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Effect of bone morphogenetic protein-2, demineralized bone matrix and systemic parathyroid hormone (1-34) on local bone formation in a rat calvaria critical-size defect model

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Aim: To determine the potential of recombinant human bone morphogenetic protein-2 (rhBMP-2) soak-loaded on to an absorbable collagen sponge (ACS) to induce local bone formation compared with the clinical reference demineralized bone matrix (DBM) and to investigate potential additive/synergistic effects of exogenous parathyroid hormone (PTH).

Methods: Critical-size (8 mm), through-through calvaria osteotomy defects in 160 adult male Sprague–Dawley rats were randomized to receive one of eight interventions: rhBMP-2/ACS, DBM, ACS, or serve as controls (empty defects) combined or not with systemic PTH. Ten animals from each group were followed for 4 and 8 wks for radiographic and histometric analysis. Multivariable analysis was used to assess the effect of experimental intervention and healing time on local bone formation.

Results: In the multivariable analysis, rhBMP-2/ACS exhibited significantly greater histologic bone formation than control ($\beta \pm SE$: 54.76 ± 5.85, $p < 0.001$) and ACS ($\beta \pm SE$: 9.14 ± 3.31, $p = 0.007$) whereas DBM showed significantly less bone formation than control ($\beta \pm SE$: -32.32 ± 8.23, $p < 0.001$). Overall, PTH did not show a significant effect on bone formation ($\beta \pm SE$: 2.72 ± 6.91, $p = 0.70$). No significant differences in histological defect closure were observed between 4 and 8 wks for all but the control group without PTH.

Conclusion: rhBMP-2/ACS significantly stimulates local bone formation whereas bone formation appears significantly limited by DBM. Systemic application of PTH provided no discernible additive/synergistic effects on local bone formation.

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Numerous clinical protocols have been introduced to reconstruct and/or regenerate alveolar bone, several associated with undesirable donor site morbidity from harvesting autogenous bone from the iliac crest, tibia, or the mandibular symphysis and ramus area. Thus, a wealth of biomaterials (bone derivatives and bone substitutes) have been developed with the intent to substitute/extend autogenous bone for alveolar augmentation, including demineralized bone matrix (DBM), also known as demineralized freeze-dried bone allograft, a widely used cadaver-sourced allogeneic bone biomaterial. Demineralization of bone exposes growth factors sequestered in the collagenous matrix, which potentially may support new bone growth/formation. However, the clinical relevance of DBM is not uncontroversial and studies using large animal platforms and clinical settings dispute the osteoinductive and osteoconductive qualities of DBM (1–3).

Bone morphogenetic proteins (BMPs) were discovered based on the osteoinductive activity concealed in the collagenous matrix of bone (4). BMPs stimulate angiogenesis and migration, proliferation, and differentiation of mesenchymal stem cells into cartilage- and bone-forming phenotypes (5). Although BMP implantation without a carrier induces bone formation, a carrier appears to be needed for efficient bone formation (6). Type I collagen matrices are considered appropriate carriers for growth factors because of rheologic properties, biocompatibility, and their resorbable nature (7). A construct combining recombinant human BMP-2 (rhBMP-2) and an absorbable (type I) collagen sponge (ACS) as a carrier has been introduced and received approval for orthopedic and craniofacial indications. Preclinical and clinical studies have demonstrated the regenerative potential of this construct (8). Nevertheless, rhBMP-2/ACS has also been associated with untoward dose-dependent side effects, including seroma formation, exuberant swelling, and bone remodeling, indicating room for optimization (9, 10).

