Bone Regeneration Using a Synthetic Matrix Containing a Parathyroid Hormone Peptide Combined with a Grafting Material

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Purpose: The aim of the present study was to test whether a newly developed synthetic matrix made of polyethylene-glycol (PEG) containing a covalently bound peptide of the parathyroid hormone (PTH_{1-34}) enhances bone regeneration compared to grafting procedures and to spontaneous healing. **Materials and Methods:** *In each of 16 rabbits used, 4 titanium cylinders were screwed into perforated slits made in the cortical bone of the calvaria. The cylinders were either left empty (control) or filled with 1 of the following: (1) PEG matrix and hydroxyapatite/tricalcium phosphate (HA/TCP) granules, (2) PEG matrix containing 100µg/mL of PTH_{1-34} and HA/TCP granules, or (3) PEG matrix containing 20µg/mL of PTH_{1-34} and HA/TCP granules. After 8 weeks, the animals were sacrificed, and ground sections were obtained for histology.* **Results:** *Quantitative histomorphometry demonstrated a significantly increased amount of newly formed bone for PTH_{1-34} compared to sites treated with PEG and HA/TCP and to empty control sites ($P < .01$; analysis of variance and subsequent pairwise Student t test). The mean percentages of mineralized bone were 19.6% ± 6.0% for 100µg/mL PTH, 18.0% ± 6.2% for 20µg/mL PTH, 12.0% ± 6.5% for PEG and HA/TCP without PTH, and 10.5% ± 3.7% for the empty control. The mean areas of bone regenerated within the cylinders were 53.5% ± 22.7% for 100 µg/mL PTH, 51.1% ± 22.6% for 20 µg/mL PTH, 34.3% ± 22.5% for PEG and HA/TCP without PTH, and 23.2% ± 10.1% for the empty control.* **Discussion:** *Human and animal trials have demonstrated that daily systemic injection of PTH increases bone mineral density. The present study showed that local administration of PTH was also effective in stimulating bone formation.* **Conclusion:** *It is concluded that this synthetic PEG hydrogel containing a covalently bound peptide of the PTH combined with HA/TCP granules significantly stimulated in situ bone augmentation in rabbits.* INT J ORAL MAXILLOFAC IMPLANTS 2007;22:258–266

Key words: bone regeneration, calcium phosphates, graft material, hydroxyapatite, parathyroid hormone, polyethylene glycols

Following tooth loss or tooth extraction, a natural process of alveolar bone resorption occurs. The result may be inadequate bone volume for the placement of endosseous dental implants.[1]

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Several methods have been described for the regeneration of lost alveolar bone.[2] These procedures typically involve the application of bone graft materials. The use of autogenous bone as grafting material appears to render the most predictable results. However, the use of this "gold standard" material is limited because of limited tissue resources and donor site morbidity.[3] Further work is necessary in the search for effective and safe alternatives to autogenous bone grafts.

The most intriguing method of enhancing the local bone volume is the induction of bone formation. In the past decade a number of preclinical and clinical studies have indicated that the use of growth factors and differentiation factors can significantly improve bone and soft tissue regeneration.[4–8] It has been shown that the regenerative potential of such factors depends to a high degree on the method of applica-

tion.[9,10] One common problem with the current application methods is the lack of control over the presentation or release kinetics of the bioactive molecules. The use of peptides or natural proteins to stimulate rapid and predictable bone formation could greatly advance the practice of bone regeneration.

Animal experiments have documented an anabolic effect of the parathyroid hormone (PTH) on both cancellous and cortical bone; this effect increases the strength of the bones.[11,12] In addition, it has been shown that the 34 amino acid domain of the PTH has biological activity similar to that of a full-length protein.[13] In humans, PTH is presently used for systemic treatment of osteoporosis. For this purpose, a dose of 20 µg/day is delivered to osteoporotic patients through daily injections in a pulsed fashion. Clinical trials have shown this therapy to be effective in increasing bone density and decreasing the risk of vertebral and nonvertebral fractures.[14] While PTH has been shown to be of substantial benefit in the systemic treatment of osteoporosis, its use for the treatment of local bone defects has not been evaluated. Local administration of the PTH, as opposed to intermittent systemic injections, would have multiple advantages.

