Effect of rhBMP-2 dose on bone formation/maturation in a rat critical-size calvarial defect model


Abstract
Background: Application of recombinant human bone morphogenetic protein-2 (rhBMP-2) has been associated with significant adverse events in craniofacial settings, including swelling and seroma formation. Recent work has demonstrated an inverse relationship between bone formation/maturation and rhBMP-2 dose, frequency/severity of adverse events increasing with rising dose.

Objective: The objective of this study was to determine the most effective dose for rhBMP-2 soak-loaded onto an absorbable collagen sponge (ACS) carrier for bone formation/maturation using an established defect model.

Methods: One hundred sixty-eight outbred male Sprague-Dawley rats, age 11–13 weeks, weight 325–375 g randomized into seven groups of 24 subdivided into groups of eight, were used to provide radiographic and light microscopy observations of bone formation/maturation and aberrant healing events at 2, 4 and 8 weeks following application of rhBMP-2/ACS into critical-size, ø8-mm, through-through, calvarial osteotomy defects for a dose of 1.25, 2.5, 5.0, 10.0 and 20.0 µg rhBMP-2/defect, or serve as ACS or sham-surgery controls.

Results: rhBMP-2 dosages ≥2.5 µg/defect showed histological defect closure >90% within 2 weeks, and complete resolution within 4 weeks. Adverse healing events including swelling, excessive bone formation or seroma formation could not be determined with certainty in this defect model. Notably ACS control sites showed complete defect closure at the 8-week healing interval.

Conclusions: rhBMP-2/ACS accelerates local bone formation in the rat critical-size through-through calvarial defect model once reaching an osteoinductive dose threshold. This threshold may already be reached at a 1.25-/2.5-µg dose in this model. No further enhancement to bone formation/maturation may be observed adding rhBMP-2 above the 2.5-µg dose. The 1.25–20.0 µg dose range did not invoke appreciable aberrant healing events.

Conflict of interest and source of funding statement
This study was supported in part by a grant from Medtronic Spine & Biologics, Memphis, TN, USA. Drs. Cristiano Susin, Jaebum Lee and Ulf M.E. Wikesjö are supported by a grant from Nobel Biocare AG. Dr. Amanda N. Buxton is an employee of Medtronic Spine & Biologics. All other authors claim no conflict of interest. The opinions expressed in this article do not represent the views of the US Department of Defense, the Department of the Army or the US Army Dental Corps. Use of any commercial products in this project does not imply endorsement by the US Government.

Manuel Pelaez1,2, Cristiano Susin1, Jaebum Lee1, Tiago Fiorini1,3, Frederick C. Bisch4, Douglas R. Dixon5, James C. McPherson III6, Amanda N. Buxton7 and Ulf M.E. Wikesjö1
1Laboratory for Applied Periodontal & Craniofacial Regeneration (LAPCR), Georgia Regents University College of Dental Medicine, Augusta, GA, USA; 2US Army Dental Activity, Fort Bragg, NC, USA; 3Section of Periodontology, School of Dentistry, Federal University, Porto Alegre, Rio Grande do Sul, Brazil; 4US Army Advanced Education Program in Periodontics, Fort Gordon, GA, USA; 5US Army Dental Activity, West Point, NY, USA; 6US Army, Department of Clinical Investigations, Ft. Gordon, GA, USA; 7Medtronic Spine & Biologics, Memphis, TN, USA

Key words: animal models; BMP-2; bone formation; bone maturation; calvarial bone; dose

Accepted for publication 30 April 2014
Regeneration of bone is vital to rehabilitation of congenital defects, defects resulting from trauma or tumour resection, and in craniofacial settings defects resulting from periodontal disease, peri-implantitis or remodelling following tooth loss. In consequence, without the establishment of necessary bone volume and geometry, implant dentistry supported prosthetic rehabilitation may become challenging, if at all possible. Current craniofacial regenerative therapy includes the use of autologous bone grafts, cadaver-sourced allogeneic/xenogeneic or synthetic bone biomaterials, biologics or combinations thereof also including barrier devices for guided tissue/bone regeneration; autologous bone grafts representing the current benchmark or gold standard. However, the efficacy of present bone grafts, biomaterials and devices is not undisputed as evidenced by a continuous search for/development of alternatives.

