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The efficacy of various alloplastic bone grafts on the healing of rat calvarial defects

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SUMMARY The objective of this study was to determine the relative efficacy of currently available alloplastic bone repair materials in the healing of rat calvarial defects histologically, histomorphometrically and biochemically. A representative material was selected from six major classes of bone repair materials and placed in 4 mm diameter calvarial defects of 6-week-old male Sprague–Dawley rats (five animals in the control and each of the six experimental groups). The outcomes were assessed after 2 months for alkaline phosphatase (ALP) activity and after 4 months of healing for histomorphometry.

The tested alloplastic implant materials did not significantly increase ALP activity or the amount of new bone formation in the healing of rat calvarial defects relative to controls ($P > 0.05$). However, when the implant material itself was included in the analysis, significant differences were observed ($P < 0.05$). Additionally, the tested materials varied in their ability to bridge the bony defect. These data suggest that the rate of bone formation cannot be increased beyond control levels, rather the advantage of implant materials may be in their efficiency in filling the defect through incorporation of the material into the healing site and rapidly bridging the wound.

Introduction

Alloplastic bone repair materials are common alternatives to autografts, but given the variety of available materials (Table 1), the research question is 'What are the relative bone healing responses to currently available alloplastic bone implant/repair materials?' Studies have been performed to compare a particular material with another (Lovelace *et al.*, 1998; Wheeler *et al.*, 1998; Richardson *et al.*, 1999; Froum *et al.*, 2002), but there has been no comprehensive study to evaluate and compare the major categories of these materials.

Grafting materials fall into one of three categories: autografts, allografts, and alloplasts (Misch and Dietsh, 1993). Autografts are taken from the host and are the only type of grafting material that possesses osteogenic, osteoinductive, and osteoconductive abilities. They are considered the 'gold standard' for the regeneration of osseous structures. However, their use is limited by the need for a second surgical site to harvest the bone, possible post-operative complications related to the harvesting procedure, and limited yield in some situations.

Allografts are taken from another individual of the same species with a different genotype, such as cadavers, relatives, or bone banks. The preparations are often frozen, freeze-dried, irradiated, or demineralized. Freezing and freeze-drying the donor bone decreases the antigenicity of the graft (Mellonig, 1996). Irradiating the donor bone decreases the chance of infection. It is believed that the process of demineralization removes the mineral content, leaving only the collagen and morphogens, thus increasing the osteoinductive potential (Rummelhart and Gray, 1989). The advantages of allografts over autografts are that they eliminate the need for a donor site during surgery and are readily available. However, the disadvantages include rejection, infection, and longer healing periods, and they typically result in less bone volumes than autografts (Misch and Dietsh, 1993).

Alloplasts are synthetic bone substitutes that are readily available and also eliminate the need for a patient donor site. Ideally, alloplastic implant materials should be biocompatible with host tissues, non-antigenic, non-carcinogenic, and non-inflammatory (Ferraro, 1979; Bissada and Hangorsky, 1980; Han and Carranza, 1984). Additionally, they should be sufficiently porous and interconnective for tissues to grow into and around the implant (osteoconduction), able to stimulate bone induction, resorbable and replaceable by bone, radio-opaque in order to be visualized radiographically, able to withstand sterilization without losing favourable qualities, stable in varying temperatures and humidity, inexpensive, and easily attainable (Alderman, 1969; Bissada and Hangorsky, 1980; Han and Carranza, 1984).

Most alloplasts are osteoconductors. The tissue response with respect to osteoconduction varies with the differences in porosity and interconnectivity of the material. The opportunity for ingrowth seems to increase as the porosity and interconnectivity increase.

Table 1 Some currently available Food and Drug Administration-approved alloplastic bone grafting materials.