Parathyroid hormone (PTH) is produced by the parathyroid glands and recombinant PTH is commercially available to treat bone disorders. Continuous endogenous exposure to PTH leads to an increase in osteoclastic density and activity; however, pulsed systemic application produces a rapid increase in bone formation markers (11). Studies in mice show that administration of PTH (1-34) for 4 wks leads to an inhibition of osteoblast apoptosis, resulting in prolonged enhanced bone formation (12). PTH injection in rats is thought to increase differentiation of osteoprogenitor cells into osteoblasts (13). Moreover, PTH increases osteoblast and osteoclast function, turnover, and remodeling, which results in increased trabecular and cortical thickness and ultimately overall bone density and bone mass (14). Although PTH has been shown to favorably impact bone metabolism and structure, only scarce information relative to any stand-alone bone augmentation, much less regeneration protocols, has been presented. Moreover, it is unclear whether PTH has any additive/synergistic effects that could be used to enhance other therapies specifically BMP- and DBM-induced bone formation. Therefore, the objective of this study was to determine the potential of rhBMP-2 to induce local bone formation compared with the clinical reference DBM, and to demonstrate any additive/synergistic effects of systemic PTH on these protocols using a rat calvaria defect model.

Material and methods

Animals and experimental design

One hundred and sixty male Sprague–Dawley outbred rats (*Rattus norvegicus*), age 11–12 wks, weight 325–375 g, obtained from a USDA-approved breeder were used. The animals were acclimatized for 7 d. They were double-housed in plastic cages labeled with cage cards and wore ear tags for identification. The 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.

The animals were randomized into two study arms of 80. One study arm received daily injections of PTH (1-34), the other did not receive PTH. Each study arm was then divided into groups of 20 animals receiving one of four test/control conditions: rhBMP-2/ACS, DBM, ACS, or serve as controls (empty defects). Ten animals from each group were euthanized at 4 and 8 wks, respectively, for radiographic and histologic analysis.

Agents and biomaterials

Rat PTH (1-34) (15 μg/kg SC; Sigma, St. Louis, MO, USA) in a water-soluble carrier was administered once daily throughout the study for animals scheduled to receive this treatment. The animals were weighed weekly and the PTH dose adjusted according to current weight of the animal. rhBMP-2/ACS (INFUSE® Bone Graft, Medtronic, Memphis, TN, USA) adjusted to 0.1 mg/mL (Dr. John M Wozney, personal communication) was used. ACS (Medtronic) was used as a stand-alone carrier control. A particulate human DBM biomaterial (LifeNet, Virginia Beach, VA, USA) was used as clinical reference. A, ø10-mm, dome-shaped titanium micro-mesh (Jeil Medical, Seoul, Korea) was used to shield the defect site from soft tissue collapse/compression.

Surgical procedures

The animals were pre-medicated using buprenorphine (0.05–0.1 mg/kg SC). Anesthesia was induced with ketamine hydrochloride (65 mg/kg IP). After induction, the dorsal surface of the animal head was shaved and disinfected using a 2% chlorhexidine solution. Animals were stabilized into a stereotaxic device (Stoelting Company, Wood Dale, IL, USA), fitted with an anesthesia nose cone, and draped. Isofluorene (1.0–3.0%/O_2) was administered to maintain a surgical plane of anesthesia.

Experienced surgeons (BS, DD, CS, JL) performed all surgeries in a surgical

vivarium. Using aseptic routines, a 3-cm midline incision was made through the skin along the sagittal suture of the skull (Fig. 1). Soft tissues and periostea were elevated and reflected. Under saline irrigation, a critical-size, ø8-mm, through-through, cranial osteotomy defect centered over the sagittal suture immediately anterior to the occipital suture was created using a diamond-coated trephine bur (Continental Diamond Tool, New Have, IN, USA). The defects were filled with either DBM (35 mg), or a ø8-mm precut ACS (soak-loaded with 50 μL saline), or rhBMP-2/ACS (50 μL rhBMP-2 at 0.1 mg/mL soaked-loaded on to an ø8-mm precut ACS for 10 min), or served as control. The sterile custom, dome-shaped titanium micromesh was placed over the defect to avoid soft tissue collapse/compression of the defect. Finally, the flaps were adapted and closed using surgical staples (Disposable Skin Staples; TFX Medicad Ltd, London, UK) ensuring everted wound margins.

The animals were placed in cages, warmed on a heating pad, and observed for distress until they were able to move about normally. Yohimbine HCl (1–2 mg/kg IP) was administered to accelerate recovery as necessary. Buprenorphine (0.05–0.1 mg/kg SC) was administered every 12 h for 48 h to control pain. Animals exhibiting signs of pain beyond that point received additional dose(s) of buprenorphine. The animals were euthanized at 4 or 8 wks using a CO_2 chamber following isoflurane induction. Block biopsies (12 × 15 mm) of the calvariae were harvested and fixed in 10% buffered formalin, the titanium mesh and overlying soft tissue being removed.