Bone morphogenetic proteins (BMPs), naturally occurring proteins sequestered in the collagenous matrix of bone, have been shown to be osteoinductive stimulating conversion of undifferentiated mesenchymal cells into osteoblastic phenotypes resulting in clinically relevant bone formation (Urist 1965, Wozney et al. 1988, Valentin-Opran et al. 2002, Wikesjö et al. 2007). First generation commercially available BMP technologies, i.e. recombinant human BMP-2 (rhBMP-2) soak-loaded onto an absorbable collagen sponge (ACS) derived from highly purified bovine tendon Type I collagen, have been established and approved for orthopaedic, spine and craniofacial indications, i.e. recombinant human BMP-2 (INFUSE®; Medtronic, Memphis, TN, USA), rhBMP-2 adjusted to 0.025, 0.05, 0.1, 0.2, or 0.4 mg/ml, was used. ACS (Medtronic) was used as a stand-alone carrier control. A ø10-mm, dome-shaped titanium micro-mesh (Jeil Medical, Seoul, Korea) was used to shield the defect sites from soft tissue collapse/compression.

**Materials and Methods**

**Animals/Pre-surgical provisions**

One hundred sixty-eight outbred male Sprague-Dawley rats, age 11–13 weeks, weight 325–375 g, obtained from a USDA-licensed vendor (Harlan Laboratory, Madison, WI, USA), were used following a protocol approved for this study by the Institutional Animal Care and Use Committee, Dwight David Eisenhower Army Medical Center, Fort Gordon, GA, USA. The animals were acclimatized for at least 7 days upon arrival. Ear-tagged animals were double-housed in plastic cages labeled with cage cards. Cages were housed in purpose-designed rooms, air-conditioned with 10–15 air changes/h; temperature 18–22°C and relative humidity 30–70%. A 12/12 h light/dark cycle was used. The animals had ad libitum access to water and a standard laboratory diet throughout the study.

**Surgical procedures**

The animals were pre-medicated using butorphanol (0.01–0.05 mg/kg SC). Anaesthesia was induced with ketamine hydrochloride (65 mg/kg IP). After induction, the skull of the animal was shaved and disinfected using a 2% chlorhexidine solution. Animals were stabilized into a stereotaxic device (Stoelting Company, Wood Dale, IL, USA), fitted

Table 1. Flowchart of study, 168 Sprague-Dawley rats divided into seven groups provided observations of bone formation/maturation and aberrant healing events at 2, 4 and 8 weeks

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 24</td>
<td>N = 24</td>
<td>1.25 µg, N = 24</td>
<td>2.5 µg, N = 24</td>
<td>5.0 µg, N = 24</td>
<td>10.0 µg, N = 24</td>
<td>20.0 µg, N = 24</td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>8 weeks</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>
with an anaesthesia nose cone, and draped. Isoflurane (1.0–3.0%) was administered to maintain a surgical plane anaesthesia with O₂ to effect. All anaesthetic procedures were performed, monitored and documented by the veterinary team using standard techniques.

Experienced teams performed the surgeries in an animal surgical theatre (Fig. 1). A 3-cm midline incision was made through skin along the sagittal suture of the skull and soft tissues and periosteum elevated and reflected. Then, under constant sterile saline irrigation, a critical-size, ø8-mm through-through cranial osteotomy centred over the mid-sagittal suture within the parietal bone was created using a diamond coated trephine bur (Continental bone was created using a diamond saw, USM). Care was taken to leave the coated trephine bur (Continental adapted and closed using surgical staples (Visistat Skin Stapler, Teleflex, Limerick, PA, USA) ensuring everted wound margins.

The defects were either filled with ø8-mm ACS soak-loaded in buffer (25.3 mM t-glutamic acid, 1.875 mM sodium chloride, 2.5% glycine, 0.5% sucrose, 0.01% (v/v) Polysorbate 80, pH 4.5), rhBMP-2 at 0.025, 0.5, 0.1, 0.2, or 0.4 mg/ml in buffer, or served as sham-surgery (empty) controls. Following manufacturer’s specifications, the wetted ACS was allowed a minimum of 15 min and not longer than 2 h to allow rhBMP-2 binding before implantation; total rhBMP-2 dose/defect 0, 1.25, 2.5, 5.0, 10.0 or 20.0 μg.

The sterile dome-shaped ø10-mm titanium mesh was then placed over the defects, experimental and control, to avoid soft tissue collapse/compression. Finally, flaps were adapted and closed using surgical staples (Visistat Skin Stapler, Teleflex, Limerick, PA, USA) ensuring everted wound margins.