Synthetic hydroxyapatite ceramics
Calcitite HA 2040 and 4060 (Sulzer Medica, Sulzer Calcitek Inc, Carlsbad, CA 92008, USA)
Orthomatrix HA-500 and HA-1000 (Lifecore Biomedical, Chaska, MN 55318, USA)
Osteogen (Implandent Ltd, Holliswood, NY 11423, USA)
Osteograf D (CeraMed Dental, Lakewood, CO 80028, USA)
Osteograf LD (CeraMed Dental)
Naturally derived hydroxyapatite ceramics
Interpore 200 (manufactured by Interpore International, distributed by Steri-Oss, Yorba-Linda, CA 92887, USA)
Osteograf N (CeraMed Dental)
Deorganified bovine bone
BioOss (Luit Pold Pharmaceuticals, Shirley, NY 11967, USA)
Calcium carbonate
BioCoral (INOTEB, Gonnery, France)
Pro-Osteon 500R (Interpore Cross International, Irvine, CA 92618, USA)
Biocompatible composite polymer
Bioplant HTR (Septodont Inc., New Castle, DE 19720, USA)
Bioactive glass
Bioglass (registered trademark of US Biomaterials Corp. but licensed to Block Drug Corp., Jersey City, NJ 07302, USA)
Biogran (Orthovita, Malvern, PA 19355, USA)
Perioglas (Block Drug Corporation)

The materials tested are indicated in bold.

Early in the process, ingrowth of fibrovascular tissue into the pores occurs. As this develops, appositional bone growth against the walls of the pores begins in a process termed 'incorporation'. This process starts from the edges of the graft inward towards the centre of the implant.

In this study, representative commercially available materials from major categories of alloplastic bone graft material were tested in a rat calvarial defect model. The hypothesis was that currently available alloplastic bone repair materials would enhance the healing of a 4 mm rat calvarial defect compared with controls (no implant) as measured histomorphometrically and biochemically.

Materials and methods

A representative material from each of six categories of alloplastic materials (Table 1) was tested in an established model of bone repair in healing a 4 mm calvarial defect (Mulliken and Glowacki, 1980; Glowacki and Mulliken, 1985). This model is advantageous over long bone defect models because it eliminates the effects of motion at the defect site and the need for internal fixation devices (Wang *et al.*, 1998). Preliminary studies have shown that an 8 mm rat calvarial defect filled with osteoinductive material, such as allogenic decalcified bone, heals in only 4 weeks (Wang and Glimcher, 1999). Because the efficacy of osteoconductive materials such as alloplasts on bone repair is usually less effective than allogenic decalcified bone, a 4 mm defect was studied in anticipation that at least one of the graft materials would achieve near-complete healing.

Seven groups of 6-week-old male Sprague–Dawley rats were used in this study (six experimental and one control), each group containing five animals. Defects were made in each half of the calvarium of each animal and filled with either implant material (experimental groups) or left empty (control group), thus totalling 10 implant sites per group. Two animals per group were sacrificed in a CO_2 chamber after a 2 month healing period, yielding four implant sites per group. One implant site was used for histological assessment while the remaining three implant sites were used for the determination of alkaline phosphatase (ALP) activity. Three animals per group were sacrificed after a 4 month healing period, yielding six implant sites for histomorphometric analysis.

Animal model

This research was conducted with the approval of the Children's Hospital Animal Care and Use Committee. The rats were anaesthetized by administration of ketamine (60 mg/kg body weight) and xylacine (15 mg/kg body weight) intramuscularly. Hair over the calvarium was shaved and cleaned with a depilator. Xylocaine (0.5 ml of 1 per cent) was injected intradermally in the middle of the top of the calvarium. Following elevation of a cranial skin flap, the subcutaneous fascia was divided, and periosteal flaps were reflected bilaterally. Two full thickness cranial defects, 4 mm in diameter, were produced with a dental bur while rinsing with sterile lactate Ringer's solution to cool and clear any remaining debris. During this process extreme care was taken not to damage the dura mater. The bone implant material was placed in the experimental groups as directed by the manufacturer, the defects were completely covered, and the skin was closed using 4-0 nylon suture.

After 60 days, two animals (four implants) per group were sacrificed in a CO_2 chamber. Three implants

were used for the ALP assay and the remaining one for histology. The implants used for the ALP assay were dissected under a dissecting microscope to avoid harvesting host bone. After decalcification of the remaining implant and processing, a histological analysis was performed. After 120 days, the remaining three animals in each group were sacrificed in a CO_2 chamber and the implant sites were processed for histomorphometric studies.

