S.M.S. Bidic[1], J.W. Calvert[2], K. Marra[3], P. Kumta[3], P. Campbell[4], R. Mitchell[5], W. Wigginton[6], J.O. Hollinger[4,7], L. Weiss[8], and M.P. Mooney[1,5*,9]

[1]Plastic Surgery, University of Pittsburgh; [2]Plastic surgery, University of California-Irvine; [3]Complex Engineered Systems, Carnegie Mellon University, Pittsburgh, PA; [4]Biomedical Health Engineering and Biological Sciences, and Bone Tissue Engineering Center, Carnegie Mellon University, Pittsburgh, PA; [5]Oral Medicine and Pathology, 329 Salk Hall, University of Pittsburgh, Pittsburgh, PA, USA, 15261; [6]Radiology, University of Pittsburgh; [7]Orthopaedic Surgery, University of Pittsburgh; [8]The Robotics Institute, Carnegie Mellon University, Pittsburgh, PA; and [9]Anthropology and Orthodontics, University of Pittsburgh; *corresponding author, mpm4@pitt.edu

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Rabbit Calvarial Wound Healing by Means of Seeded Caprotite® Scaffolds

ABSTRACT

Autologous bone is the most successful bone-grafting material; however, limited supply and donor site morbidity are problematic. Synthetic bone substitutes are effective, but healing is slow and unpredictable. Osseous wound healing may be enhanced if bone substitutes are combined with autologous bone marrow cells. To test this hypothesis, we created 40 calvarial defects in 20 12-week-old New Zealand White rabbits, divided into four groups: (1) unrepaired controls, (2) autologous bone grafts, (3) unseeded Caprotite® (a polymer-ceramic composite) grafts, and (4) Caprotite® grafts seeded with autologous bone marrow stromal cells. CT scans were obtained at 0, 6, and 12 weeks post-operatively, and defects were harvested for histology. Defects repaired with autologous bone had significantly ($p < 0.05$) more bone than the other three groups, although seeded Caprotite® defects showed different wound-healing sequelae. Results suggest that seeded Caprotite® scaffolds did not significantly enhance osseous defect healing compared with controls.

KEY WORDS: Caprotite®, calvaria, rabbits, scaffolds, bone cells.

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INTRODUCTION

Oncologic surgery, trauma, and congenital disorders often leave patients with large bony defects. Autologous bone is the most successful bone-grafting material; however, limited supply and donor site morbidity require the use of bone substitutes (Whang *et al*., 1999; Murphy *et al*., 2000; Service, 2000; Shea *et al*., 2000; Einhorn and Lee, 2001; Hutmacher, 2001; Mackenzie *et al*., 2001). Demineralized bone matrix (Chakkalakal *et al*., 2001), hydroxyapatite granules and scaffolds (Liljensten *et al*., 2000; Ma *et al*., 2001), organic sponges (Goldstein *et al*., 1999), synthetic sponges (Marra *et al*., 1999a,b), porous ceramics (Ignatius *et al*., 2001; Pilliar *et al*., 2001), and collagen discs (Winn *et al*., 1999a) have all been used lately as bone substitutes or vehicles to deliver bone cells or growth factors. Our group has developed a bone substitute called Caprotite® (81% PLGA [poly(D,L-lactic-co glycolic acid)], 9% PCL [poly(caprolactone)], and 10% hydroxyapatite), which represents an improvement on current technology by combining the controlled degradation rates of synthetic polymers with the strength and biocompatibility of hydroxyapatite (Marra *et al*., 1999a,b; Calvert *et al*., 2000).

The bone substitutes described previously have been used either in isolation to repair bone, or as a vehicle to carry signaling molecules, cells, or genetic codes to the healing wound site. Although each therapeutic approach has its costs and benefits, bone substitutes used as delivery vehicles are generally thought to be more effective and to enhance osseous wound healing by increasing osteoblast proliferation, microcirculation, and/or collagen synthesis within the defect (Bonadio *et al*., 1999; de Bruijn *et al*., 1999; Winn *et al*., 1999a,b, 2000; Shea *et al*., 2000; Mayr-Wohlfart *et al*., 2001).

