Bone morphogenetic protein-2 enhances bone formation when delivered by a synthetic matrix containing hydroxyapatite/tricalciumphosphate

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Abstract

PURPOSE: The aim of the present study was to test whether or not a synthetic matrix consisting of a polyethylene glycol (PEG) hydrogel containing recombinant human bone morphogenetic protein-2 (rhBMP-2) combined with grafting materials enhances bone regeneration compared with grafting alone or empty control sites. MATERIAL AND METHODS: In each of 10 rabbits, four titanium cylinders were screwed in perforated slits made in the external cortical bones of the calvaria. The following four treatment modalities were randomly allocated: (1) empty control, (2) a combination of a PEG matrix and hydroxyapatite/tricalciumphosphate (HA/TCP) granules and a combination of a PEG matrix containing either 10 microg/ml (3) or 30 microg/ml (4) of BMP-2 and HA/TCP granules. After 8 weeks, the animals were sacrificed and ground sections were obtained for histological analysis. For statistical analysis repeated measures ANOVA and subsequent pairwise Student's t-test were applied (P<0.01). RESULTS: Histomorphometric analysis showed an average area fraction of newly formed bone of 13.96 +/- 5.98% for the empty control, 15.16 +/- 7.95% for the PEG and HA/TCP group, 26.32 +/- 8.56% for the group containing 10 microg rhBMP-2/ml, and 30.15 +/- 7.63% for the group containing 30 microg rhBMP-2/ml. Statistical analysis revealed significantly more newly formed bone in the two rhBMP-2 groups compared with the PEG and HA/TCP group and with the empty control. Regarding the surface fraction of the HA/TCP graft particles covered with newly formed bone the addition of rhBMP-2 revealed a more than two-fold increase compared with cylinders containing HA/TCP granules without rhBMP-2. This difference reached statistical significance. CONCLUSIONS: It is concluded that rhBMP-2 significantly enhances bone regeneration in rabbits when delivered by a synthetic matrix containing HA/TCP. This synthetic PEG matrix containing HA/TCP granules apparently fulfills a number of criteria required for an ideal carrier system for rhBMP-2.
BMP-2 enhances bone formation when delivered by a synthetic matrix containing HA/TCP

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Key words: Bone morphogenetic protein, polyethylene glycol, carrier material, bone regeneration, hydroxyapatite, calcium phosphates

Running title: The effect of BMP-2 on bone formation

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Abstract

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**Materials and Methods:** In each of 10 rabbits, 4 titanium cylinders were screwed in perforated slits made in the external cortical bones of the calvaria. The following 4 treatment modalities were randomly allocated: (1) empty control, (2) a combination of PEG matrix and hydroxyapatite/tricalciumphosphate (HA/TCP) granules and a combination of PEG matrix containing either 10µg/ml (3) or 30µg/ml (4) of BMP-2 and HA/TCP granules. After 8 weeks, the animals were sacrificed and ground sections were obtained for histological analysis. For statistical analysis repeated measures ANOVA and subsequent pairwise Student's t-test were applied (p<0.01).

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**Conclusion:** It is concluded that rhBMP-2 significantly enhances bone regeneration in rabbits when delivered by a synthetic matrix containing HA/TCP. This synthetic polyethylene-glycol matrix containing HA/TCP granules apparently fulfills a number of criteria required for an ideal carrier system for rhBMP-2.
Introduction

Bone morphogenetic proteins, a subfamily of the transforming growth factor-ß superfamily, were discovered based on the bone-inductive activity sheltered in bone matrix (Urist 1965). One of these proteins, recombinant human bone morphogenetic protein-2 (rhBMP-2) has been shown to induce bone formation in a variety of indications. A large number of animal and human studies have documented the successful use of rhBMP-2 to induce and enhance bone regeneration for mandibular resection defects (Toriumi et al. 1991; Boyne 1999), for cleft palate defects (Boyne et al. 1998), for alveolar ridge defects (Cochran et al. 1999; Barboza et al. 2000; Jung et al. 2003), and for sinus floor augmentations (Nevins et al. 1996; Boyne et al. 1997). In addition, it has been shown that dental implants placed in bone induced by rhBMP-2 osseointegrate and respond to long-term functional loading much like dental implants placed in native alveolar bone (Jovanovic et al. 2003).