Histomorphometric analysis

For the histomorphometric analysis, three animals per group were sacrificed and the implant sites were harvested after a 4 month healing period (six implant sites per group). A 4 month healing period was selected with the expectation that a good range of bone healing between the groups would be achieved. Beyond this time, complete healing could have occurred at some sites, making comparisons difficult (Wang and Glimcher, 2000).

After harvesting and decalcification, the specimens were cut into anterior and posterior halves and embedded in paraffin. Representative sections (6 μm thick) were taken every six sections (approximately 36 μm apart). The sections were stained with haematoxylin and eosin. Computer images from the representative sections were captured using the Optimus 5.2 computer program (Bothell, Washington, USA). The area of new bone formation was calculated in pixels and compared with the area of the total defect to obtain the percentage area of new bone. Eight slides were taken for each implant site and an average value was calculated. To determine the location of the bone formation in the defect, an area in the central portion of the defect was analysed (2 mm wide × the thickness of the cranium at the defect edges) in the same manner to obtain an average percentage area of new bone in the central portions of the defect. Implant particles in direct contact with the new bone were included in the analysis to determine the amount of healing when incorporation of implant material and new bone was considered.

Biochemical analysis

Two animals per group were sacrificed after a 2 month healing period and three implant sites were harvested for biochemical analysis of ALP activity (one implant was used for histological comparison). A 2 month healing period was chosen with the objective of measuring osteoblastic activity during healing.

ALP activity was used as an index of osteoblastic activity. The implants were cut and homogenized in 2 ml of ice-cold 0.15 M NaCl, 30 mM $NaHCO_3$, pH 7.8, containing 0.5 per cent Nonidet P-40 detergent (Fluka Biochemika, Buchs, Switzerland), and incubated for 1 hour on ice. The mixture was then centrifuged at 100 000 ***g*** for 30 minutes at 4°C and the supernatants were assayed for ALP activity after 1 hour of incubation at 37°C using *p*-nitrophenylphosphate (*p*-NPP, Sigma, St. Louis, Missouri, USA) as a substrate. Absorbance measurements at 410 nm were converted into moles of *p*-nitrophenal released per litre, using $\varepsilon_{410} = 12\,500/M/cm$, and the time of incubation was used to provide the rate of substrate hydrolysis in moles/l/minute. One unit of ALP was defined as the enzyme activity that liberates 1 μmol of *p*-NP under the assay conditions. The final ALP activity was expressed as units of activity per milligram of total protein in the sample.

Alloplastic materials

Representative material from each of six categories of alloplastic materials (Table 1) was evaluated. In brief the materials tested were: (1) Orthomatrix™ HA-500, a synthetic hydroxyapatite ceramic that is round in shape with a diameter of 250–420 μm; (2) Interpore™ 200, a porous coral-derived hydroxyapatite ceramic; (3) Bio-Oss™, a deorganified bovine bone; (4) Pro-Osteon™ 500R, a resorbable calcium carbonate with a 2–10 μm outer layer of calcium phosphate that is designed to resorb slowly and delay the exposure of the quickly resorbing calcium carbonate substructure; (5) Bioplant™ HTR, a composite polymer 550 μm in size with a 350 μm internal pore. The particles are egg-shaped and made of a combination of three biocompatible polymer layers. The innermost layer is polymethylmethacrylate, providing strength; the middle layer is polyhydroxyl-ethylmethacrylate with a thin layer of barium sulphate, giving the material radio-opacity; and the outer layer is calcium hydroxide carbonate to attract osteoprogenitor cells. (6) Biogran™, a glass particle material with a 300–355 μm diameter designed to control the location of bone formation. Each particle contains a hollow calcium phosphate chamber providing a protective environment for cellular differentiation and subsequent bone growth. Resorption of the glass particle eventually occurs as the bone fills the chamber.

Statistical analysis

Intergroup comparisons were undertaken using one-way analysis of variance (ANOVA) with Bonferroni correction. Statistical significance was established at $P < 0.05$. Correlation between ALP activity and histomorphometric analysis was determined using Pearson's correlation coefficient.

Results

Histological evaluation at 2 months showed minimal bone growth in the defect area with no bridging of the defect in any group (data not shown). Additionally, there was minimal incorporation of implant material with new bone. Histological results at 4 months are shown in Figure 1.