Post-surgery procedures

The animals were moved to a recovery area post-surgery. Thermal support and monitoring was continued until complete recovery indicated by the ability to remain sternal and ambulate. The animals were then returned to their cages and received buprenorphine (0.05–0.1 mg/kg, SC) every 12 h for 48 h to control pain. Activity level, behaviour, appearance, body temperature, body condition, anorexia and appearance of surgical sites were used to assess the continued need for analgesics. The animals were observed twice daily for 48 h for any abnormalities, and treatments were documented. Surgical staples were removed at 10–14 days.

Euthanasia

Attending veterinarian performed scheduled euthanasia at 2, 4 and 8 weeks using intracardiac injection of a concentrated sodium pentobarbital solution (Euthasol® 150 mg/kg IV; Delmarva Laboratories, Midlothian, VA, USA) following isoflurane anaesthesia induction. The calvarias were harvested and fixed in 10% buffered formalin solution upon removal of the titanium mesh and overlying soft tissue.

Radiographic analysis

Digital radiographs were exposed using a Faxitron LX-60 (Faxitron X-Ray LLC, Lincolnshire, IL, USA). Before control and test specimens were evaluated, the following calibration steps were performed. First, all automatic image enhancement controls were disabled. Pilot scans of control bone samples were used to determine the baseline radiographic settings (10 s and 26 kV) for optimal and constant image capture. To standardize the analysis, an ø8-mm defect was created in a strip of radiographic Teflon (Applied Plastics Technology Inc., Bristol, RI, USA) to calibrate the defect dimensionally under radiographic analysis. This served to confirm both internal and outer dimensional measurements during histometric analysis, and was used to standardize circle measurement from sample to sample. Using an image-analysis software (Image J, National Institutes of Health, Bethesda, MD, USA) a ø7.5-mm circle was constructed to define the defect area and the fraction area fill was calculated for each defect.

Histotechnical preparation

The calvarial biopsies were demineralized utilizing a decalcifying solution (Cal-Ex Decalcifying Solution, Fisher Scientific, Pittsburgh, PA, USA). The biopsies were sectioned perpendicular to the sagittal suture through the centre of the defect for the histometric analysis using a razorblade. Demineralized specimens placed in cassettes were washed, dehydrated and embedded in paraffin, and then sectioned at 4 μm thickness using a 2030 microtome (BioCut, Leica, Rechert-Jung, Nussloch, Germany). Three central sections per defect site were stained using haematoxylin & cosin for the histometric and histological analysis, and three sections were stained using Mason’s green trichrome for histological analysis only and were used as a cross-reference to identify degrees of bone mineralization.

Histopathological observations

Two masked examiners (MP, UW) conducted the histopathological evaluation including woven/lamellar bone, residual ACS, fatty/red/fibrovascular marrow, cortex formation, absence/presence of a haematoma, or seroma formation.

Histometric analysis

Two masked examiners (MP, UW) established the landmarks and extent of bone formation using polarized and incandescent light microscopy (BX 51, Olympus America, Inc., Melville, NY, USA). Then a cali-

---

Fig. 1. Osteotomies were prepared under aseptic conditions including sterile saline irrigation using an ø8-mm trephine bur. The ensuing ø8-mm critical-size defect was filled with ACS, rhBMP-2/ACS, or left empty (sham-surgery). A ø10-mm titanium mesh device was placed to cover the defect and the soft tissues were then adapted and closed using surgical staples.
brated examiner (MP) performed the histometric analysis using incandescent and polarized light microscopy (BX 51, Olympus America, Inc.), a microscope digital camera system (Retiga 4000R QImaging, Burnaby, BC, Canada), and a PC-based image analysis system (Image-Pro Plus®, Media Cybernetic, Silver Spring, MD, USA). The following parameters were recorded for the most central section of each defect:

- Defect width: distance between the defect margins;
- Defect fill (linear): accumulated length of new bone formation between the defect margins; and
- Defect fill (surface): total area of newly formed bone between the defect margins; and
- Bone density: percent mineralized tissue within the area of the newly formed bone.

**Statistical analysis**

Statistical analysis was performed using statistical software (Stata 11.1 for Mac, Stata Corporation, College Station, TX, USA). One-way ANOVA was used to compare group estimates of bone formation within experimental periods. Radiographic and histological bone formation was used as outcomes. Significance was set at 5% and p-values were adjusted for multiple comparisons using the Bonferroni correction.