Various biomaterials have been tested as carriers for rhBMP-2 in craniofacial preclinical models (Wikesjö et al. 2003). These include an absorbable collagen sponge (ACS) (Boyne et al. 1997; Howell et al. 1997), decalcified bone matrix (Toriumi et al. 1991; Sigurdsson et al. 1996), hyaluronan (Hunt et al. 2001), deproteinized bovine bone matrix (Jung et al. 2003), hydroxyapatite (Koempel et al. 1998), calcium phosphates (Wikesjö et al. 2002), polylactic acids (Weber et al. 2002) and polymethylmethacrylate (Boyne & Shabahang 2001).

In spite of the large amount of preclinical and clinical studies with various biomaterials, rhBMP-2 has not yet found the way into clinical practice in dentistry. Economic reasons and problems encountered with the lack of an ideal carrier system might be responsible for this. ACS is the most frequently used carrier system for rhBMP-2 and has been extensively tested (Howell et al. 1997; Cochran et al. 1999). Collagen can bind BMPs to some degree and is readily infiltrated by cells and remodeled into bone tissue (Lutolf et al. 2003). However, like other natural polymers, it has some limitations in clinical use, primarily because of the lack of space maintenance, the difficulty to engineer its properties, and its immunogenicity (Sigurdsson et al. 1996; Barboza et al. 2000). In order to improve the space maintenance of ACS the use of barrier membranes for guided bone regeneration have successfully been
applied (Wikesjö et al. 2003). In contrast, recent experimental studies reported that bone formation following application of rhBMP-2 in conjunction with barrier membranes was delayed and results were hampered, when the membrane was exposed and subsequent infections occurred (Cochran et al. 1999; Jovanovic et al. 2003).

A synthetic carrier material which enables vascular and cellular ingrowth and which is space-providing would, therefore, be desirable to define the spatial configuration of the induced bone. In recent animal studies it was demonstrated that hydrogels made of polyethylene glycol (PEG) fulfill a number of criteria required to serve as in situ forming matrix for optimal cell ingrowth and retention of bioactive proteins (Lutolf et al. 2003; Jung et al. [a]). In one study it was shown that the release kinetics of rhBMP-2 could be controlled by entrapping the rhBMP-2 into the synthetic PEG matrix (Lutolf et al. 2003). The results showed efficient and highly localized bone formation. Although this material is a promising candidate for a successful carrier material the gel might not have sufficient mechanical stability to provide space for bone regeneration in larger defects. Therefore, it was suggested to combine the PEG material with a grafting material exhibiting osteoconductive properties (Hartman et al. 2005; Jung et al. [b]).

The aim of the present study was to test whether or not a synthetic matrix made of polyethylene glycol (PEG) containing rhBMP-2 combined with hydroxyapatite /tricalciumphosphate granules enhances bone regeneration. The hypothesis was that the rhBMP-2 leads to more bone formation than the grafting material alone or than empty control sites.
Materials and Methods

Animals

The present animal investigation was evaluated and approved by the responsible Animal Research Ethics Committee at the University of Zurich, Switzerland. 10 adult (12 months old) New Zealand White rabbits, weighing between 3 and 4 kg, were used in the present study. The animals were kept in a purpose-designed room for experimental animals and were fed a standard laboratory diet.

Synthetic matrix and bone morphogenetic proteins

The present study employed a polyethylene glycol-based hydrogel as a synthetic matrix and carrier system for rhBMP-2. Preparation of the gel was performed similar to previously published protocols (Elbert et al. 2001; Jung et al. [a]). In brief, the elastic gel network was formed by reacting of stoichiometric amounts of a 4-arm PEG with acrylate endgroups and a linear PEG with thiol endgroups (Nektar Therapeutics, Huntsville AL) in an aqueous buffer system (2 mM HCl).

For the control gels a 9 amino acid cys-RGD peptide (Bachem, Bubendorf, Switzerland) was added to the PEG-acrylate solution. The BMP containing test gels were identical to the control gels with the exception that 10 or 30 µg of rhBMP-2 in a 2 mM HCl solution were mixed into the PEG solution (2 mM HCl). The final concentrations amounted to 350 µg/g gel for the cys-RGD and to 10 or 30 µg/ml gel for the rhBMP-2, respectively.