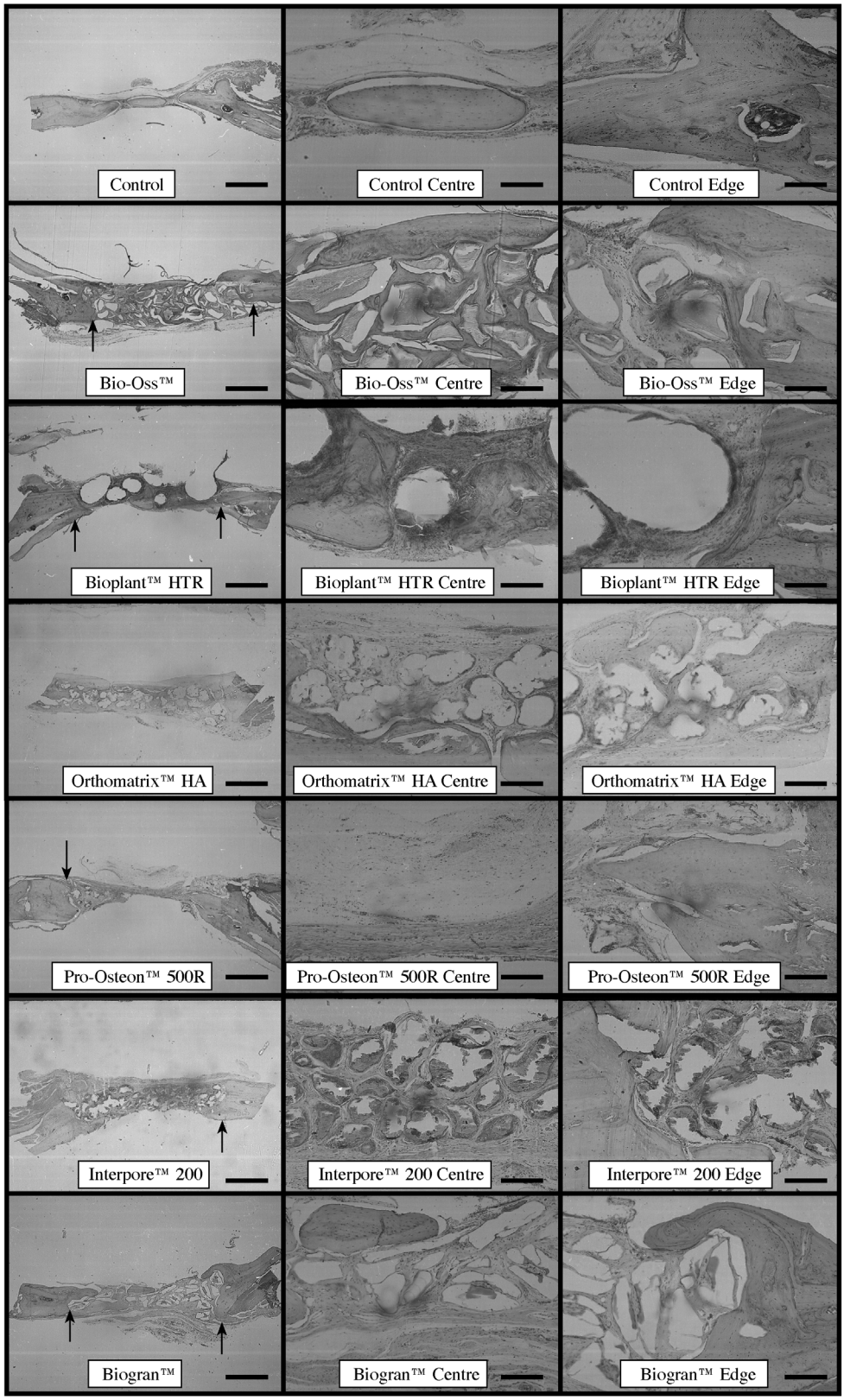

Figure 1 Comparison of alloplastic bone repair materials after 4 months of healing. The control group showed incomplete bridging of the defect with new bone formation at the edges and the centre (the arrows indicate the edges of the original host bone). All other groups showed a mixture of new bone and implant material in the defect, with the exception of the Pro-Osteon™ 500R group which had very little implant material remaining. Other materials exhibited shrinkage artefacts and loss of material during histological processing resulting in voids. All sections were stained with haematoxylin and eosin. Left column: $\times 20$ magnification, bar = 1 mm; centre and right columns: $\times 100$ magnification, bar = 200 μm.