Examiner reliability for the histometric evaluation was assessed using the Concordance Correlation Coefficient. This coefficient ranges between 0 and 1 and values close to 1 mean high reliability. The concordance coefficient was ≥0.92 for different parameters of bone formation demonstrating a high reliability for the examiner.

**Results**

**Clinical observations**

Healing was generally uneventful; no animals were lost following surgery. Two specimens from the 4-week 20-µg rhBMP-2 group were excluded from the analysis due to the titanium mesh being encased in mineralized tissue resulting in fracture of the newly formed bone with mesh removal. One specimen from the 8-week 2.5-µg rhBMP-2 group and one from the 4-week sham-surgery control were excluded due to fracturing of specimens during harvesting.

**Radiographic observations**

**Sham-surgery control**

The sham-surgery control exhibited limited bone formation along the osteotomy perimeter at 2 weeks, some specimens showing faint radiopacity in the centre of the defect (Fig. 2). Four-wk specimens showed somewhat accentuated peripheral mineralization as well as radiopaque shapes of varying morphology more centrally. Eight-week specimens did not substantially deviate from the 2- and 4-week observations.

**rhBMP-2/ACS**

All rhBMP-2 specimens showed diffuse radiopacity along the perimeter of the osteotomy sites as well as more centrally at 2 weeks (Fig. 2). At 4 weeks, a honeycombed structured radiopacity typically obliterated the osteotomy site (Fig. 2). There were no remarkable differences between rhBMP-2 doses ranging from 1.25 through 20.0 µg.

**Radiographic bone fill**

Results of the radiographic analysis are shown in Fig. 3 and Table 2.
rhBMP-2/ACS groups exhibited significantly greater bone fill than the sham-surgery control irrespective of dose and observation interval. The ACS carrier control exhibited significantly less bone fill than rhBMP-2/ACS groups at 2 and 4 weeks; whereas no significant differences were observed at 8 weeks. At 2 weeks, bone fill was significantly lower for the low rhBMP-2 dose compared with higher doses. No significant differences were observed among rhBMP-2 doses at 4 and 8 weeks. Within group comparisons generally showed significant differences in radiographic bone fill between 2 and 4 weeks with no significant differences thereafter; the ACS carrier control showing significant radiographic bone fill following 4 weeks of healing.

Histological observations

Sham-surgery control
Representative photomicrographs for the sham-surgery control are shown in Fig. 4. Two-week sham-surgery controls were characterized by osteogenic (woven) bone formation limited to the defect margins and by fibrovascular tissue. Three specimens showed residual haematomas. Four-week specimens exhibited a combination of woven and lamellar bone, red marrow but no residual haematomas, bone formation expanding from the defect margins, two defects approximating 50% fill. Eight-week specimens showed a combination of woven and lamellar bone, cortex formation and fatty marrow in one specimen; another specimen also showing a haematoma. Narrow bone formation never reached 50% of the defect width.

ACS carrier control
Representative photomicrographs of the ACS carrier control are shown in Fig. 5. At 2 weeks, three ACS carrier control specimens demonstrated no bone formation, five specimens showing woven bone formation emerging from the defect margins. All specimens showed large amounts of residual ACS. Four specimens showed residual hematomas. Four-week specimens exhibited a combination of woven and lamellar bone, red marrow and large amounts of residual ACS; thin newly formed bone bridging the defect in two specimens. Eight-week specimens showed a combination of woven and lamellar bone, cortex formation and fatty marrow,

![Graphs showing radiographic and histological bone fill](image)

Table 2. Mean (± SD) radiographic bone fill (%) according to group and observation interval

<table>
<thead>
<tr>
<th></th>
<th>Sham-surgery</th>
<th>ACS control</th>
<th>rhBMP-2/ACS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.25 µg</td>
<td>2.5 µg</td>
<td>5.0 µg</td>
</tr>
<tr>
<td>2 weeks</td>
<td>21.7 ± 5.8Aa</td>
<td>27.3 ± 9.5Aa</td>
<td>60.2 ± 14.8Ba</td>
</tr>
<tr>
<td>4 weeks</td>
<td>37.5 ± 11.1Ab</td>
<td>36.10 ± 9.3Aa</td>
<td>85.1 ± 10.7Bb</td>
</tr>
<tr>
<td>8 weeks</td>
<td>25.8 ± 7.9Aa</td>
<td>92.9 ± 6.2Bb</td>
<td>100.0 ± 0.1Bc</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Different capital letters indicate significant differences (p < 0.05) among experimental groups within each time period. Different lowercase letters indicate significant differences (p < 0.05) among time periods within experimental groups.
new bone formation bridging, or almost bridging, the defect. Neither
hematomas nor residual ACS were observed.