Surgical procedure

Anesthesia of the 10 animals was initiated by injection of 65 mg/kg of ketamine and 4 mg/kg of xylazine and maintained with isoflurane/O₂. The surgical procedure and the augmentation device have been comprehensively described in a previous paper (Jung et al. [b]). At the cranium of each rabbit a full thickness flap was elevated to expose the right and the left parietal and frontal bones. Four evenly distributed 6 mm diameter circular slits with 1 mm sink depth and perforations of the external cortical plate inside the slits were prepared. Subsequently, a specially designed cylinder made of c.p. titanium with a machined surface was screwed in each of the slits obtaining primary stability. The cylinders
measured 7 mm in height and 7 mm in outer diameter and exhibited a screw design towards the bone site and a small shoulder for a titanium lid towards the covering skin flap (Fig.1).

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 the RGD modified PEG matrix and 0.15 g of hydroxyapatite (HA) / tricalciumphosphate (TCP) granules (Straumann Bone Ceramic®; Institut Straumann AG, Basel, Switzerland). The granules were highly porous and had a size range of 500–1000 μm. The 2 remaining cylinders served as test sites and were augmented with a combination of the RGD modified PEG matrix containing either 10 μg/ml (test 1) or 30 μg/ml (test 2) of rhBMP-2 and the HA/TCP granules. The cylinders were assigned in a random systematic manner. For the first animal the treatment modalities were randomly chosen for the four sites. In all subsequent animals the sequence of the treatment modalities was kept but the locations were stepwise rotated by one position in clockwise direction. For the cylinders containing PEG and the granules the PEG matrix in its liquid form was mixed with the HA/TCP granules for about 10 seconds. Subsequently, this putty-like mixture was placed into the determined cylinders. Within 60 seconds, the PEG gels set and thus stabilized the HA/TCP granules. The cylinders were left open towards the bone but were closed with a titanium lid towards the covering skin-periosteal flap (Fig.2). 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 Ketamin. The skull containing all 4 cylinders was removed and placed in 40% ethanol.

**Histologic preparation**

The samples were dehydrated in a graded series of increasing ethanol concentrations. Thereafter, they were embedded in methylmethacrylate without being decalcified according to standard procedures (Schenk et al. 1984). The specimens were sectioned in the frontal plane through the middle of the cylinders. Sections of 200μm thickness were obtained, ground and polished to a uniform thickness of 60-80μm. The specimens were surface-stained with toluidine blue (Schenk et al. 1984).
Histomorphometry

Quantitative evaluation of bone regeneration was assessed by applying standard morphometrical techniques (Weibel 1980; Gundersen et al. 1988). Measurements were carried out directly in the light microscope at a magnification of 160x, using an optically superimposed eyepiece test grid composed of 100 points and 10 cycloid lines (Schenk & Olah 1980). The number of test points overlying the profiles of the different components (i.e. mineralized bone tissue, non-mineralized tissue and graft particles) were counted. They are defined and symbolized according to the standard nomenclature of the International Society for Stereology (Exner 1987). The graft to bone contact was calculated by the number of intersections between graft particles and the outlines of either mineralized bone or non-mineralized tissue.

Statistical analysis

Mean values and standard deviation were calculated for the area fraction of new bone formation within the cylinders and for the graft to bone contact. Values were displayed as box-plots ranging from the 25th (lower quartile) to the 75th (upper quartile) percentile including the mean and whiskers showing the minimum and maximum values. For statistical analysis repeated measures ANOVA and subsequent pairwise Student's t-test with corrected p-values according to Bonferroni 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 weights were noted, and no postoperative infections were observed. Upon specimen retrieval one cylinder from the control group with HA/TCP without rhBMP-2 was found to be dislocated from the skull bone because of loss of fixation and was embedded in soft connected tissue. This cylinder was, therefore, excluded from further analysis. The remaining 39 cylinders were found to be stable and in the same position as at the time of placement.

Descriptive histology

The external and internal cortical plates of the cranium were clearly visible on the histological specimens. The threaded base of all cylinders was well integrated in the external cortical plate of the skull. In rare situations new bone formation was found on the outside of the cylinders apparently climbing from the external skull base upwards along the outside wall of the cylinder.