Table 2 Mean percentage area (± standard deviation) of new bone or new bone plus implant in the centre and total defect (n = 30).

Implant group	New bone in total defect	New bone + implant in total defect	New bone in centre of defect	New bone + implant in centre of defect
Pro-Osteon 500R	34.7 ± 8.0	39.8 ± 6.7	11.5 ± 8.2*[b]	11.9 ± 8.6
Interpore 200	38.5 ± 9.3	67.1 ± 8.1**	19.7 ± 10.0[b]	21.2 ± 9.6
Orthomatrix HA	45.3 ± 6.2	67.7 ± 7.0**	43.6 ± 8.6[a]	57.2 ± 8.6**
Biogran	30.6 ± 9.7	33.1 ± 8.0	32.0 ± 10.1	32.3 ± 13.8
Bioplant HTR	37.1 ± 8.0	69.4 ± 7.1**	25.6 ± 6.8	62.8 ± 7.6**
Bio-Oss	47.4 ± 7.1	80.1 ± 5.1**	39.1 ± 7.4[a]	81.1 ± 6.3**
Control	42.2 ± 6.4	42.2 ± 6.4	30.4 ± 23.1	30.4 ± 23.1

*P < 0.05; **P < 0.01 compared with the control.
[a]P < 0.05 compared with [b] groups.

Analysis of the percentage area of new bone in the total defect (Table 2) showed a range of 16.9 per cent of new bone formation between the groups with the least and most new bone formed. When compared with the control group, these differences were not significant. However, if implant particles in direct contact with new bone were included, significant differences were found. Bio-Oss™ had the largest increase, followed by Bioplant™ HTR, Orthomatrix™ HA, and Interpore™ 200. Biogran™ and Pro-Osteon™ 500R did not display implant–bone incorporation and thus their values remained relatively the same.

Analysis of new bone growth in the 2 mm central portions of the defects (Table 2) revealed large variations among groups. The Bio-Oss™ and Orthomatrix™ HA groups displayed larger amounts of new bone growth when compared with the control group, but these mean differences were not statistically significant. If the two groups with the most new bone (Orthomatrix™ HA and Bio-Oss™) were compared with the two groups with the least amount of new bone (Pro-Osteon™ 500R and Interpore™ 200), a statistical difference was observed. Additionally, if implant particles in direct contact with new bone were included in the analysis, the Bio-Oss™, Bioplant™ HTR, and Orthomatrix™ HA groups all significantly increased their values and were statistically significant. The Pro-Osteon™ 500R group had significantly less bone formation in the central region compared with the control group.

ALP activity was measured at the 2 month healing period (Table 3). There were no significant differences between the experimental groups and the controls (P > 0.05), but the Pro-Osteon™ 500R group was significantly less than the controls (P = 0.07).

There was a strong correlation (r = 0.8) between the percentage area of new bone growth in the defect assessed at 4 months and ALP activity assessed at 2 months (Figure 2). The Bio-Oss™ group had the highest amount of ALP activity and also the largest percentage area of new bone growth. The control group had the second highest amount of ALP activity and its percentage of new bone growth was also among the highest. Although Orthomatrix™ HA had lower ALP activities (ranked third) than Bio-Oss™ and the control, its percentage of new bone growth was essentially as high as Bio-Oss™. Bioplant™ HTR and Interpore™ 200 had similar amounts of new bone (both less than the control). Pro-Osteon™ 500R and Biogran™ had the

Table 3 Alkaline phosphatase activity in a 4 mm defect after a 2 month healing period.

Implant group	Alkaline phosphatase activity (μmol/l/minute/mg protein ± standard deviation)
Pro-Osteon 500R	29.2 ± 0.4*
Interpore 200	30.9 ± 0.6
Orthomatrix HA	43.0 ± 1.0
Biogran	33.7 ± 1.0
Bioplant HTR	40.2 ± 1.3
Bio-Oss	59.5 ± 0.5
Control	53.9 ± 1.4

*Not significantly different compared with the control at P < 0.05 but significantly different at P = 0.07.