The present study was designed to investigate the efficacy of Caprotite® as a synthetic bone substitute and delivery vehicle to facilitate osseous wound healing. The delivery of autologous bone cells was chosen as a logical first study because of the ease of cell harvesting and previous data on cell attachment, cell proliferation, and scaffold mineralization rates *in vitro* (Marra *et al*., 1999a,b; Calvert *et al*., 2000). Based on the studies cited above, it was hypothesized that Caprotite® scaffolds seeded with autologous bone marrow stromal cells would heal calvarial defects faster than unseeded defects in a rabbit model.

MATERIALS & METHODS

Sample

Forty bilateral defects were made in the parietal bones of 20 12-week-old New Zealand White male rabbits (*Oryctolagus cuniculus*). The rabbits were obtained from Myrtle's Rabbitry (Thompson Station, TN). This study has

been reviewed and approved by the University of Pittsburgh, Institutional Animal Care and Use Committee (IACUC). The defects were randomly divided into 4 groups of 10 each:

- Control Group — unrepaired defects, surgical control group;
- AutoGraft Group — defects repaired with autologous parietal bone grafts, "clinical gold standard" control group;
- Unseeded Caprotite® Group — defects repaired with unseeded Caprotite® disks, scaffold control group; and
- Seeded Caprotite® Group — defects repaired with Caprotite® disks seeded with autologous, osteogenic bone marrow stromal cell, experimental scaffold group.

Caprotite® Manufacturing

The technical details of Caprotite® manufacturing have been published previously (Marra *et al*., 1999a,b). In short, the scaffold materials were poly(caprolactone) (Aldrich [*M*w 65 kDa]), poly(D,L-lactic acid-*co*-glycolic acid) (*M*w 40 kDa-65 kDa [65:35], Aldrich, Chicago, IL), hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$, Aldrich, Chicago, IL), and $CHCl_3$ (Fisher, Indianapolis, IN). Polymer scaffolds were prepared by means of a particulate-leaching technique (Mikos *et al*., 1996). Sieved NaCl (particle size, 150-250 μm) and hydroxyapatite (particle size, ~ 10 μm) were suspended in solution and sonicated for 60 sec. Polymer scaffolds (3-6 mm thick) were cut into 8-mm-diameter discs and pressed to a thickness of 1 mm. The discs were immersed in distilled water to dissolve the NaCl. Porosity was 80% (as controlled by the amount of NaCl incorporated) (Figs. 1A, 1B).

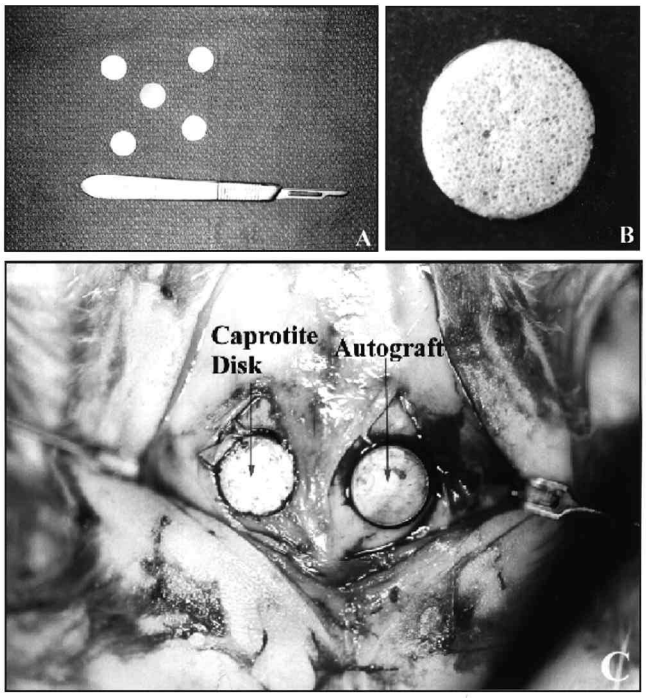

Figure 1. Caprotite® disks, 8 mm in diameter, before seeding and implantation **(A).** Closer view of a disk with 80% porosity and 150-200-mm pores **(B).** Superior, intra-operative view of a rabbit calvaria showing the *in situ* location of autograft and Caprotite® disk **(C).**

