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Validity of radiographic evaluations of bone formation in a rat calvaria osteotomy defect model

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Abstract

Objective: The objective of this study was to evaluate the validity of radiographic evaluations of bone formation in a critical-size rat calvaria osteotomy defect model.

Methods: Bilateral, critical-size (∅ 6 mm) calvaria osteotomy defects in 30 adult Sprague–Dawley rats treated with a rat platelet-rich plasma preparation or control treatments were evaluated by radiographic and histometric measures following a 4- or 8-week healing interval. Standardized radiographic images of the rat calvaria gross specimens were used to assess bone formation within the defect sites by visual evaluation of the grey scale by three masked examiners. The most central portion of each defect site was subject to histometric analysis using a PC-based image analysis system. Kappa statistics and percentage agreement between the radiographic and histometric analysis were estimated.

Results: Radiographic evaluations of bone formation are associated with significant weaknesses poorly representing actual healing events; κ statistics (0.17) denoting slight agreement beyond chance. Perfect agreement between the histologic and radiographic analysis for defect sites showing complete and partial histologic bone fill was achieved 63% and 50% of the time, respectively. Agreement reached only 20% for sites with no/limited bone fill. When no/limited and partial bone fill occurred, the radiographic analysis tended to overestimate bone fill and underestimate bone fill when complete closure of the defect sites was observed in the histologic analysis.

Conclusion: Low accuracy was observed when radiographic evaluations were employed in identifying and characterizing bone fill in the rat calvaria osteotomy defects. Assessment of bone healing in animal models aiming at treatment recommendations for clinical application must not solely be based on radiographic analysis, but should be confirmed using histologic observations.

Key words: bone formation; calvaria; comparative methods; histology; radiography; rats

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With the expanding use of implant dentistry to rehabilitate edentulous and partially edentulous patients, effective bone augmentation protocols and biomaterials have become increasingly important. Rodent, canine, and nonhuman primate models have thus been developed and increasingly used to evaluate the biologic potential and application of osteogenic, osteoconductive, or osteoinductive candidate technologies for craniofacial indications before clinical introduction. Small animal/rodent models are routinely used as screening systems to evaluate the biologic potential of new technologies. Large animal models have been employed to provide insight into their clinical potential and application.

Standardized cranial and mandibular osteotomy defects in the rat or rabbit are widely used in craniofacial research to evaluate bone formation (Kaban & Glowacki 1981, Hollinger et al. 1989, Dahlin et al. 1991, 1994, Kleinschmidt et al. 1993, Marden et al. 1993, Sandberg et al. 1993, Winn et al. 1999, Kim et al. 2001, Lu & Rabie 2002, 2004, Carvalho et al. 2004). The critical-size calvaria osteotomy defect includes defect dimensions precluding spontaneous bone healing during the lifetime of the animal unless implanted with an osteogenic,

osteoconductive, or osteoinductive technology. An 8 mm diameter calvaria osteotomy defect has been suggested suitable to evaluate candidate biomaterials for bone regeneration and constitutes a critical-size defect in the rat (Schmitz & Hollinger 1986, Hollinger & Kleinschmidt 1990). Others have defined and used smaller rat calvaria critical-size defects (Turnbull & Freeman 1974, Mulliken & Glowacki 1980, Glowacki et al. 1981, Bosch et al. 1995, 1996, 1998, Bohning et al. 1999, Blom et al. 2001, Cacciafesta et al. 2001).

Evaluation of the healing process of critical-size calvaria osteotomy defects can be performed by gross specimen assessment (Mulliken & Glowacki 1980, Alberius et al. 1992, Bosch et al. 1998, Donos et al. 2004), radiographic techniques (Cacciafesta et al. 2001, Kim et al. 2001, Verna et al. 2002), and histologic and histometric evaluations (Bosch et al. 1995, 1996, 1998, Thaller et al. 1998, Bohning et al. 1999, Blom et al. 2001). Radiographic evaluations have the potential advantage of being less costly and time consuming than the ''gold standard'' protocol of histologic analysis; however, their validity has not been fully explored. Few studies have examined the accuracy of assessment of bone formation using standardized conventional radiographs compared with histometric analysis. The objective of this study was to evaluate the validity of radiographic evaluations of bone formation in a critical-size rat calvaria osteotomy defect model.