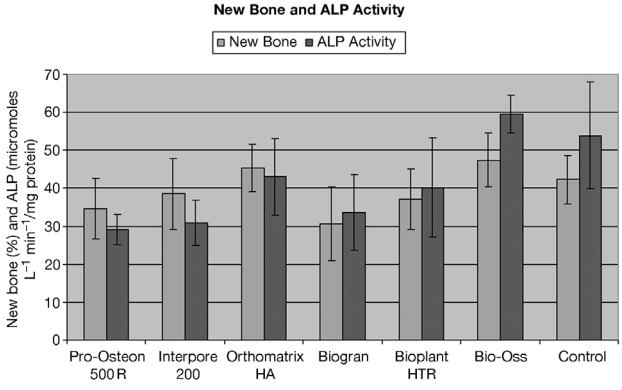

Figure 2 New bone and alkaline phosphatase (ALP) activity. ALP activity measured at 2 months was found to be strongly correlated (r = 0.8) with the percentage area of new bone growth in the defect assessed at 4 months.

lowest ALP activity and percentage of new bone growth.

Discussion

An established model of bone repair was used to assess and compare the efficacy of currently available US Food and Drug Administration (FDA)-approved alloplastic bone materials after 2 and 4 month healing periods by histomorphometric and biochemical analysis. There were no statistically significant differences in the amounts of bone formation between the treatment and control groups. Thus, it was concluded that the alloplastic materials tested did not significantly increase the amount of new bone formation in a 4 mm defect after 4 months of healing. This result is consistent with other calvarial defect repair experiments with a polymer–ceramic composite that showed that osseous defect healing was not significantly different than controls (Bidic *et al.*, 2003). In a tooth extraction socket model of bone healing, a similar result was found where neither bioactive glass nor demineralized freeze-dried bone was able to increase bone fill and healing significantly compared with controls (Froum *et al.*, 2002).

As there was also no significant increase in ALP activity between the groups, this may suggest that the rate of bone repair cannot be increased beyond control levels, rather the advantage of implant materials could be in their efficiency of filling the bone defect through incorporation of the material into the healing site. In doing so, these materials could facilitate bridging of the defect for cellular migration and bone fill. Bridging of the defect seems to be important to overall healing (Wang and Glimcher, 2000). In the 2 mm central portion of the defect, the amount of healing tissues filling the defect was significantly increased in the Bio-Oss™, Bioplant™ HTR, and Orthomatrix™ HA groups compared with the controls, because their particles were incorporated with the adjacent new bone. In the total defect, the Bio-Oss™, Bioplant™ HTR, Orthomatrix™ HA, and Interpore™ 200 groups largely increased their values when the incorporated material was included in the assessment. The Pro-Osteon™ 500R and Biogran™ groups showed little or no incorporation of implant and bone, thus their values remained the same.

Although no significant increase in ALP activity between the groups was found at the 2 month assessment, a strong correlation ($r = 0.8$) between ALP activity and the percentage area of new bone growth in the defect assessed at 4 months was found (Figure 2). This could indicate that some materials allow or promote continuation of ALP activity to produce relatively more new bone growth by 4 months.

As the bridging of a defect is important to the quality of defect healing, the percentage area of new bone in the central portion of the defect was evaluated to determine which materials were able to facilitate bone growth in the defect's central areas, thus suggesting an ability to enhance bridging (Table 2). None of the treatment groups showed significantly more bone formation compared with the control group which had a large variation in the amount of new bone formed. However, there was a significant difference between the two groups with the most bone formation (Orthomatrix™ HA and Bio-Oss™) and the two groups with the least amount of bone formation (Pro-Osteon™ 500R and Interpore™ 200). The low values for these two groups suggest little or no enhancement of defect bridging. In fact, the Pro-Osteon™ 500R group had significantly less bone formation relative to the control group, suggesting that this material inhibited or had a different sequence of new bone formation in the centre of the defect.