Radiographic analysis

Radiographs were obtained using a digital imaging instrument (Faxitron X-Ray, Wheeling, IL, USA) following initial calibration. The block biopsies were placed and stabilized into Petri dishes positioned 41.7 cm from the ionizing source. A projected red laser grid was used to assure consistent perpendicular positioning. Using imaging software (Image J; National Institutes of Health, Bethesda, MD, USA) an 8-mm custom circle was constructed to define the total defect area and the fraction area fill was calculated using gray scale values for each specimen. The radiographic gray scale value was normalized to the averaged gray scale value of 10 representative control calvariae.

Histotechnical preparation

The calvariae were sectioned perpendicular to the sagittal suture, producing a plane of analysis through the center of the defect. Specimens were demineralized (Cal-Ex Decalcifying Solution; Fisher Scientific, Pittsburgh, PA, USA), trimmed, dehydrated in a graded ethanol series, embedded in paraffin, and sectioned at 4 μm (Bio-Cut, Leica, Reichert-Jung, Nussloch, Germany). Three central sections per defect stained with hematoxylin and eosin were used for the histometric/histologic analysis. Additionally, three central sections stained with Mason's green trichrome were used for cross-reference.

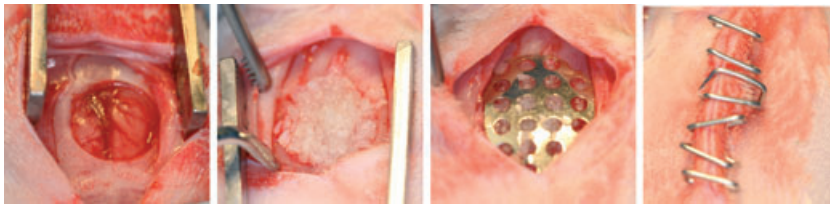

Fig. 1. Osteotomies were prepared under sterile saline irrigation using a ø8-mm trephine bur. The ensuing ø8-mm critical-size defect was filled with demineralized bone matrix, absorbable collagen sponge, recombinant human bone morphogenetic protein-2/absorbable collagen sponge, or left empty (control). A custom ø10-mm titanium mesh was placed over each defect and the soft tissues were adapted and closed using surgical staples.

Histometric analysis

Two masked examiners (BS, UW) established defect borders and extent of bone formation using polarized and incandescent light microscopy (BX 51, Olympus America, Melville, NY, USA). A calibrated examiner (BS) performed the histometric analysis using incandescent and polarized light microscopy (BX 51, Olympus America), 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 most central section was used to assess defect closure and percentage bone formation was calculated by dividing the linear amount of new bone by the defect width.

Statistical analysis

Statistical analysis was performed using statistical software (Stata 11.2 for Mac; Stata Corporation, College Station, TX, USA). Multiple linear regression was used to model the effect of the experimental conditions on bone formation. Histometric bone formation was the primary outcome of the study, and radiographic bone formation was the secondary outcome. Significance was set at 5% and *p*-values were adjusted for multiple comparisons. Means (± SD) are presented in Tables 1 and 2 and beta coefficients (± SE) in Table 3. Examiner reliability for the histometric evaluation was assessed by duplicate measurements of 20 sections 1 wk apart using the concordance correlation coefficient. This coefficient ranges between 0 and 1 and values close to 1 mean high reliability. The concordance correlation coefficient was 0.99 for bone formation demonstrating a high reliability for the examiner.