The staining allowed a clear distinction between the graft material and the regenerated bone. New bone formation within the cylinder space seemed to occur from the skull. The area closer to the covering lid showed a lower bone density than the area closer to the skull. Apparently, perforations through the external cortical plate served as the pathways through which new bone formation invaded the cylinder space.

Tissue fill inside the cylinders varied strongly between the experimental groups. The empty control group demonstrated more or less tissue formation occupying about half the cylinder volume (Fig. 3). The groups containing HA/TCP granules revealed an even distribution of the granules within the entire cylinder, indicating that the PEG gels stabilized the granules within the cylinder. A large variability of granule sizes was observed within a given cylinder as well as between cylinders. In the HA/TCP containing cylinders newly formed bone trabeculae frequently bridged the spaces between the granules (Fig. 4). Within the area delimited by the regenerated bone a high degree of bone to graft contact was discernible. This indicated osteoconductive properties of the HA/TCP. To the naked eye the mount of newly formed bone appeared to be considerably higher in cylinders treated with rhBMP-2 (Fig. 5 and 6).
Histomorphometry

Quantitative histomorphometry revealed that both groups containing rhBMP-2 showed a significantly increased amount of newly formed bone within the cylinders compared to the control groups. Average area fractions of newly formed bone after 8 weeks amounted to 13.96±5.98% for the empty control group, to 15.16±7.95% for the HA/TCP group, to 26.32±8.56% for the group containing 10µg rhBMP-2/ml, and to 30.15±7.63% for group containing 30µg rhBMP-2/ml. Statistical analysis revealed that the area fraction of newly formed bone in the empty controls was not statistically significantly different from the group containing HA/TCP granules without rhBMP-2. Similarly, the difference in area fraction of newly formed bone in the two BMP-containing groups did not reach statistical significance. However, highly statistical significant difference in area fraction of newly formed bone was observed for both two groups containing BMP (10µg/ml and 30µg/ml) compared to two groups without the growth factor (control and HA/TCP alone) (Table 1 and Fig. 7).

The surface fraction of bone substitute particles covered with newly formed bone is shown in Table 2 and Figure 8. The addition of rhBMP-2 to the HA/TCP granules revealed a more than two fold increase in bone to graft contact. Compared to the HA/TCP granules alone (22.95±17.35) the addition of 10µg/ml (47.51±18.74) and 30µg/ml rhBMP-2 (55.18±19.67) revealed a highly statistically significant difference. No statistical significant difference was detected between the two BMP treated sites.
Discussion

The present study demonstrated that the combination of a polyethylene glycol (PEG) matrix containing rhBMP-2 together with HA/TCP granules significantly increased the amount of bone regeneration. This was documented by significantly more bone formation and by significantly more bone to graft contact compared to control sites treated without rhBMP-2.

A large variety of carrier and matrix systems have been tested for the application of rhBMP-2 in animal models (Wikesjö et al. 2003). It has been suggested that a successful candidate carrier system should provide optimal conditions for vascular and cellular in growth, and for release kinetics of growth factors (Brekke & Toth 1998). The carrier system should further be reproducible, nonimmunogenic, moldable, and space providing (Wikesjö et al. 2003). In recent animal studies it has been shown that fibrin as a carrier system for growth factors fulfills a number of criteria for an ideal matrix (Bruder & Fox 1999; Schmoekel et al. 2004a; Jung et al. 2005). In addition to optimal cellular in growth properties, fibrin contains a variety of adhesion sites for cells. Furthermore, remodeling of the fibrin matrix takes place by cellular proteolytic activities during cell in growth. Nonetheless, the fibrin matrix is a product derived from human blood, and therefore, carries the potential risk for transmission of agents.