To determine the primary location of new bone formation, the amount of new bone formed in the central portion was compared with the total bone formation (Table 2). Despite large differences in the amounts of bone formation, the Orthomatrix™ HA and Biogran™ groups had as much bone growth (1.7 and 1.4 per cent less, respectively) occurring in the central area as in the overall area. The differences for the Bio-Oss™, control and Bioplant™ HTR groups were similar (8.3, 11.8, 11.6 per cent, respectively) The Interpore™ 200 and Pro-Osteon™ 500R groups had considerably less bone in the central areas than in the total area (19.0 and 23.2 per cent less, respectively).

Some areas displayed bone growth around the implant material with direct contact to bone, which suggests incorporation of implant particles with bone. In other areas, there was a layer of soft tissue between the implant and new bone, suggesting that different pathways of bone repair/formation were present.

In all groups, including the control group, a thickening of bone at the bony margin of the defect was observed, which was attributed to thickening of the cambium layer of the periosteum. Thickening occurs due to proliferation and differentiation of the undifferentiated cells from the periosteum, endostium, and bone marrow. The periosteum is lifted from the bone surface by underlying hyperplastic cells, which form an elevated ring around the defect margin. As these precursor cells divide and differentiate to osteoblasts, new bone grows into the defect site in an attempt to bridge the gap. Later, woven bone is laid down and eventually becomes more organized into lamellar bone (Shapiro, 1988). The control group showed high intragroup variability of new bone formation. New bone was present in the centre of the defect in some samples. However, the defects were not completely bridged. In other samples, fibrous tissue was seen in the centre of the defect. The new bone that formed was lamellar bone, seen with mature osteocytes. The Bio-Oss™ (deorganified bovine bone) group had

new bone growth directly around the implant and in the centre of the defect. There were some minute areas of fibrous tissue around the implant, but nearly complete bridging of the defect occurred with the amalgamation of the Bio-Oss™ implant and new bone.

Bioplant™ HTR is designed with an outer layer of calcium hydroxide carbonate to give the material osteopromotive properties. This layer has been shown to form a calcium carbonate–apatite bond with new bone (Ashman, 1984, 1992). In some specimens, direct contact of new bone with the implant was observed, supporting this observation. However, in other areas there was a layer of fibrous tissue between the Bioplant™ HTR particles and the new bone.

The Orthomatrix™ HA-500 (synthetic hydroxyapatite ceramic) group showed nearly complete bridging of the defect, with most of the bone growth occurring on the dura side of the defect and less between the implant particles. The centre of the defect was occupied mostly with fibrous tissue between the implant particles. The implant in the Pro-Osteon™ 500R (resorbable calcium carbonate) group was found to have mostly disappeared at 4 months, leaving fibrous tissue in the majority of the defect. Some lamellar bone was seen at the defect edges, but no bridging of the defect was noted. The Interpore™ 200 (coral-derived hydroxyapatite ceramic) group formed lamellar bone at the defect margins and around the implant particles, but in the centre of the defect the implant particles were surrounded by fibrous tissue and some bone. The Biogran™ (glass particles) group showed defects filled with a mixture of new bone and implant particles. Some remnants of implant particles were still present but most had disappeared either through the decalcification procedure in histological processing or through natural resorption of the material. Small areas of new bone were observed extending from the defect edges into the middle portion of the defect, but no bone was present in the 'growth chambers'. Thus, it seems that this material was unable to direct the location of new bone formation.

Conclusions

An established model of bone repair was used to assess and compare the efficacy of currently available FDA-approved alloplastic bone materials in the healing of 4 mm rat calvarial defects after 2 and 4 month healing periods. Biochemical and histomorphometric analyses were performed. The tested alloplastic implant materials did not significantly increase ALP activity assessed at 2 months or the amount of new bone formation in the healing of rat calvarial defects relative to controls ($P > 0.05$). However, when the implant material itself was included in the analysis, significant differences were observed ($P < 0.05$). Additionally there was a strong correlation ($r = 0.8$) between ALP activity at 2 months and new bone growth in the defect assessed at 4 months. The tested materials varied in their ability to bridge the bony defect and their incorporation into the newly forming bone. These factors contributed to the quality of healing in the defect. Some of the differences between the tested materials were subtle as tested in the model of a healing defect, but may become more pronounced in a non-healing defect.

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