Material and Methods

This study utilizes histometric and radiographic data obtained in previous studies evaluating the biological potential of a platelet-rich plasma (PRP) preparation in a rat calvaria osteotomy defect model (Fig. 1; for detail see Pryor et al. 2005a, b). Thirty, 22-week-old, male Sprague–Dawley rats, weight approximately 525 g, were used following a protocol approved by the local Institutional Animal Care and Use Committee.

Experimental procedures

Anaesthesia and pain control followed recommended routines for the species. The animal's head was shaved, washed with a disinfectant, and stabilized by a nose cone apparatus (Euthanex Corp., Palmer, PA, USA). A midline incision was made from the nasofrontal area to the external occipital protuberance along the mid-sagittal suture. Skin and underlying tissues including the temporal muscle were reflected bilaterally to expose the full extent of the calvaria. In each animal, one calvarial through-and-through osteotomy, 6.0 mm in diameter, was trephined into the dorsal portion of the parietal bone on each side of the mid-sagittal suture using a dental handpiece and a trephine bur (#11-31-0050; Ace Surgical Supply Co. Inc., Brockton, MA, USA) under irrigation with sterile saline. The trephined bone was removed from the surgical field. Using aseptic techniques, 42.4 µl of the PRP preparation was used to soak-load a precut ($\varnothing$ 6×1.5 mm) absorbable collagen sponge (ACS; CollaCote®, Sulzer Dental Inc., Carlsbad, CA, USA). The PRP/ACS construct or ACS alone was implanted into contralateral calvaria osteotomy defects in 18 animals. Twelve animals received ACS without the PRP preparation *versus* sham surgery in contralateral calvaria osteotomy defects. The wound margins were closed using autoclips (Autoclip® Wound Closing System, Stoelting Co., Wood Dale, IL, USA). A bacitracin–neomycin–polymyxin ointment (Vetropolycin® Ophthalmic Ointment, Pharmaderm, Melville, NY, USA) was applied to the eyes of the animal. The animal was monitored until anaesthesia recovery. Animals were sacrificed at 4 (13 animals) and 8 (17 animals) weeks post-surgery by CO_2 inhalation. The cranial bone including the bilateral defects was removed in total, rinsed in water, and placed into 10% buffered formalin.

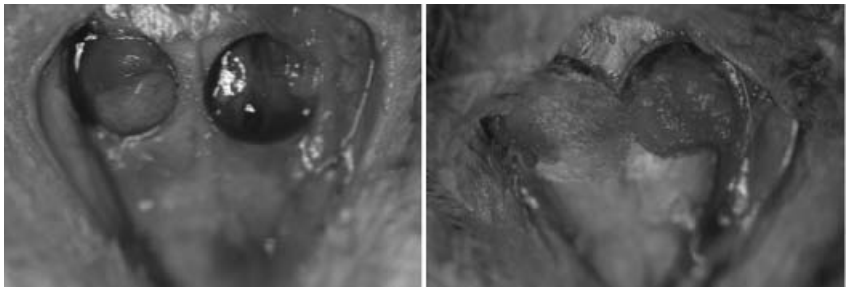

Fig. 1. Bilateral critical-size 6 mm rat calvaria osteotomy defects before (left) and after implantation of a platelet-rich plasma (PRP) preparation in an absorbable collagen sponge (ACS) carrier or the ACS carrier without PRP (control). Reproduced with permission from Pryor et al. 2005a.

Radiographic processing and analysis

Standardized radiographic images of the rat calvaria gross specimens were obtained using a dental radiographic unit (70 kVp, 7 mA for 0.083 s; Gendex 770, Gendex Corporation, Des Plaines, IL, USA) and No. 2 Kodak Ultra-speed X-ray film (Eastman Kodak Company, Rochester, NY, USA). The calvaria gross specimens, recorded individually, were placed on the radiographic film, the defect area placed flat on the film and the X-ray source directed perpendicular to the film/gross specimen. The X-ray source–film distance was 12 in. Radiographs were processed in an automatic dental film processor (A/T 2000, Air Techniques, Hicksville, NY, USA). The radiographs (analog films) were transformed into digitized images using a film scanner (620ST AcerScan, San Jose, CA, USA) at 1200 dpi, which was connected to a standard PC. There was no attempt to adjust or calibrate the optical density of the radiographic images. The distinction between mineralized and non-mineralized tissues was made for each defect by visual evaluation of the grey scale in each image by three masked examiners. The defects were classified and scored according to the following criteria:

- 0 = *No/limited bone fill*: the osteotomy defect remained radiolucent with the exception of limited new bone apposition at the defect margins;
- 1 = *Partial bone fill*: the osteotomy defect was partially radiopaque exhibiting regions of radiolucencies and radiopacities suggestive of new bone formation from the defect margins without establishing bone continuity;
- 2 = *Complete bone fill*: The osteotomy defect showed radiopacity/osseous continuity between the defect margins.

Complete agreement was reached most of the time. When the classifica-

tion of the osteotomy defect was not unanimous, the defect was reevaluated to achieve consensus by two or all three evaluators.

Histotechnical processing and histometric analysis

Calvaria specimens were fixed in 10% buffered formalin for 3–5 days, and decalcified in formic acid and sodium citrate for 24 h. The specimens were washed with tap water, dehydrated with ascending concentrations of ethyl alcohol, cleared in xylene, and infiltrated with paraffin. Serial sections (7 μm) parallel to the mid-sagittal suture were cut from the centre of each osteotomy defect using a microtome (RM2155, Leica Microsystems GmbH, Nussloch, Germany) and stained with haematoxylin and eosin.

The most central portion of each osteotomy defect, selecting the section displaying widest extension, was identified and subject to histologic and histometric analysis (Koo et al. 2004). On the average three central sections were used for the histologic evaluation representing approximately 80 μm of the central aspect of the defect. The sections were viewed and analysed for new bone formation by one calibrated examiner (M. E. P) using incandescent and polarized light microscopy (BX 60, Olympus America Inc., Melville, NY, USA). The following linear measurements were recorded for the most central section for each defect using a microscope digital camera system (DP10, Olympus America Inc.) and a PC-based image analysis system (Image-Pro Plus™, Media Cybernetic, Silver Springs, MD, USA):

- *defect width:* the distance between the margins of the original osteotomy defect;
- *bone fill:* the length of newly formed bone along an axis bridging the gap between the osteotomy defect margins.

Percentage bone fill was calculated based on defect width and bone fill measurements. The osteotomy defects were classified and scored according to the following criteria:

- 0 = *No/limited bone fill*: $\leq$ 25% of the defect width was filled with bone;
- 1 = *Partial bone fill*: >25% and $\leq$ 90% of the defect width was filled with bone;
- 2 = *Complete bone fill*: >90% of the defect width was filled with bone.

Statistical analysis

Frequency tables and graphs were used to explore agreement between the radiographic and histologic analysis. Unweighted κ statistics was calculated to test perfect agreement between the two methods. Kappa measures agreement beyond what should be expected to occur only by chance given random guessing (i.e., it provides a chance-corrected measure of agreement). The correlation between the two methods was also evaluated using the Spearman rank test.

Kappa statistics was used to assess the reliability of the examiners. The reproducibility of the examiner that performed the histometric analysis (M. E. P) was assessed by repeated evaluation of randomly selected defect sites. Excellent intra-examiner reproducibility was achieved ($\kappa = 1.0$; $p < 0.001$). The pooled reproducibility for the radiographic analysis was assessed for the three examiners. Excellent reproducibility was also observed for this outcome ($\kappa = 0.90$; $p < 0.001$).

Results

Figures 2 and 3 show radiographic/photomicrograph composites of rat calvaria osteotomy defect sites in this study. The results of the analysis of category agreement of defect bone fill between the radiographic and histologic analysis are presented in Table 1. Radiographic and histologic scores reached perfect agreement for 49.1% of the defect sites. The unweighted κ statistics, which discounts agreement due to chance, was 0.17 ($\pm$ SE 0.10) denoting slight agreement beyond chance. Whereas perfect agreement was low, a one- and two-category difference between radiographic and histologic scores occurred for 49.1% and 1.9% of the defect sites, respectively. Overall the correlation between the two methods was weak (Spearman's rank correlation = 0.43).

Perfect agreement between the histologic and radiographic analysis for defect sites showing complete and partial histologic bone fill was achieved 63% and 50% of the time, respectively. Agreement reached only 20% for sites with no/limited bone fill. When no/limited and partial bone fill occurred, the radiographic analysis tended to overestimate bone fill and underestimate bone fill when complete closure of the defect sites was observed in the histologic analysis.