Results

Clinical observations

No adverse events were observed during the healing interval. Seven animals succumbed due to anesthesia

Table 1. Histometric defect closure according to experimental intervention and healing time

	4 wk		8 wk		
	Mean	SD	Mean	SD	*p* Value
w/o PTH					
Control	33.08A	18.82	57.86A	24.02	0.03
DBM	13.52A	34.98	9.46B	7.00	0.74
ACS	87.21C	12.27	91.34C	10.95	0.41
rhBMP-2	99.57C	1.37	97.12C	9.10	0.52
w/PTH					
Control	42.96A	14.01	49.64A	21.81	0.43
DBM	20.38A	17.03	14.62B	14.60	0.48
ACS	71.09B	20.56	76.24C	22.93	0.62
rhBMP-2	100.00C	0.00	100.00C	0.00	1.00

ACS, absorbable collagen sponge; DBM, demineralized bone matrix; PTH, parathyroid hormone; rhBMP-2, recombinant human bone morphogenetic protein-2; w/, with; w/o, without.

Table 2. Radiographic defect closure according to experimental intervention and healing time

	4 wk		8 wk		
	Mean	SD	Mean	SD	*p* Value
w/o PTH					
Control	26.65A	16.70	47.79A	9.36	0.001
DBM	21.52A	11.47	51.93A	21.97	0.001
ACS	74.88C	15.13	85.29BC	17.31	0.19
rhBMP-2	98.26D	5.48	98.75C	3.44	0.81
w/PTH					
Control	35.27A	7.36	42.08A	14.14	0.19
DBM	35.89A	9.77	56.09A	20.65	0.01
ACS	56.12B	18.72	73.74B	18.43	0.06
rhBMP-2	98.84D	2.29	96.59C	7.27	0.36

ACS, absorbable collagen sponge; DBM, demineralized bone matrix; PTH, parathyroid hormone; rhBMP-2, recombinant human bone morphogenetic protein-2; w/, with; w/o, without.

complications. Thus, three animals in the 4-wk control group, one in the 8-wk DBM^{PTH} group, one each in the 4- and 8-wk ACS groups, and one in the 4-wk ACS^{PTH} group were lost to analysis.

Histologic observations

Representative photomicrographs are shown in Fig. 2. Controls showed limited bone formation at 4 and 8 wks. However, one animal in the 4-wk group showed partial reestablishment of the cortical plates and one animal in the 8-wk group demonstrated a blend of lamellar and woven bone formation. At 4 wks, $control^{PTH}$ sites showed a minimal increase in osteogenic bone formation compared with control sites without PTH. At 8 wks, there were no noticeable differences between control sites irrespective of PTH status.

Defect sites receiving DBM showed, with one exception, limited bone formation emerging from the defect margins at 4 wks. Irregular-shaped DBM particles were distributed throughout the defect without appreciable evidence of bone metabolic activity; osteoblastic or osteoclastic cells were not observed. The DBM particles appeared fragmented suggestive of initial biodegradation. At 4 wks, sites receiving DBM^{PTH} showed somewhat greater osteogenic bone formation compared with sites receiving stand-alone DBM. Defect sites receiving DBM and DBM^{PTH} showed a limited increase in bone formation without other appreciable histopathologic differences at 8 wks.

Defect sites receiving ACS showed large amounts of residual ACS invested in fibrovascular tissue and bone at 4 wks. Internal and external cortical plates were either partially or completely reestablished with bone formation ranging from woven to lamellar bone. Complete bone fill was observed in few sites. Sites receiving ACS^{PTH} exhibited somewhat lesser osteogenic bone formation and larger amounts of residual ACS. Generally, at 8 wks, the specimens exhibited small to medium marrow spaces with fibrovascular tissue. The cortical plates were reestablished, including woven and lamellar bone.

Defect sites receiving rhBMP-2/ACS showed robust bone formation and the internal and external cortical plates were reestablished, including trabecular bone with cell-rich fibrovascular tissue and establishing fatty marrow at 4 and 8 wks. There was apparently more residual ACS present in sites receiving rhBMP-2/ACS^{PTH} compared with sites receiving rhBMP-2/ACS alone. One site in the rhBMP-2/ACS group showed delayed bone formation exhibiting considerable residual ACS at 4 wks.