In order to have a completely reproducible and nonimmunogenic carrier system mimicking some of the properties of fibrin, a synthetic matrix made of PEG has been investigated (Lutolf et al. 2003; Jung et al. [a]). A previous in vitro and in vivo study documented that PEG combines the advantages of synthetic materials and of native protein-based materials (Lutolf et al. 2003). It was shown that the use of PEG containing rhBMP-2 resulted in complete bone coverage of critical size cranial defects in rats similar to the use of a collagen matrix containing rhBMP-2. In contrast to this enzymatically degradable PEG material the present study used a hydrolytically degradable PEG matrix, which was first investigated in a recent animal study (Jung et al. [a]). It was demonstrated that this PEG matrix containing a covalently bound peptide of the parathyroid hormone (PTH1-34) beneficially affected bone regeneration to an amount similar to the use of autogenous bone.
In all of these studies using PEG as a matrix system for bioactive factors the matrix has been additionally optimized by incorporation of the peptide Arg-Gly-Asp (RGD peptide). The effect of RGD peptide on cell adhesion was first identified more than 20 years ago (Pierschbacher et al. 1984). In order to prove the cell adhesion properties of RGD incorporated into the PEG matrix a recent in vitro study was performed (Lutolf et al. 2003). It was shown that human fibroblasts migrated radially from cell clusters into the surrounding PEG matrix only if the matrix contained RGD. This improved cell attraction cause by the RGD peptide might also have an effect on bone formation. In a variety of experimental studies it was demonstrated that the addition of RGD to implant surfaces increases bone formation significantly compared to uncoated implants (Schaffner et al. 1999; Elmengaard et al. 2005; Schliephake et al. 2005). However, the present study revealed no significant increased bone formation in sites treated with PEG containing RGD without rhBMP-2 compared to the empty control sites. This might be explained by the fact that indeed undifferentiated cells grow into the cylinder but they might experience sufficient signals in order to become osteoblast cells. The possible synergistic effect of RGD and rhBMP-2 in the test cylinder need to be further investigated.

An additional criterion for an ideal carrier material is that it is able to provide the space for regeneration. While it is recognized that collagen is one of the best researched carrier material, different studies have documented inadequate bone regeneration when collagen is used alone as a bone graft substitute (Barboza et al. 2000; Howell et al 1997). Increased regeneration volume has been shown when collagen was combined with grafting materials consisting of HA/TCP (Miranda et al. 2005; Arosarena et al. 2005). It has been concluded that HA/TCP combined with collagen appeared to be a suitable carrier for rhBMP-2. The presently used PEG gel without granules has been successfully used in self-containing bone defects (Jung et al. [a]). Because the hydrogel alone might have insufficient mechanical properties for larger bone augmentation procedures, the combination of the PEG matrix with HA/TCP granules was tested in the present study. It could be shown that the combination of the PEG matrix with HA/TCP and rhBMP-2 was effective in augmenting the entire test cylinders. This was documented be a two-fold increase of newly formed bone in the rhBMP-2 treated sites compared to the control sites. Although, the higher rhBMP-2 concentration (30μg/ml) showed slightly higher amounts
of newly formed bone compared to the lower concentration (10μg/ml) a statistically significant difference could not be observed. This indicates that a 3-fold increase of the rhBMP-2 concentration within the PEG matrix did not significantly improve bone regeneration in this augmentation model. A recent study using a similar animal model with two titanium cylinders on the rabbit skull compared the effect of demineralized bovine bone mineral and autogenous bone on bone augmentation within the cylinders (Slotte et al. 2003). For augmentation with autogenous bone 17.3% and for demineralized bovine bone mineral 19.9% newly formed bone was found within the cylinders after 12 weeks of healing (Slotte et al. 2003). In the present study both rhBMP-2 concentrations revealed a higher amount of newly formed bone (26.3 and 30.2%, respectively) after 8 weeks. Hence, within a shorter time period more bone was regenerated by the use of rhBMP-2 applied in a PEG matrix containing HA/TCP compared to a study using either autogenous bone or demineralized bovine bone mineral. From a clinical point of view, it may be speculated that the investigated grafting material can be used for bone defects that are nowadays recommended to augment with autogenous bone grafts. This in combination with the shorter healing time would represent an important step forward in simplifying bone augmentation procedures.

The osteoconductive properties of the HA/TCP granules in combination with the PEG hydrogel could be observed in all groups. Considerable amounts of new bone had formed in direct contact with the HA/TCP granules. Furthermore, the rhBMP-2 treated sites showed a 2 to 2.5 fold increased bone to graft contact compared to sites treated with HA/TCP without rhBMP-2. This is in agreement with a randomized clinical trial showing a similar two-fold increase of bone to graft contact when a demineralized bovine bone mineral was combined with rhBMP-2 (Jung et al. 2003). Comparable amounts of graft to bone contacts could be detected between the control cylinders with HA/TCP (22.95%) and a recent study using demineralized bovine bone minerals (24.93%) for augmenting cylinders in a similar animal model (Slotte et al. 2003).