Hydrogels made of polyethylene glycol (PEG) can serve as an in situ forming matrix for optimal cell ingrowth and retention of bioactive proteins.[15] In a recent in vivo and in vitro study, it was demonstrated that the release kinetics of bone morphogenetic protein (rhBMP-2) can be controlled by entrapping the rhBMP-2 in this synthetic matrix. This enabled highly efficient and highly localized bone regeneration.[15]

The combination of a PTH peptide bound to a synthetic PEG matrix has recently been shown to be effective in stimulating bone regeneration around dental implants in the dog mandible.[16] The use of a PEG matrix containing a PTH peptide in combination with grafting materials for local bone augmentation has not been investigated yet.

The aim of the present study was to test whether a newly developed synthetic matrix made of PEG containing a covalently bound peptide of the parathyroid hormone (PTH_{1-34}) combined with hydroxyapatite/tricalcium phosphate (HA/TCP) granules enhances bone regeneration compared to grafting procedures with HA/TCP and to spontaneous healing in rabbits.

MATERIALS AND METHODS

Animals

Sixteen adult (12-month-old) New Zealand White rabbits, weighing between 3 and 4 kg, were used in the present study. The animals were kept in a pur-

pose-designed room for experimental animals and were fed a standard laboratory diet. The study was evaluated and accepted by the responsible Veterinary Authority.

Synthetic Matrix and Bioactive Peptides

The synthetic matrix used in the present study was a PEG-based hydrogel. This gel was formed by reacting a 4-arm PEG with acrylate endgroups with a linear PEG with thiol endgroups (Nektar Therapeutics, Huntsville, AL) in an aqueous buffer system (triethanolamine/HCl).[17] The PEG termini connect through a highly self-selective addition reaction, forming an elastic gel network. Immediately before application, both PEG solutions were sterile-filtered and mixed with HA/TCP granules. For the activated gels, a 35 amino acid peptide of the PTH (cys-PTH_{1-34}) and the 9 amino acid cys-RGD peptide (Bachem, Bubendorf, Switzerland) were added first to the PEG-acrylate solution, resulting in the formation of covalent bonds between the cystein residues and the PEG acrylate. The final concentrations for the peptides were 350 µg/g for cys-RGD gel and 20 or 100 µg/g for cys-PTH_{1-34} gel.

Surgical Procedure

Anesthesia of the animals was initiated by injection of 65 mg/kg of ketamine and 4 mg/kg of xylazine and maintained with isofluorane/oxygen (O_2). The surgical area was first shaved and disinfected with iodophor to allow for aseptic surgical conditions. A straight incision was made from the nasal bone to the midsaggital crest. The soft tissues were reflected, and the periosteum was elevated. In the right and left parietal and frontal bones, 4 evenly distributed 6-mm-diameter circular slits were prepared with a trephine bur under copious irrigation with sterile saline. The trephine drill was modified by a custom-made depth stop, which allowed the slits to be prepared with a standardized 1-mm sink depth. The external cortical plate inside this circle was not removed but was perforated with 5 evenly distributed holes using a round bur with a diameter of 1 mm (Fig 1). Subsequently, a specially designed cylinder made of commercially pure titanium was screwed in each of the circular slits; primary stability was obtained. The inside surface of each cylinder was machined titanium. The cylinders measured 7 mm in height and 7 mm in outer diameter. The apical end of the cylinder was threaded like a screw; the crestal end featured a small shoulder for a titanium lid (Fig 2).