Histometric results

Bone formation encompassed 33.08% of the critical-size defects for the control at 4 wks to reach 57.87% at 8 wks (Table 1). Sites receiving DBM showed limited bone formation at both observation intervals averaging less than 15%. In contrast, bone formation encompassed 87.21 and 91.34% for sites receiving ACS at 4 and 8 wks. Complete defect closure was observed for most sites receiving rhBMP-2/ACS without discernible differences between 4 and 8 wks. At 4 wks, no significant differences were observed between control and DBM groups; however, DBM yielded significantly less bone formation than controls at 8 wks. The ACS and rhBMP-2/ACS groups were significantly different

Table 3. Multivariable analysis of the effect of experimental interventions and healing time on histometric and radiographic defect closure

	Histometric analysis			Radiographic analysis		
	β coefficient	SE	*p* Value	β coefficient	SE	*p* Value
PTH						
w/o	Reference			Reference		
w/	2.72	6.91	0.70	2.18	4.29	0.61
Intervention						
Sham-surgery	Reference			Reference		
DBM	−32.32	8.23	<0.001	0.24	5.52	0.97
ACS	45.62	6.32	<0.001	43.60	5.09	<0.001
rhBMP-2/ACS	54.76	5.85	<0.001	62.01	3.89	<0.001
Healing period						
4 wk	Reference			Reference		
8 wk	3.44	2.92	0.24	12.87	2.35	<0.001
Interactions						
PTH − DBM	4.33	10.03	0.67	6.88	7.10	0.33
PTH − ACS	−18.21	8.96	0.04	−17.46	7.06	0.01
PTH − rhBMP-2/ACS	−1.06	7.10	0.88	−2.97	5.10	0.56

ACS, absorbable collagen sponge; DBM, demineralized bone matrix; PTH, parathyroid hormone; rhBMP-2, recombinant human bone morphogenetic protein-2; w/, with; w/o, without.

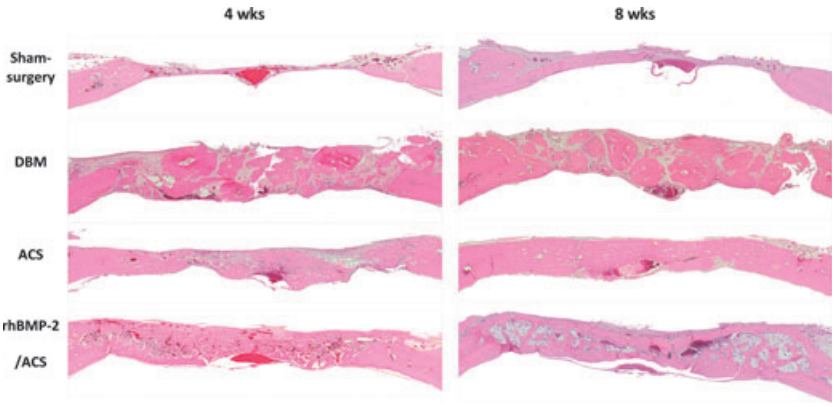

Fig. 2. Histologic representation of experimental groups at 4 and 8 wks. ACS, absorbable collagen sponge; DBM, demineralized bone matrix; rhBMP-2, recombinant human bone morphogenetic protein-2.

from the other groups, but no significant differences could be observed between them irrespective of the experimental period.

PTH exhibited no meaningful additive effects on local bone formation. Defect closure in the control^{PTH} averaged 42.96 and 49.64% at 4 and 8 wks. DBM^{PTH} sites showed the least bone formation among the groups treated with PTH reaching 20% or less at 4 and 8 wks while the ACS^{PTH} group encompassed 71.09% at 4 wks and 76% at 8 wks. rhBMP-2/ACS^{PTH} reached almost 100% defect closure irrespective of observation interval.

Radiographic observations

Representative radiographs from the 4- and 8-wk observation intervals are shown in Fig. 3. Control and DBM sites showed limited bone formation along the osteotomy perimeter at 4 wks (Table 2). At 8 wks, control and DBM defects showed significantly enhanced bone formation compared with at 4 wks. The ACS groups exhibited significantly greater bone formation at 4 and 8 wks compared with control and DBM groups. The rhBMP-2/ACS group showed nearly complete defect closure at 4 and 8 wks. PTH did not exhibit a significant effect on defect closure.

