (FRIOS Algipore, Dentsply Friadent, Mannheim, Germany) was compared to an identical, experimental material carrying the P-15 peptide. The materials are prepared by the hydrothermal conversion of the original calcium carbonate of the algae in the presence of ammonium phosphate at about 700 °C. This process preserves the porosity of the algae [14]. Both materials were in a particulate form with a particle size range of 0.3–2 mm and pores in the range of 5–10 μm. The peptide P-15, GTPGPQGIAGQRGVV, is synthesized by solid phase procedures and absorbed on hydroxyapatite in a saturable manner [9].

Selection of the study animal. The adult domestic pig was the animal of choice, because it is particularly suitable for studies of bone healing or bone restructuring. Its morphological and anatomical features form the prerequisite for the transfer of the results to humans. As a large mammal it is suitable for experimental bone surgery [12]. Tissue blood circulation, circulatory processes, fracture healing, and the rate of new bone formation in the adult pig correlate to those of humans [15]. During the study, the use of standardized surgical procedures and equivalent localization ensured the greatest possible comparability between the experimental groups. The surgery caused only minor pain to the animals and did not impair their ability to move.

Number of animals and formation of the test groups. Sixteen adult female pigs were included in this study.[1] Four test groups were formed and examined at four different times (2 weeks, 4 weeks, 12 weeks, and 26 weeks).

Particulated autogenous bone served as a control group. The evaluated algae-derived hydroxyapatite and the identical material carrying the P-15 peptide each formed one test group. Autogenous bone has osteogenic capacity. The cells within the donor graft synthesize new bone at the implantation site. To evaluate if this behaviour may contribute to a faster regeneration of a defect, in a fourth group 25% autogenous bone was added to the hydroxyapatite/P-15.

There were four animals available per test period, each of which had nine bony defects. The following material combinations were randomly selected per removal time:

Group A	Autogenous bone	9
Group B	adHA	9
Group C	adHA /P-15	9
Group D	adHA /P-15 + 25% autogenous bone	9
Total		36 defects per removal time

[1] The study was approved by the Local Animal Committee of the Government of Midfrankonia, Ansbach, Germany (approval nr. 621-2532.31-5/00).

One additional defect was placed in one animal per removal time. This defect was not filled with any material and served as a control for the model.

Type, conduct, and duration of procedures. All surgical procedures were performed using intubation anaesthesia. The animals were given a perioperative antibiosis 1 h preoperatively and for 2 days postoperatively (streptomycin, 0.5 g/day, Gruenenthal, Stolberg, Germany). An incision was first made to the skin and the periosteum of the skull which created access to the neurocranium. Using a trephine drill (1 × 1 cm, Roland Schmid, Fuerth, Germany) nine identical bony defects were created. The size of the defects (10 mm diameter, 10 mm depth) met the dimension requirements for a critical size defect in porcine species [16,17]. Without the use of a suitable augmentation material such defects are not completely regenerated with bone, but are partially filled with connective tissue [18]. The internal plate of the neurocranium remained completely intact during the procedure. The bone harvested with the trephine drill was used for filling the defects in group A. To produce particles of a defined size, it was crushed in a bone mill (Quetin Dental Products, Leimen, Germany) [19]. According to the manufacturer's guidelines, venous blood was added to the hydroxyapatite materials. The periosteum and skin over the defects were finally sutured in two layers (Vicryl 3.0; Vicryl 1.0; Ethicon GmbH, KG, Norderstedt, Germany).

Polychromatic fluorescence labelling. Starting with the 12th postoperative day, a polychromatic fluorescence labelling was performed. Sequential administration of fluorescent dyes allows one to follow the direction and the topographic localization of new bone formation. During the mineralization process, the fluorescent dyes are incorporated in the matrix of the front of mineralization by chelation [20]. Alizarine-complex (day 12; 30 mg/kg body weight, Sigma–Aldrich GmbH; Taufkirchen, Germany), calcein blue (day 19; 30 mg/kg body weight, Sigma–Aldrich), and xylenol orange (day 28; 20 mg/kg body weight, Sigma–Aldrich) were applied. The animals received intramuscular injections of the fluorescent dyes in a 2% $NaHCO_3$ solution.

Removal and preparation of the specimens. Following the protocol, the animals were sacrificed to allow recovery of the material. The pigs were sedated by an intramuscular injection of azaperone (1 mg/kg) and midazolam (1 mg/kg) in the neck. They were then euthanized by an intravascular injection of 20% pentobarbital solution into a ear vein until cardiac arrest occurred.

The skull caps of the sacrificed animals were removed and immediately frozen at −80 °C. Before preparing the specimens, an X-ray was taken (40–45 kV, 0.25 mA, and 5 min) in a Faxitron cabinet X-ray unit (Faxitron Cabinet X-ray Systems, Illinois, USA) in order to detect the defects. The individual bone defects were separated using a standard cutting system (Exakt Apparatebau GmbH, Norderstedt, Germany). Immersion fixation was carried out using 1.4% paraformaldehyde at 4 °C to render the organic matrix insoluble. The specimens were dehydrated in an ascending alcohol series at room temperature in a dehydration unit (Shandon Citadel 1000, Shandon GmbH, Frankfurt, Germany). Xylol was used as an intermediary fixation. Technovit 9100 (Heraeus Kulzer, Kulzer Division, Werheim, Germany), a special resin which allows quantitative evidence of bone matrix proteins and is also suitable for the cutting and grinding technique according to Donath and Breuner [21], was used for embedding. The method combines the morphological superiority of plastic embedded bone tissue with the advantages of specific enzyme histochemical and immunochemical markers. It permits good preservation of morphological details, the survival of antigenic determinants, and the retention of enzyme activity [22].

Microradiography. Microradiography detects bone boundaries with accuracy and allows one to obtain images from an entire defect, making it possible to measure areas of bone growth [23]. To produce microradiographs, the undecalcified resin embedded sections were reduced to 150–180 μm using a grinding unit (Exakt Apparatebau GmbH, Norderstedt). Subsequently the samples were irradiated in the Faxitron cabinet X-ray unit using 11 kV tube voltage and 0.25 mA for