Each animal received 4 cylinders. One of the cylinders served as a first control and was left empty. Another cylinder served as a second control and was augmented with a combination of an unfunctional-

Fig 1 Four evenly distributed 6-mm circular slits with a sink depth of 1 mm were prepared with a trephine drill. The external cortical plate was perforated with 5 holes.
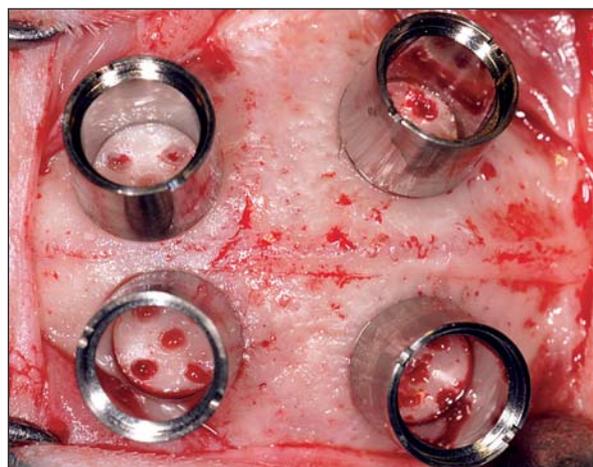
Fig 2 Four titanium cylinders with a screw design toward the bone site were screwed into each of the slits, and primary stability was obtained.
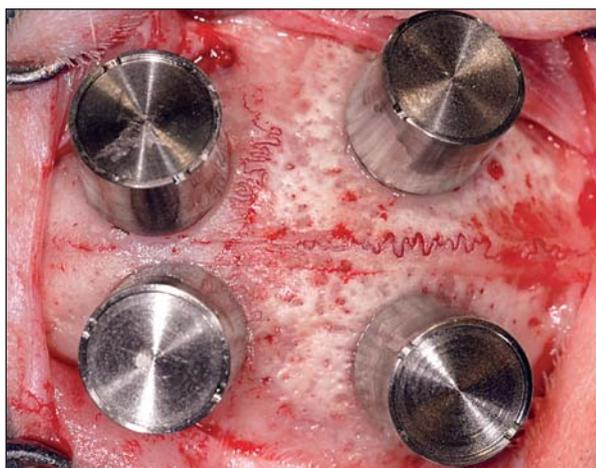
Fig 3 After augmentation, the 4 cylinders were closed with a titanium lid.

ized PEG matrix and a standardized amount of HA/TCP granules (Straumann Bone Ceramic; Institut Straumann, Basel, Switzerland). The granules were highly porous and had a size range of 500 to 1,000 μm. The 2 remaining cylinders served as test sites. One was augmented with a combination of the PEG matrix containing 100 μg/mL of cys-PTH$_{1-34}$ and the HA/TCP granules (test 1). The other was augmented with a combination of the PEG matrix containing 20 μg/mL of cys-PTH$_{1-34}$ and the HA/TCP granules (test 2). The cylinders were randomly assigned for each animal in a clockwise direction. For the cylinders containing PEG and the HA/TCP granules. The in situ gelating PEG matrix was applied extraorally in liquid form to the HA/TCP granules and mixed for about 10 seconds. Subsequently, this putty-like mixture was applied to the determined cylinders. Within 60 seconds, the PEG gels set and thus stabilized the HA/TCP granules. The cylinders were left

open toward the bone side but were closed with a titanium lid toward the covering skin-periosteal flap (Fig 3). The periosteum and the cutaneous flap were adapted and sutured for primary healing.

Eight weeks later, the rabbits were sedated with barbiturates and sacrificed by an overdose of ketamine. The skulls, which contained the cylinders, were removed and placed in 40% ethanol.