Multivariable analysis

In the multivariable analysis for the histometric results (Table 3), both ACS (β ± SE: 45.62 ± 6.32, $p < 0.001$) and rhBMP-2/ACS (β ± SE: 54.76 ± 5.85, $p < 0.001$) exhibited significantly greater bone formation than control, and rhBMP-2/ACS yielded a small but significantly greater bone formation compared with ACS (β ± SE: 9.14 ± 3.31, $p = 0.007$). In contrast, DBM showed significantly less bone formation than control (β ± SE: −32.32 ± 8.23, $p < 0.001$). Overall, PTH did not show a significant effect on bone formation (β ± SE: 2.72 ± 6.91, $p = 0.70$), PTH actually exhibited a borderline significant negative effect on local bone formation for the ACS^{PTH} group (β ± SE: −18.21 ± 8.96, $p = 0.04$). No significant differences were observed between 4 and 8 wks (β ± SE: 3.44 ± 2.92, $p = 0.24$).

Overall, similar results were observed in the multivariable analysis for the histometric and radiographic analysis. Contrary to the histometric findings, DBM did not show reduced defect closure when compared with controls. A small but significant overall improvement in defect closure was observed between 4 and 8 wks.

Discussion

The objective of this study was to determine the potential of rhBMP-2/ACS to induce local bone formation compared to a clinical reference, DBM, and to investigate any additive/synergistic effects of systemic PTH using an established rat calvaria defect model. rhBMP-2/ACS supported complete resolution of the critical-size, through-through calvarial defects while DBM apparently inhibited local bone formation. PTH did not improve local bone formation

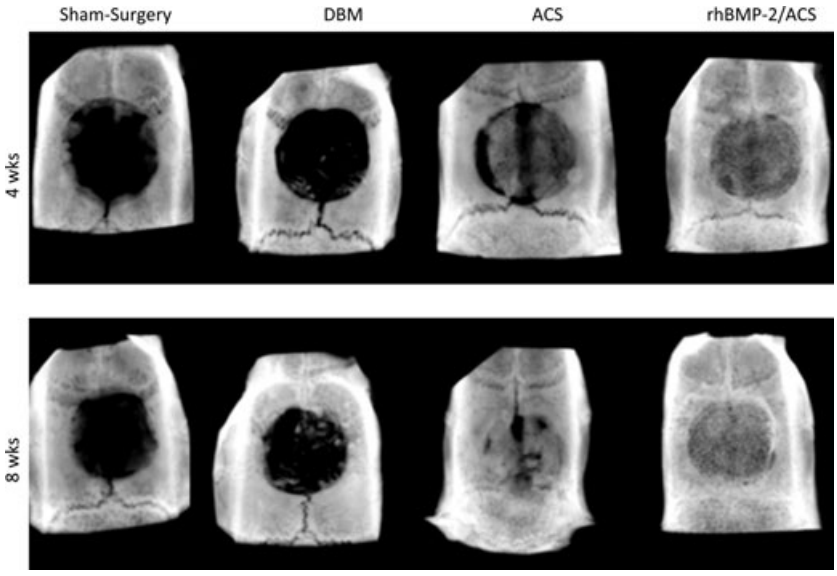

Fig. 3. Radiographic representation of experimental groups at 4 and 8 wks. ACS, absorbable collagen sponge; DBM, demineralized bone matrix; rhBMP-2, recombinant human bone morphogenetic protein-2.

showing limited perspectives as an additive/synergistic treatment to other bone augmentation protocols.