The present study used rhBMP-2 concentrations of 10 and 30μg/ml. This is at least 10 times less compared to the majority of experimental studies and about 50 to 100 times less compared to clinical studies using rhBMP-2 for bone regeneration (Boyne et al. 1997; Higuchi et al. 1999; Cochran et al. 2003).
It is known that the kinetics of protein release can greatly influence bone regeneration (Schmoekel et al. 2004b). Different studies reported a prolonged release of rhBMP-2 from hydrogels either made of PEG or gelatin (Hong et al. 1998; Lutolf et al. 2003). The presently used PEG hydrogel revealed an in vitro degradation of 13 days (Jung et al. [a]). Although the release kinetics of rhBMP-2 from the presently used PEG gel has not yet been evaluated, based on the above data a sustained release over 1 to 2 weeks might be anticipated. The slow and local release of rhBMP-2 might be critical in reducing the concentrations to a level, which is acceptable for clinical applications.

It is concluded that rhBMP-2 significantly enhances bone regeneration in rabbits when delivered by a synthetic matrix containing HA/TCP. This synthetic polyethylene-glycol matrix containing HA/TCP granules apparently fulfills a number of criteria required for an ideal carrier system for rhBMP-2.

Acknowledgements

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### Tables

#### Table 1  Fraction of mineralized bone

<table>
<thead>
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<th>Condition</th>
<th>Number of samples</th>
<th>Mean (%)</th>
<th>SD</th>
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<tr>
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<td>9</td>
<td>13.96</td>
<td>5.98</td>
</tr>
<tr>
<td>PEG</td>
<td>10</td>
<td>15.16</td>
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#### Table 2  Bone to graft contact

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<th>Number of samples</th>
<th>Mean (%)</th>
<th>SD</th>
</tr>
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<tr>
<td>PEG</td>
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<td>PEG/BMP 30</td>
<td>10</td>
<td>55.18</td>
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</table>
Figures

Fig. 1: Four titanium cylinders with a screw design towards the bone site were screwed in each of the slits obtaining primary stability.

Fig. 2: After augmentation with the respective treatment modalities, the four cylinders will be closed with a titanium lid towards the covering skin-periosteal flap.

Fig. 3: Histological section of an empty control cylinder after 8 weeks of healing (Toluidine blue staining; new bone = light blue)

Fig. 4: Histological section of a control cylinder augmented with a combination of PEG matrix and HA/TCP granules after 8 weeks of healing (Toluidine blue staining; new bone = light blue, HA/TCP granules = dark grey)

Fig. 5: Histological section of a test cylinder augmented with a combination of PEG matrix containing 10 µg/ml rhBMP-2 and HA/TCP granules after 8 weeks of healing (Toluidine blue staining; new bone = light blue, HA/TCP granules = dark grey)

Fig. 6: Histological section of a test cylinder augmented with a combination of PEG matrix containing 30 µg/ml rhBMP-2 and HA/TCP granules after 8 weeks of healing (Toluidine blue staining; new bone = light blue, HA/TCP granules = dark grey)

Fig. 7 and 8: Values of area fraction of newly formed bone (Fig. 7) and bone to graft contact (Fig. 8) are displayed as box-plots ranging from the 25th to the 75th percentile including the mean and whiskers showing the minimal and maximal values (Statistically significant different *=p<0.05 and **=p<0.01)
Fig. 7

Area fraction of newly formed bone (%)

empty     0 µg BMP     10 µg BMP     30 µg BMP

0,00 1,00 2,00 3,00 4,00
treatment
Fig. 8

Bone to graft contact (%)

0 µg BMP  10 µg BMP  30 µg BMP

0.00  20.00  40.00  60.00  80.00

**  *  

Bone to graft contact (%) for different BMP concentrations (0 µg, 10 µg, and 30 µg). The graph shows the variability and central tendency of bone graft contact in each condition.