\textit{rhBMP-2/ACS}

Representative photomicrographs from the 2.5 and 20.0 μg rhBMP-2/ACS
groups are shown in Fig. 6. At 2 weeks all specimens with one excep-
tion irrespective of rhBMP-2 dose showed considerable bone formation
closing or almost closing the defect. The newly formed bone showed fea-
tures of woven bone as well as emerging lamellar structures in a few
specimens. All specimens showed considerable residual ACS. Red but
not fatty marrow was observed. Haematomas appeared equally distrib-
ted among the groups. A majority of 4-week specimens showed defect
closure. The newly formed bone exhibited a blend of woven and lamel-
lar bone and red and fatty marrow.

Cortex formation was observed for all rhBMP-2 doses, however, least
featured in the rhBMP-2/ACS 20-μg group. Residual ACS was observed
in all groups as well as haematomas. All defects with one exception
(rhBMP-2/ACS 20.0 μg) were closed at 8 weeks including woven and
lamellar bone, red and fatty marrow, also including cortex formation irre-
spective of rhBMP-2 dose. Residual ACS was observed in some samples,
haematomas were not observed.

\textbf{Histometric observations}

The results of the histometric analy-

sis are shown in Fig. 3 and Tables 3,
4 and 5. Similar observations were
made for the histological (Table 3)
and radiographic (Table 2) bone fill
analyses with no significant differ-
ences among groups at any experi-
mental interval when the rhBMP-2
dose was 2.5 μg or higher. Notewor-
thy, radiographic bone fill for the
ACS control trailed histological
bone fill, which was already robust
by 4 weeks. Albeit not statistically
significant, histological evidence of
bone formation was somewhat lower
for the rhBMP-2 1.25-μg dose at 2
weeks.

No significant differences in bone
area were observed among rhBMP-
2/ACS groups throughout the study
with the exception of the rhBMP-2
1.25-μg dose at 2 weeks which had
significantly smaller bone area than
higher doses. Whereas the sham-sur-
gery control represented a signifi-
cantly smaller bone area than the
rhBMP-2/ACS groups, no significant
differences were observed between
the ACS control and rhBMP-2/ACS
groups at 8 weeks.

Moreover, no significant differ-
ences in bone density were observed
among experimental groups at 2 weeks.
Limited bone formation in the
sham-surgery group exhibited signifi-
cantly increased bone density com-
pared with the other groups. At
8 weeks the sham-surgery and ACS
controls exhibited significantly greater
bone density than the rhBMP-2/ACS
groups.

\textbf{Discussion}

The objective of this study was to
determine the most effective dose
for rhBMP-2 soak-loaded onto the
ACS carrier for bone formation/
maturity using the rat critical-size
through-through calvarial defect
model. rhBMP-2 dosages of 2.5 μg
or higher showed defect closure
approaching or greater than 90%
within 2 weeks, and near complete
resolution within 4 weeks. Adverse
healing events including swelling,
excessive bone formation or seroma
formation could not be determined
with certainty in this defect model.
Notably ACS control sites showed
complete defect closure at the 8-week
healing interval.

This study used a rat critical-size
through-through calvarial defect model
(Muschler et al. 2010, Spicer et al.
2012); in context a critical-size defect
defined as the smallest size intraosseous
wound that will not spontaneously
heal over the natural or experimental
lifetime of the animal. This model
appears a favoured platform for screen-
ing candidate osteoconductive and

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd
Osteoinductive regenerative technologies such as devices for guided tissue regeneration, biomaterials serving as osteoconductive conduits, tissue engineering constructs including matrix, growth and differentiation factors, or combinations thereof prior to pivotal evaluation in discriminating large animal models and ultimately clinical trials (Han et al. 2005, Pang et al. 2005, Pryor et al. 2005a,b, Hong et al. 2006, Pohling et al. 2006, Choi et al. 2010, Yun et al. 2010, Bateman et al. 2012, Stancoven et al. 2012, Herberg et al. 2014).