Histologic Preparation

The samples were dehydrated in a graded series of ethanols. Thereafter, they were embedded in methyl methacrylate without being decalcified according to standard procedures.[18] In order to facilitate the infiltration of the methyl methacrylate into the tissues, the titanium lid of the cylinders was lifted up slightly or removed. The specimens were sectioned in the frontal plane through the middle of the cylinders. Sections of 200 μm thickness were obtained, ground, and polished to a uniform thickness of 60 to 80 μm. The specimens were surface-stained with toluidine blue.[18]

Histomorphometry

Quantitative evaluation of bone regeneration was assessed by applying standard morphometrical techniques.[19,20] Measurements were carried out directly in the light microscope at a magnification of 160× using an optically superimposed eyepiece test grid composed of 100 points and 10 cycloid lines.[20,21] The number of test points overlying the profiles of the different components (ie, mineralized bone tissue, nonmineralized tissue, and graft particles) were counted. They were defined and symbolized according to the standard nomenclature of the International Society for Stereology.[22] The graft-to-bone contact was calculated in the lower, middle, and

upper thirds of the cylinder by the number of intersections between graft particles and the outlines of either mineralized bone or nonmineralized tissue.

In addition, a quantitative evaluation of the area of bone regeneration within the cylinders was carried out in order to have a more clinically relevant parameter. Digital images were obtained and processed with an image analysis program (Adobe Photoshop 7.0.1; San Jose, CA). Measurements were carried out directly in the digital images at a magnification of 12.5×. Thereafter, the numbers of pixels within the total cylinder area were counted. The borders of the newly formed bone, including bone marrow and integrated graft particles within the cylinders, were manually marked on the computer screen using a digital pen. Subsequently, the pixels within this marked area were counted by the software. The area of bone regeneration was calculated as follows: Area of bone regeneration (%) = (pixel number of the bone area/total pixel number of the cylinder) × 100.

Statistical Analysis

Mean values and standard deviations were calculated for the amount of bone formation within the cylinders for both evaluation methods (point measurement and area of bone regeneration) and for graft-to-bone contact. The results are displayed as box plots. For statistical analysis repeated measures analysis of variance (ANOVA) and subsequent pairwise Student t tests with corrected P values according to Holm's were used to detect the differences between the 4 treatment modalities.

RESULTS

During the experiment, all animals showed uneventful healing of the area of surgery. No reductions in body weight were noted, and no postoperative infections were observed. Upon specimen retrieval it was discovered that 3 cylinders had been dislocated from the skull bone because of loss of fixation and were embedded in soft connective tissue. These 3 cylinders, 2 from test sites and 1 from a control site, were excluded from further analysis. The remaining 61 cylinders were found to be stable and in the same position as at placement.

Descriptive Histology

Qualitative histological evaluation revealed varying amounts of newly formed bone with no signs of inflammation in all cylinders. In the empty control cylinders, the augmented tissue consisted of slender bone trabeculae and large marrow spaces. Adjacent to the inner walls of these cylinders the bone trabec-
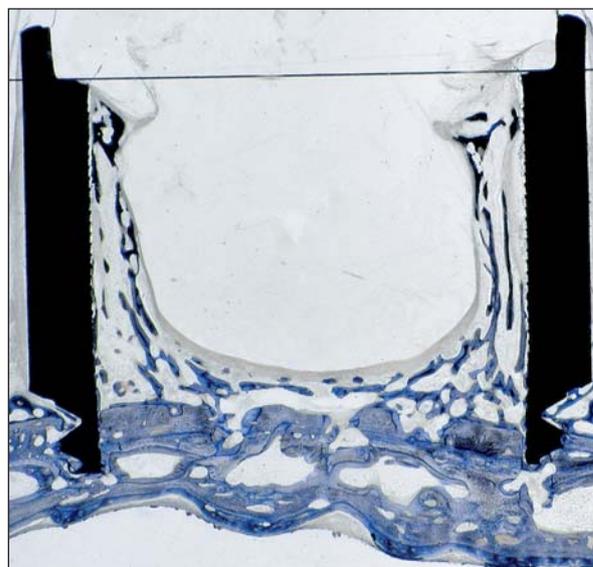
Fig 4 Histologic section of an empty control cylinder after 8 weeks of healing (new bone = light blue; toluidine blue; original magnification × 10).

ulae were oriented in a parallel manner. Various degrees of direct contact with the titanium surface of the machined cylinders were observed (Fig 4).