Notably, perfect agreement between the histologic and radiographic analysis slightly improved from 43% for defect sites subject to a 4-week healing interval to 53% for sites subject to an 8-week interval (Fig. 4). Nevertheless, the κ statistics were 0.17 ($\pm$ SE 0.13) and 0.20 ($\pm$ SE 0.14) for the 4- and 8-week healing intervals, respectively, demonstrating low agreement beyond chance.

Discussion

The validity of outcome measures in experimental models used to evaluate

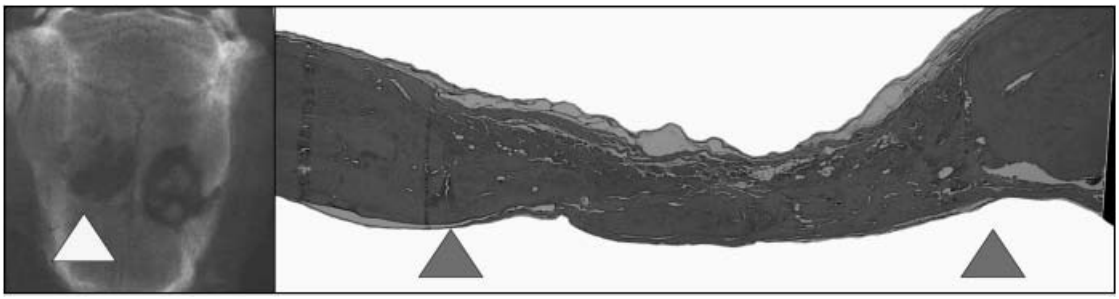

Fig. 2. Standardized radiographic image and photomicrograph of a critical-size 6 mm rat calvaria osteotomy defect representing a one-category difference between the radiographic and histologic observations; the radiographic image was scored to represent partial bone fill whereas the photomicrograph shows complete bone fill.

Fig. 3. Standardized radiographic image and photomicrograph of a critical-size 6 mm rat calvaria osteotomy defect representing a one-category difference between the radiographic and histologic observations; the radiographic image was scored to represent complete bone fill whereas the photomicrograph shows partial bone fill.

Table 1. Category agreement (%) of bone fill in critical-size 6 mm rat calvaria osteotomy defects as evaluated using radiographic and histologic analysis

	Bone fill	Radiographic analysis		
		no/limited	partial	complete
Histologic analysis	No/limited	2 (3.8%)	8 (15.1%)	0 (0.0%)
	Partial	3 (5.7%)	12 (22.6%)	9 (17.0%)
	Complete	1 (1.9%)	6 (11.3%)	12 (22.6%)

Perfect agreement: 49.1%.
One-category disagreement: 49.1%.
Two-category disagreement: 1.9%.

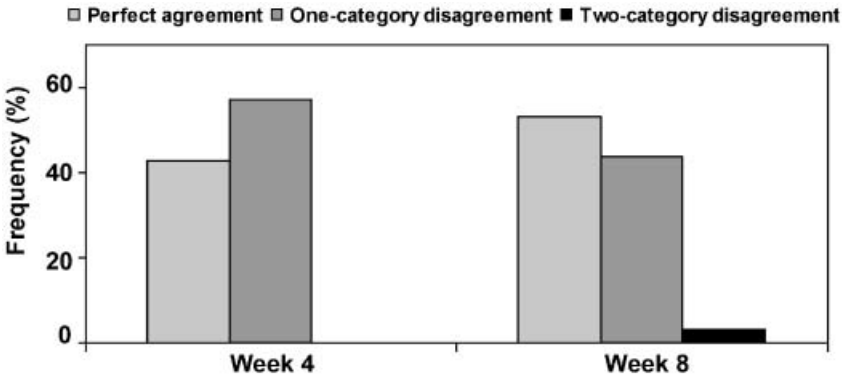

Fig. 4. Category agreement (%) of bone fill in critical-size 6 mm rat calvaria osteotomy defects as evaluated using radiographic and histologic analysis following a 4- or 8-week healing interval.

bone regeneration is an important consideration. The objective of this study was to evaluate the validity of radiographic evaluations of bone formation in a widely used critical-size rat calvaria osteotomy defect model. The results demonstrate that radiographic evaluations of bone formation are associated with significant weaknesses, and, as such, poorly represent actual healing events. When no/limited and partial bone fill occurred, the radiographic analysis tended to overestimate bone fill and underestimate bone fill when complete closure of the defect sites was observed in the histologic analysis. The validity of the radiographic evaluation somewhat increased with extended healing intervals likely associated with increasing mineralization of bone matrix at the same time further emphasizing that radiographic measures commands low diagnostic accuracy of early bone formation in this experimental model.