The modification of the original rat calvarial defect model used herein included insertion of a ø10-mm titanium mesh to prevent soft tissue collapse into the defect space compromising bone formation and to ensure wound stability. This modification of the defect model exhibits baseline data of the original model, that is a defect that will not fill with new bone following sham-surgery procedures within the lifetime of the animal also shown in a previous study (Stancoven et al. 2012).

The sham-surgery control showed 37%, 38%, and 32% histological defect fill at 2, 4 and 8 weeks respectively. Similar observations were made in the radiographic analysis (35%, 48% and 39%). Two-week specimens were characterized by osteogenic (woven) bone formation limited to

![Fig. 6. Representative photomicrographs from sites receiving BMP-2/ACS at 2 (top pair), 4 (centre pair) and at 8 weeks (each pair represents the 2.5-µg/top and 20-µg/ bottom dose). Note no remarkable differences in bone formation between the 2.5 and 20.0 µg dose.](image)

### Table 3. Mean (± SD) histological bone fill (%) according to group and observation interval

<table>
<thead>
<tr>
<th></th>
<th>Sham-surgery</th>
<th>ACS control</th>
<th>rhBMP-2/ACS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.25 µg</td>
<td>2.5 µg</td>
<td>5.0 µg</td>
<td>10.0 µg</td>
</tr>
<tr>
<td>2 weeks</td>
<td>36.8 ± 19.6Aa</td>
<td>15.1 ± 26.4Aa</td>
<td>77.4 ± 35.4Baa</td>
<td>93.1 ± 11.4Baa</td>
</tr>
<tr>
<td>4 weeks</td>
<td>38.3 ± 19.1Aa</td>
<td>70.3 ± 22.5Bb</td>
<td>96.9 ± 6.8Ca</td>
<td>100.0 ± 0.0Ca</td>
</tr>
<tr>
<td>8 weeks</td>
<td>32.1 ± 10.3Aa</td>
<td>97.3 ± 3.1Bc</td>
<td>100.0 ± 0.0Ba</td>
<td>100.0 ± 0.0Ba</td>
</tr>
<tr>
<td>p-value</td>
<td>0.76</td>
<td>&lt;0.001</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Different capital letters indicate significant differences (p < 0.05) among experimental groups within each time period.
Different lowercase letters indicate significant differences (p < 0.05) among time periods within experimental groups.

### Table 4. Mean (± SD) histological bone area (mm²) according to group and observation interval

<table>
<thead>
<tr>
<th></th>
<th>Sham-surgery</th>
<th>ACS control</th>
<th>rhBMP-2/ACS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.25 µg</td>
<td>2.5 µg</td>
<td>5.0 µg</td>
<td>10.0 µg</td>
</tr>
<tr>
<td>2 weeks</td>
<td>0.7 ± 0.6Aa</td>
<td>0.2 ± 0.3Aa</td>
<td>2.0 ± 1.2Aa</td>
<td>3.5 ± 1.1Aa</td>
</tr>
<tr>
<td>4 weeks</td>
<td>1.0 ± 0.5Aa</td>
<td>1.9 ± 1.5Aa</td>
<td>5.2 ± 0.9Ba</td>
<td>6.9 ± 1.4Bb</td>
</tr>
<tr>
<td>8 weeks</td>
<td>1.1 ± 0.4Aa</td>
<td>5.5 ± 2.0BCb</td>
<td>8.4 ± 1.8Ca</td>
<td>7.7 ± 0.7BCb</td>
</tr>
<tr>
<td>p-value</td>
<td>0.21</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Different capital letters indicate significant differences (p < 0.05) among experimental groups within each time period.
Different lowercase letters indicate significant differences (p < 0.05) among time periods within experimental groups.

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd
the defect margins and fibrovascular tissue. Four-week specimens showed a combination of woven and lamellar bone and red marrow. Cortical formation was not observed until the 8-week observation interval. Others have shown more limited bone formation in sham-surgery controls using this defect model and healing intervals of 4 and 8 weeks ranging from 1% to 18% (Ahn et al. 2003, Pang et al. 2005, Jung et al. 2006, Lee et al. 2010). These studies did not use a space-providing device thus native bone formation may have been compromised from soft tissue collapse into the defect site impairing the native osteogenic potential. A previous study using the titanium mesh as in this study showed similar (34%) defect fill at 4 weeks and somewhat greater fill (58%) at 8 weeks (Stancoven et al. 2012). In still another study using non-reinforced PTFE barriers placed towards the dura and the periosteal flap, histological defect fill approximated 10% and 24% at 4 and 8 weeks, respectively, the barrier collapsing into the defect apparently compromising local bone formation (Yun et al. 2010).