Within the control cylinders containing the unfunctionalized PEG matrix and the HA/TCP granules, the amount of newly formed bone varied greatly. In contrast to the empty cylinders bone growth was not predominantly along the titanium walls but was typically in contact with the bone substitute material. Most of the graft particles were intact and evenly distributed within the augmented tissue. In the upper third of the cylinders, the HA/TCP granules were mainly surrounded by nonmineralized tissue (Fig 5). In the 2 test groups significantly more newly formed bone could be detected. Some newly formed bone reached the upper third of the cylinder (Figs 6 and 7).

Histomorphometry

The quantitative histomorphometric analysis revealed that both PTH groups showed a significantly increased amount of newly formed bone compared to sites treated with the unfunctionalized PEG and HA/TCP granules and to the empty control sites. The addition of PTH to the PEG matrix revealed both a 1.6-fold increase in the percentage of mineralized bone and in area of bone regeneration within the cylinders. Compared to spontaneous healing, an even more pronounced increase of 1.9-fold for percentage of mineralized bone and of 2.3-fold for area of bone regeneration was detected with the use of PTH (Figs 8 and 9).
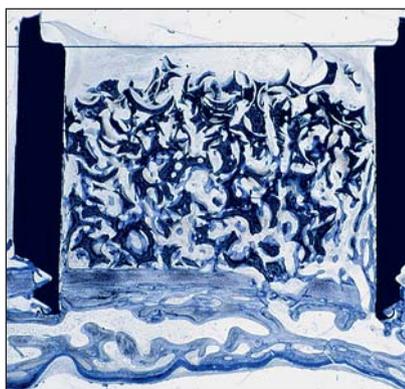
Fig 5 Histologic section of a control cylinder augmented with a combination of PEG matrix and HA/TCP granules after 8 weeks of healing (new bone = light blue, HA/TCP granules = dark gray; toluidine blue staining; original magnification ×10).
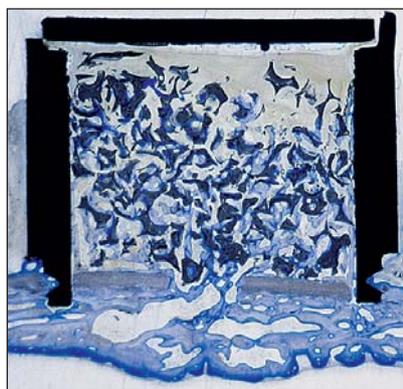
Fig 6 Histologic section of a test cylinder augmented with a combination of PEG matrix (20 µg/mL) and HA/TCP granules after 8 weeks of healing (new bone = light blue; HA/TCP granules = dark grey; toluidine blue; original magnification ×10).