Radiographic evaluations have been used to evaluate the effect of various treatment concepts on bone formation. Cacciafesta et al. (2001) used a microcomputer tomography (micro-CT) to evaluate bone formation in a rat calvaria osteotomy defect model following systemic application of recombinant human growth hormone. Bone formation was significantly greater in the experimental group than in the control. Kim et al. (2001) evaluated the effect of PRP on bone formation in a rabbit calvaria osteotomy defect model using a cross-sectional CT. When combined with a cancellous bovine bone mineral biomaterial, PRP increased local bone formation. Verna et al. (2002) used micro-CT to evaluate bone formation following application of perforated and occlusive ePTFE membranes in a rat calvaria osteotomy defect model. Patterns of mineralization showed a difference between perforated and occlusive membranes. These studies all employed radiographic analysis without a histologic component. Although there may be differences in discrimination between radiographic techniques, it appears from the present study and studies using onlay models (Marechal et al. 2005) that radiographic observations should be viewed with caution, and that observations of bone healing in experimental models such as herein or in more complex models should be confirmed using histologic observations.

Radiographic bone formation has not been consistently evaluated in animal models such as herein. A few studies have employed scoring systems or indices to assess bone formation. Cacciafesta et al. (2001) used a dichotomous scoring system to assess bone fill in a rat calvaria defect model following systemic application of recombinant human growth hormone. Marx et al. (1998) used a Graft Maturity Index to visually assess osseous maturity in mandibular resection defects at 2, 4, and 6

months following implantation of a PRP construct. Similarly, Turnbull & Freeman (1974), Takagi & Urist (1982), and Hollinger et al. (1989) made arbitrary visual assessments of grey level contrasts to identify bone formation in calvaria osteotomy defect sites. In the present study, distinction between mineralized and non-mineralized tissues for each defect was achieved by consensus and scored using three categories, the radiographic evaluations being performed by three experienced masked examiners. The radiographic assessments distinguished patterns of mineralization; however, when the results from the radiographic assessments were compared with histologic observations, this study suggests that radiographic measures yield a low degree of diagnostic accuracy for analysis of early bone formation.

Kappa statistics was used to establish the level of agreement between standardized radiographs and histologic sections in the assessment of bone formation. This statistics is more appropriate than the simple percentage agreement score for analysing reproducibility in that it indicates the proportion of agreement beyond that expected by chance only (Cohen 1960). The level of agreement for the 4- and 8-week observations was 0.17 and 0.20, respectively, indicating slight/low agreement (Landis & Koch 1977). It is important to acknowledge that the κ statistic is influenced by the number of categories, thus the lower the number of categories the more difficult it is to achieve a high κ statistic (Maclure & Willett 1987). Thus, the use of more categories could have positively influenced the reproducibility.

In conclusion, the results suggest that radiographic evaluations of bone fill in rat calvaria osteotomy defects yield a low level of accuracy. Evaluation of bone formation in animal models aiming at treatment recommendations for clinical application must not solely be based on radiographic analysis, but should be confirmed using histologic observations.

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Clinical Relevance

Scientific rationale for study: Radiographic assessments are common surrogate endpoints in experimental studies on bone healing. We herein explore the validity of radiographic assessments of bone healing in a rat calvaria osteotomy defect model widely used to evaluate devices, bone biomaterials, and biologic factors ultimately intended for clinical application. The radiographic assessment is compared with a histologic evaluation revealing actual bone formation.

Principal findings: It is shown that radiographic assessments of bone healing poorly reflect actual healing events.

Practical implications: The observations in this non-complex animal model suggest that radiographic assessments should not be considered a reliable endpoint in experimental studies screening candidate technologies for skeletal reconstruction.

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