The ACS control exhibited 15%, 70% and 97% histological defect fill at 2, 4 and 8 weeks. Corresponding radiographic observations were 41%, 48% and 94%, three sites showed no bone formation at 2 weeks, with five sites showing woven bone formation emerging from the defect margins.

Four-week specimens exhibited a combination of woven and lamellar bone including large amounts of residual ACS, complete defect fill observed in two sites. Cortical formation was not observed until 8 weeks with complete or almost complete defect fill and no residual ACS. The observations herein corroborate that presented in previous studies showing sites receiving ACS demonstrating complete or almost complete defect fill within 4 and 8 weeks with or without a space-providing titanium mesh (Pryor et al. 2005a,b, Stancoven et al. 2012), whereas others have shown histological defect fill encompassing less than 30% (Ahn et al. 2003, Pang et al. 2005, Lee et al. 2010) at these observation intervals. Notably, while the ACS does not possess relevant osteoconductive properties in large animal models (Choi et al. 1993, Sigurdsson et al. 1997), it appears to possess substantial latent osteoconductive properties in the apparently more reactive rat critical-size through-through calvarial defect model. This observation has consequences for the selection of observation intervals using this model and the ACS but may also apply to other candidate carrier technologies.

In this study, the histometric analysis revealed greater than 90% bone fill for rhBMP-2/ACS groups (rhBMP-2 dose ≥2.5 µg) within 2 weeks exhibiting features of woven bone, red marrow, and in a few specimens emerging lamellar structures. Considerable residual ACS was observed in all specimens. Complete or almost complete bone fill was seen in all sites at 4 weeks without striking differences in bone volume and density between doses ranging from 1.25 to 20.0 µg. Four-week specimens exhibited a blend of woven and lamellar bone, red and fatty marrow and residual ACS. Cortex formation was observed for all doses, however, least featured in the high-dose 20-µg group (suggestive of delayed bone maturation). All defects, with one exception (20.0 µg), were closed at 8 weeks with woven and lamellar bone, red and fatty marrow, also including cortex formation and residual ACS in some specimens irrespective of rhBMP-2 dose. These observations contrast that observed in the sham-surgery and ACS controls significantly trailing bone formation compared with sites receiving rhBMP-2/ACS irrespective of dose. Consistent bone formation/maturation among rhBMP-2 dose variations in this study suggest that similar dose range using the ACS carrier should also be pursued in large animal models taking into account relative immature bone formation/delayed maturation observed in a previous study using a 100, 200 and 400 µg rhBMP-2 dose applied to 2-cc canine critical-size, supraalveolar, peri-implant defects (Tatikis et al. 2002) prior to clinical evaluation.

Aberrant healing events including swelling, excessive bone or seroma formation were not discernable in this study irrespective of rhBMP-2 dose. These observations can be species, site, dose and carrier matrix dependent. Aberrant healing events following application of BMPs in this model have not been reported beyond transient soft tissue oedema (Bae et al. 2012). Absence of aberrant healing events contrasts to that observed in previous studies using ACS or an anodized dental implant as delivery systems for rhBMP-2 all showing advanced seroma formations (Wikesjö et al. 2003, 2004, 2008a,b, Jovanovic et al. 2007, Leknes et al. 2008). An inverse relationship between seroma formation, bone remodelling and dose was observed in the canine critical-size, supraalveolar, peri-implant defect model, the high dose showing advanced seroma formation and excessive bone remodelling (Leknes et al. 2008, Wikesjö et al. 2008a). Similar observations were made as rhBMP-2 coated dental implants were placed into primary osteo-

| Table 5. Mean (± SD) histological bone density (%) according to group and observation interval |
|---------------------------------|-------------------|-------------------|----------------|-------------------|-------------------|-------------------|
|                                 | Sham-surgery      | ACS control       | rhBMP-2/ACS     | p-value           |
|                                 |                   |                   | 1.25 µg         |                   |                   |                   |
| 2 weeks                         | 24.9 ± 26.4Aa     | 24.9 ± 26.4Aa     | 25.5 ± 14.5Aa   | <0.001            |
| 4 weeks                         | 35.1 ± 10.6Ba     | 39.6 ± 12.8Bb     | 34.3 ± 9.4Ba    | <0.001            |
| 8 weeks                         | 68.2 ± 11.5Ab     | 57.4 ± 8.4Bb      | 51.0 ± 12.3Bb   | <0.001            |
| p-value                         | <0.001            | <0.001            | <0.001          | <0.001            |