Fig 7 Histologic section of a test cylinder augmented with a combination of PEG matrix containing 100 µg/mL and HA/TCP granules after 8 weeks of healing (new bone = light blue, HA/TCP granules = dark grey; toluidine blue; original magnification ×10).
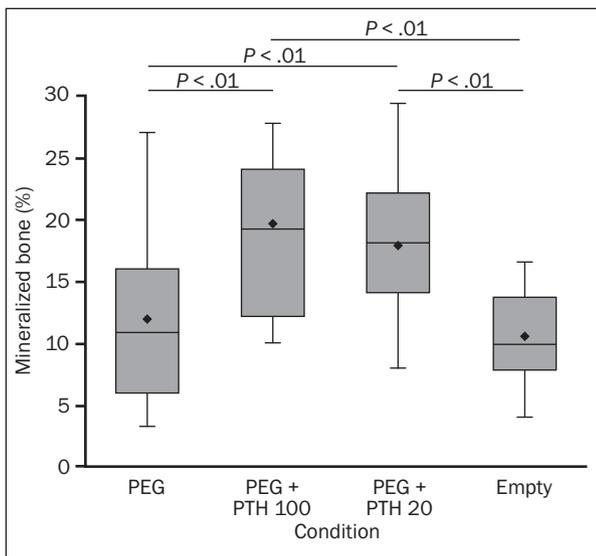
Fig 8 The percentage of mineralized bone generated within the cylinders in each group is displayed. The midline indicates the median value, the diamond indicates the mean value, and the whiskers indicate the maximum and minimum values.
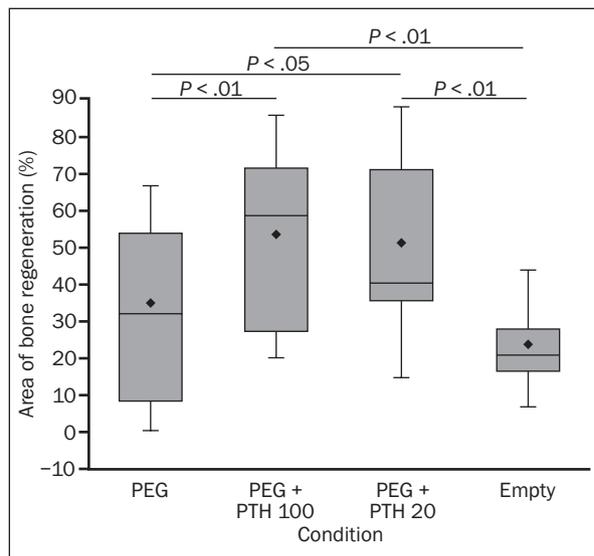
Fig 9 The percentage of bone regenerated with respect to the total area within the cylinder is displayed for each group. The midline indicates the median value, the diamond indicates the mean value, and the whiskers indicate the maximum and minimum value.

The percentage of mineralized bone evaluated by point measurements within the total cylinder for each group is shown in Table 1. The addition of PTH was correlated with an overall increase of the area of bone regeneration. In the 2 test cylinders 53.5% ± 22.7% (100 µg/mL PTH) and 51.1% ± 22.6% (20 µg/mL PTH) of the total area was regenerated bone tissue, including bone marrow and integrated graft particles. For the PEG alone and the empty control cylinders, significantly less bone tissue was regenerated (Fig 9, Table 2). The unfunctionalized PEG group revealed consistently but not significantly higher values for percentage of mineralized bone and area of bone regenerated compared to the empty controls.

No effect of the higher PTH concentration could be detected when the cylinders augmented with 20 or 100 µg/mL of the PTH peptide were compared.

The percentage of the surface of the bone substitute particles covered with newly formed bone is shown in Table 3. Higher values of graft-to-bone contact were found in the lower third of the cylinder in all groups compared to the values in the middle and the upper third. Although the PTH groups revealed higher values of graft-to-bone contact in the lower and the middle thirds, no significant difference between the unfunctionalized PEG group and the test groups could be detected (Fig 10). All groups showed large standard deviations.

Table 1	Percentage of Mineralized Bone		
Condition	No.of samples	Mean (%)	SD
PEG	16	12.0	6.5
PEG + PTH 100	16	19.6	6.0
PEG + PTH 20	14	18.0	6.2
Empty	15	10.5	3.7

Percentage of test points overlying mineralized bone compared to the total number of test points overall (n = 100).