The rat calvariae, critical-size, through-through osteotomy defect appears the preferred model to screen candidate osteoconductive and osteoinductive technologies whether devices, biomaterials serving as osteoconductive scaffolds, biologics including matrix, growth, and differentiation factors, or combinations thereof before pivotal evaluation in discriminating well-characterized large animal models and ultimately clinical settings (15). This study used a modification of this model to evaluate the osteoconductive/osteoinductive effect of rhBMP-2/ACS, DBM, and any additive/synergistic effect of systemic PTH on local bone formation. A ø10-mm, dome-shaped titanium mesh was used to ensure space provision and wound stability. The control groups exhibited 34 and 58% histometric defect closure at 4 and 8 wks, respectively. In contrast, Ahn and co-workers (16) noted 8% defect closure at 8 wks, and Jung and co-workers (17) reported 1% defect closure at 4 and 8 wks. Still others report a limited defect closure approximating 15 and 18% at 8 wks (18, 19). These studies, however, did not use a space-providing device thus native bone formation may have been compromised from soft tissue collapse into the defect and may not represent the true (native) osteogenic potential of this surgically induced defect. Indeed, in a study using nonreinforced polytetrafluoroethylene barriers placed towards the dura and periosteal flap, the barriers were found collapsed/compressed into the defect compromising essential space provision for local bone formation; defect closure approximating 10 and 24% at 4 and 8 wks (20). The impact of tissue/cell occlusion on bone formation in this model is unknown; the titanium mesh used in this study featured large macro-pores allowing ingrowth of cells from adjoining tissues. Nevertheless, it appears from the results in the control and previous reports that whereas tissue/cell occlusion is not a requirement for bone formation (21), it might enhance regeneration outcomes at least in periodontal settings (22).

In this study, a commercial human DBM preparation was used; nevertheless, defect closure approximating 14 and 10% at 4 and 8 wks was observed compared with 33 and 58% for the control. The histologic evaluation substantiated that the DBM biomaterial was biocompatible. Human DBM has been suggested to be effective in this defect model, closing most of the osteotomy defect when used with a barrier device (23), while others have shown reduced or delayed bone formation using human DBM in the rat calvaria defect model approximating 15 and 32% at 4 and 8 wks (20). Still others have observed variable bone formation for commercial human DBM preparations, some preparations achieving up to a five-fold greater bone formation (24), whereas selection of defect size may explain total or partial defect closure using rat-derived DBM combined or not with expanded polytetrafluoroethylene barriers in ø5-mm rat calvaria defects (25). Still, differences in outcomes between studies can only be speculated upon. The limited effects of DBM (1–3) may have several explanations. Importantly, critical bone inductive factors may not be present in pharmacologic relevant quantities even for optimally verified preparations; DBM typically includes 80 μg crude BMP/kg. Thirty-five mg DBM was used to fill the calvaria defects in the present study. This dose/concentration may not be sufficient to generate appreciable/relevant effects in craniofacial and other skeletal settings in large and small animal models and much less in patients while being osteoinductive in highly reactive ectopic rodent models (4, 26, 27). Others have suggested rationales for failing osteoinduction to include DBM donor age (28), variability in commercial preparations (28), and particle size (29). Notably, in the present study the DBM preparation did not only fail to display osteoinductive properties, it also failed to enhance osteogenic bone formation, in other words it did not reveal any osteoconductive qualities; bone formation halted to below baseline levels compared with control at 4 and 8 wks.

Defect closure encompassed 87 and 91% at 4 and 8 wks for sites receiving the bovine collagen type I ACS control in the present study. This observation becomes even more intriguing when compared with defect closure, or lack thereof, following the use of human collagen type I DBM biomaterial discussed above. While most studies have

shown histometric defect closure generally encompassing less than 30% for the ACS (16, 18, 19), this study corroborates other previous work showing that some sites receiving ACS demonstrated complete defect fill within 4 and 8 wks, although it should be recognized that ø6-mm defects were used in these studies (30). Again, differences between studies likely represents an effect of the space-providing titanium mesh device protecting the apparently, at least in this model, osteoconductive ACS. In perspective, it is necessary to realize that the ACS does not posses relevant osteoconductive properties in large animal models (31). The histologic observations further verified fragments of residual ACS at both 4 and 8 wks. This finding is consistent with previous work observing residual ACS when used as a carrier for rhBMP-2 in the canine supraalveolar periodontal defect model at 8 wks (32).

The histometric analysis revealed complete or almost complete defect closure at 4 and 8 wks for sites receiving rhBMP-2/ACS. These sites demonstrated reestablished cortical plates to the original contour of the calvaria without obvious aberrant reactions, including seroma formation shown following application of rhBMP-2 using discriminating large animal craniofacial models (33, 34). Possibly a more favorable dose was used in the present study compared with that observed in the large animal models. The low-dose rhBMP-2/ACS implant produced significant bone formation at 4 and 8 wks compared with the DBM preparation, which apparently inhibited bone formation. These results closely corroborate other studies that have evaluated rhBMP-2/ACS and DBM using the supraalveolar critical-size peri-implant defect model (3, 31).