Different capital letters indicate significant differences (p < 0.05) among experimental groups within each time period. Different lowercase letters indicate significant differences (p < 0.05) among time periods within experimental groups.
mies also using a canine model (Wikesjö et al. 2008b). Also, the application of rhBMP-2/ACS promoted advanced seroma formation in saddle-type defects in dogs in presence/absence of guided tissue regeneration barriers (Jovanovic et al. 2007) as well as using the canine critical-size, supraalveolar, peri-implant defect model (Wikesjö et al. 2003, 2004) suggesting that rhBMP-2 dose and not carrier technology play a primary role in genesis of these aberrant healing reactions.

In conclusion, rhBMP-2/ACS accelerates local bone formation in the rat critical-size through-through calvarial defect model once reaching an osteoinductive dose threshold. This threshold may already be reached at the 1.25-/2.5-μg-dose interval in this model. No further enhancement to bone formation/maturation may be observed adding rhBMP-2 above the 2.5-μg dose. The 1.25–20.0-μg dose range did not involve discernable aberrant healing events in this defect model. The maturation rate of rhBMP-2 induced bone appears to trail that of native bone although no meaningful differences were noted within the selected dose range.

References


Electrospun scaffolds formed from collagen, gelatin or other biodegradable materials have been developed for use in tissue engineering applications. These scaffolds may be used to deliver drugs or growth factors, or to support cell proliferation and differentiation. The use of electrospinning to fabricate scaffolds for tissue engineering has several advantages over traditional methods of tissue engineering, such as improved cell attachment and proliferation, increased oxygen and nutrient delivery, and more uniform distribution of growth factors. Additionally, electrospinning allows for the creation of scaffolds with a wide range of porosities and pore sizes, which can be tailored to specific tissue engineering applications.

The electrospinning process involves the use of a high-voltage electrical field to draw a polymer solution into a jet that is then deposited on a collector to form a continuous fiber. The parameters that can be used to control the morphology of the electrospun fibers include the voltage, the distance between the spinneret and the collector, the flow rate of the polymer solution, the type and concentration of the solvent, and the environmental conditions (such as humidity and temperature). By manipulating these parameters, it is possible to create fibers with a wide range of diameters and shapes, and to control the spatial distribution of fibers on the collector.

The mechanical properties of electrospun fibers can be controlled by adjusting the electrospinning parameters. For example, increasing the distance between the spinneret and the collector can result in fibers with higher Young's modulus and lower elongation at break, while decreasing the voltage can result in fibers with lower Young's modulus and higher elongation at break. The use of multiple fibers in an electrospun mat can also change the mechanical properties, allowing for a range of properties to be achieved.

In conclusion, electrospinning is a useful technique for fabricating scaffolds for tissue engineering, offering the ability to control the morphology and mechanical properties of the fibers. These scaffolds can be tailored to specific tissue engineering applications, providing a promising approach for the future of tissue engineering.
Bone formation at recombinant human bone morphogenetic protein-2-coated titanium implants in the posterior mandible (Type II bone) in dogs. *Journal of Clinical Periodontology* 35, 985–991.


**Clinical Relevance**

**Scientific rationale for the study:** Application of recombinant human bone morphogenetic protein-2 (rhBMP-2) for alveolar ridge or sinus augmentation has been associated with significant adverse events, in particular swelling and in experimental settings seroma formation. Recent work has demonstrated an inverse relationship between bone formation/maturation and rhBMP-2 dose; frequency/severity of adverse events increasing with rising dose.

**Principal findings:** Using a rat critical-size, through-through, calvarial osteotomy defect model and an rhBMP-2 dose of 1.25, 2.5, 5.0, 10.0 and 20.0 μg/defect compared with carrier and sham-surgery controls, it was shown histologically that rhBMP-2 at doses ≥2.5 μg induced defect closure >90% within 2 weeks, and complete resolution within 4 weeks. Adverse healing events including swelling, excessive bone formation or seroma formation could not be determined with certainty in this defect model.

**Practical implications:** The results suggest that significantly lower rhBMP-2 doses should be explored in discriminating large animal models to provide relevant translational observations for clinical evaluation.