Table 2	Area of Bone Regeneration		
Condition	No. of samples	Mean (%)	SD
PEG	16	34.3	22.5
PEG + PTH 100	16	53.5	22.7
PEG + PTH 20	14	51.1	22.6
Empty	15	23.2	10.1

Table 3	Graft-to-Bone Contact						
		Lower third		Middle third		Upper third	
Condition	No. of samples	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
PEG	16	32.7	29.7	23.5	24.1	6.7	12.9
PEG+PTH 100	16	47.4	21.6	31.1	22.5	7.1	8.8
PEG+PTH 20	14	48.8	19.9	23.6	20.5	2.5	7.1

DISCUSSION

The present study demonstrated that the combination of a newly developed synthetic matrix made of PEG containing a covalently bound peptide of the PTH_{1-34} combined with HA/TCP granules significantly increased the amount of bone regeneration. This was documented by the significantly greater amount of bone formation found at these sites compared to sites treated with PEG and HA/TCP granules but without PTH and to empty control sites.

The local application of PTH for the regeneration of bone defects is new. One recent animal study investigated the use of a synthetic, RGD-modified PEG matrix containing a covalently bound peptide of PTH_{1-34} for bone regeneration around dental implants in the dog mandible.[16] It could be demonstrated that the PEG matrix containing PTH_{1-34} beneficially affected bone regeneration and that the amount of bone regenerated was similar to that achieved using autogenous bone. In past years, systemic PTH treatment has demonstrated anabolic effects on bone healing in humans and animals.[23–25] The osteogenic potential of PTH was first described in 1932.[23] Single daily injections of bovine parathyroid extract stimulated osteoblast formation and bone formation in rat pups. Since then, the main features of PTH's osteogenic action have been established by consistent results from animal experiments using the fully bioactive synthetic PTH_{1-34} fragment.[26,27] The anabolic effect of PTH has been associated with increases in osteogenic cell proliferation and differentiation and prolonged osteoblast survival.[28,29] New evidence indicates that PTH directly inhibits SOST (sclerostin) transcription in vivo and in vitro.[30] SOST is the gene for sclerostin, a bone mor-
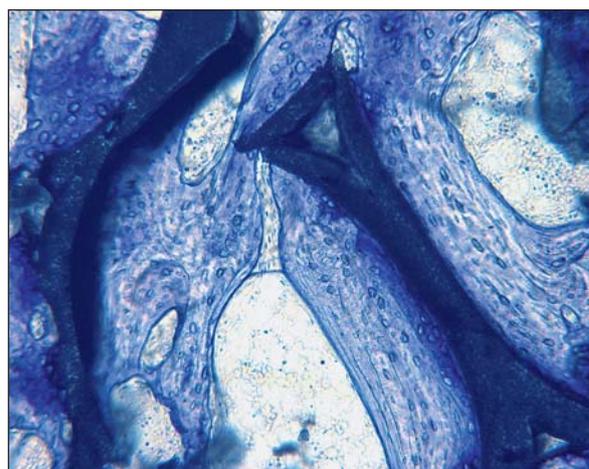
Fig 10 Histologic section of a test cylinder augmented with a combination of PEG matrix containing 100 µg/mL and HA/TCP granules. A high percentage of the surfaces of the granules is in direct contact with newly formed bone (new bone = light blue, HA/TCP granules = dark gray; toluidine blue; original magnification ×160).

phogenetic protein (BMP) antagonist involved in the restriction of new bone formation and in the inactivation of the bone formation.[31]

Human trials have demonstrated anabolic effects of PTH treatment in both women and men with osteoporosis.[14,32] Daily subcutaneous administration of 20 µg PTH_{1-34} decreased the risk of vertebral and nonvertebral fractures and increased the total-body bone mineral density.[14] Animal experiments have documented that intermittent injection of PTH can enhance guided bone regeneration and can increase

mechanical strength and density of new bone after distraction osteogenesis.[33,34] All these studies have in common that the PTH was mainly administered by daily systemic injections of small doses. It would be of great advantage to be able to beneficially effect local bone formation not by systemic but by local application of PTH. This would allow circumvention of the problems arising from the complex pharmacokinetic behavior associated with its systemic application.