Systemic PTH contributed minimal, if any, additive/synergistic effects on defect closure irrespective of local treatment. Radiographic and histologic bone formation was slightly improved in the control^{PTH} and DBM^{PTH} groups at 4 and 8 wks compared with control and DBM groups without PTH. However, these differences were not statistically significant. These observations corroborate a recent study using a rat femur critical-size defect model; intermittent PTH limitedly enhanced local bone formation within an 8-wk healing interval following sham-surgery (35). In a previous study, when PTH was added to sham-surgery in the rat cranial critical-size defect, as in the present study, limited statistically significant improvements were observed (20). Again, the results may have been influenced by collapse of occlusive expanded polytetrafluoroethylene membranes into the defect site circumvented in the present study using the titanium mesh space-providing device.

In the present study, ACS^{PTH} compared with ACS showed no additive or synergistic effects. As rhBMP-2 resulted in complete bone fill of most defects, it was not possible to evaluate PTH effects in the experimental groups receiving rhBMP-2. However, pulsed systemic PTH has been shown to increase bone mineral density and volume in a rat femur critical-size model implanted with rhBMP-2 soak-loaded on to a polyglycolic acid/gelatin composite compared to that of rhBMP-2 without PTH (35). A commonly reported clinical daily dose for PTH administered intermittently via a subcutaneous route is 20–40 μg/kg/d (36). Studies in rats have used PTH dosages of 60–200 μg/kg/d, and showed increased mechanical strength and callus volume in an adult rat fracture model (37). PTH administered to each animal in the present study amounted to 15 μg/kg/d, which has been reported an effective dose for new bone formation in the rat calvaria critical-size defect model (20). PTH-enhanced osteogenesis appears mediated by the BMP pathway (38), which could possibly enhance the osteoinductive potential of rhBMP-2 leading to a decrease in side effects by decreasing the necessary dose to achieve relevant bone formation. The understanding of mechanisms that govern systemic PTH enhanced osteogenic bone formation remains incomplete and not an objective of the present study. However, shorter observation intervals may reveal PTH accelerated bone formation/maturation that was not possible to appreciate using 4- and 8-wk healing intervals and should be addressed in future study as well as PTH dose ranging.

Evaluation of defect closure included both radiographic and histometric parameters. Although radiographic evaluation has the potential advantage of being less costly and time consuming compared with the "gold standard" histologic evaluation (39), there are limitations. Radiographic analysis cannot be used effectively evaluating bone formation evaluating radiopaque biomaterials (20). Although the radiographic evaluation considers the entire defect area and histometric evaluation uses the most central cross-section of the critical-size defect there are particular observations that should be considered. When no/limited and partial defect closure occurred using histology, the radiographic analysis tended to underestimate bone formation in the control and overestimate bone formation in the DBM groups. When complete or near-complete defect closure was observed using histology, radiographic analysis tended to underestimate bone formation in the ACS and rhBMP-2/ACS groups. However, it must be noted that when comparing the radiographic and histometric evaluation, the same trend for bone formation was noted between test and control groups at 4- and 8-wk intervals. Nevertheless, critical biologic trends, cellular/molecular events, observed in histologic evaluations may never be appreciated in radiographic surrogates.

In conclusion, rhBMP-2/ACS significantly stimulates local bone formation whereas bone formation appears significantly limited following use of the clinical reference DBM. Systemic application of PTH provides no discernible additive/synergistic effect on local bone formation and does not appear a suitable candidate for the optimization of other bone augmentation therapies.

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Conflict of interest statement

The authors declare no conflict of interest. However, it should be noted that Dr. Wikesjö was part of the team at Wyeth Research (Genetics Institute) that provided the evidence and background for the development and registration of recombinant human bone morphogenetic protein-2 (rhBMP-2; brand name INFUSE).

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