The PEG-based hydrogel used in the present study was optimized by the addition of pendant oligopeptide ligands for cell adhesion (RGD peptides).[35] The addition of RGD peptides to a PEG matrix has had a significant influence on cellular ingrowth.[15] Spindle-shaped fibroblasts migrated radially out into the surrounding PEG matrix only when the RGD ligand was present. Invasion was absent in networks lacking RGD as a result of the lack of biological recognition of the PEG.[36]

In the present study the synthetic PEG hydrogel was applied in a liquid form and turned into an elastic gel after setting in situ. It has been demonstrated previously that this gel can be successfully used for self-containing bone defects.[16] For bone augmentation, however, the gel might have insufficient mechanical properties. Hence, the present study investigated the use of a combination of HA/TCP granules with the PEG matrix in an augmentation model. The HA/TCP granules were mixed with the liquid form of the PEG in order to achieve an even distribution of the particles within the matrix. Qualitative histologic observations revealed that this aim was reached after 8 weeks. The results of the present study indicated a favorable distribution of the granules within the PEG matrix. In addition, the PEG and the HA/TCP granules completely occupied the cylinder space indicating maintenance of the initially obtained volume. Furthermore, the osteoconductive properties of the HA/TCP granules in combination with the PEG could be observed in both groups. Considerable amounts of new bone had formed in direct contact with the HA/TCP granules, particularly in the lower two thirds of the cylinders. These histologic findings were in accordance with a previous rabbit study using similar titanium cylinders with grafting materials by Slotte and associates.[37] They reported that in the upper third of the cylinder the bovine bone mineral was surrounded by soft connective tissue, while in the lower two thirds, mainly mineralized bone enclosed the graft material. With respect to the lower two thirds of the cylinders in the present study, the percentage of graft-to-bone contact in all PEG- and HA/TCP-treated groups exceeded the percentage of area filled with newly formed bone. This

implies a particular affinity between graft and bone.[38] Although there was a difference in graft-to-bone contact between the PTH-treated sites and the control sites without PTH, statistical significance was not reached. The range of values of graft-to-bone contact in the present study was similar to or lower than those reported in other rabbit studies using different grafting materials after 8 weeks of healing.[38–40] This might be due to the area selected for measurement. In the present study graft-to-bone contact was evaluated within the total cylinder area, whereas in 2 of the other studies only the central section of the specimen was selected for quantitative assessment.[38,40]

Equal amounts of newly formed bone were found in the cylinders augmented with 20 and 100 µg/mL of the PTH peptide. This indicated that the higher concentration of PTH did not significantly improve bone regeneration in this augmentation model. In addition, 20 µg/mL of the PTH_{1-34} has been demonstrated to be effective in treating peri-implant bone defect in the dog mandible.[16] The percentage of mineralized bone achieved using a combination of synthetic PEG matrix containing PTH_{1-34} with HA/TCP granules (18.0% to 19.6%) was similar to the percentages achieved using autogenous bone (17.3%) and bovine bone mineral (19.9%) after 12 weeks of healing.[37] In the present study the total area within the cylinders was assessed, whereas in the aforementioned study[37] only the area of augmented tissue within the cylinders was evaluated. Taking into account the volume of the entire cylinder regardless of the area occupied by tissue will lead to lower percentage values compared to an assessment where only the area of regenerated tissue is measured.

The model used in the present study was evaluated and compared to untreated rabbit calvaria in a recent experiment.[41] It has been concluded that the bilateral use of the parietal bones represents a reliable model for experimental guided bone augmentation regarding blood supply and bone quality. However, further research is necessary to evaluate the difference in bone regeneration between 2 and 4 titanium cylinders placed on the rabbit skull. Four cylinders would offer great advantages in reducing the sample size for animal experiments.

It is concluded that this synthetic PEG hydrogel containing a covalently bound peptide of PTH combined with HA/TCP granules significantly stimulates in situ bone augmentation in rabbits.

ACKNOWLEDGMENTS

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