Surgery

Bone marrow stromal cell harvesting

All rabbits were anesthetized with an intramuscular (IM) injection (0.59 mL/kg) of a solution of 91% ketamine hydrochloride (Ketaject, 100 mg/mL, Aveco, Fort Dodge, IA, USA) and 9% xylazine (Xylaject, 20 mg/mL, Mobay Corp, Shawnee, KN, USA). The rabbits were placed in the supine position, and both thighs were prepared for surgery. The femoral diaphysis was exposed, and a small defect was created by means of a drill and cutting bur. An 18-gauge angiocath and 10-cc syringe with Dulbecco's Modified Eagle's medium (DMEM) (GIBCO Laboratories, Albany, NY, USA) was used to flush the bone marrow out.

The bone marrow plugs were then mixed with a total volume of 10 mL DMEM in a 15-mL centrifuge tube to create a single cell suspension. The suspension was then centrifuged (800 *g*, 3 min) and the supernatant discarded. A 5-mL quantity of DMEM was then added to create a suspension from the cell pellet. The viability of the cells was > 90% as checked by the tryptan blue dye exclusion method, and the number of nucleated cells was controlled to more than 1 x 10^8/mL. Caprotite® disks were seeded by being individually soaked in this cell suspension under slight negative pressure for 10 min.

Calvarial defect creation

Following bone marrow harvesting, the scalps were prepared for sterile surgery. The calvariae were exposed through a mid-line skin incision. The periosteum was reflected laterally and bilateral, and 8-mm-diameter defects (Hollinger and Kleinschmidt, 1990) were made in the parietal bones by means of a standardized trephine cutting bur and drill (Fig. 1C).

The defects were randomly assigned to the four groups. In the control group, the defects were not repaired, and the skin was simply closed by 5-0 Vicryl suture (Ethicon, Somerville, NJ, USA). In the autograft group, the defects were repaired by immediate transfer of the bone graft from the contralateral side into the defect (Fig. 1C), and the skin incision was closed. In the unseeded Caprotite® group, we repaired the defects by placing an unseeded disk into the defect (Fig. 1C) and closing the skin incision. In the seeded Caprotite® Group, we repaired the defects by placing a disk, seeded with bone marrow stromal cells, into the defect. The skin incision was then closed. All rabbits received IM injections (2.5 mg/kg) of an antibiotic (Baytril, Bayer Corp., Shawnee Mission, KS, USA).

Data Collection

3D computed tomography (CT)

Serial 3D-CT scans were obtained from all rabbits at 0, 6, and 12 wks post-operatively (Fig. 2). Twelve wks was chosen as the end-point of the study because of the cessation of wound healing and bone formation in cranial defects by this time (Hollinger and Kleinschmidt, 1990).

All rabbits were tranquilized with an IM injection (10 mg/kg) of ketamine. The scans were taken in the sagittal plane by means of a GE HiSpeed Advantage Scanner (DFOV = 24.0 - 18.0 cm; mA = 120 - 150; kV = 120) at a thickness of 1 mm. The defects were automatically and manually traced, reconstructed, and the indirect defect volumes were calculated by means of Allegro Software (ISG Technologies, Atlanta, GA, USA) on a Sun Workstation. All measurements were taken by one individual who was blinded as to

group identity. Intra-observer, repeated-measurement reliability was calculated ($r = 0.978$; $p < 0.001$) on a randomly drawn sample (20%) of rabbits.

Histological processing

All rabbits were killed at 12 wks post-operatively with an IV overdose (45 mg/kg) of pentobarbital. The calvarial defects were harvested, fixed in 70% ethyl-alcohol (ETOH), and embedded in polymethylmethacrylate (JB-4 Plus, Polysciences, Inc., Warrington, PA, USA). The specimens were sectioned in the coronal plane (Fig. 2) to a thickness of 150 to 250 μm and ground and polished to a uniform thickness of 40 μm. The specimens were stained with Masson's trichrome for conventional qualitative bright-field light microscopy.

Statistical Analysis

Mean defect areas and standard deviations were calculated and compared among groups by a 4 x 3 (group by time post-op), two-way analysis of variance (ANOVA), with a repeated-measure design. Intergroup differences were assessed by Tukey's multiple-comparison test (SPSSPC v10, Chicago, IL, USA). Mean differences were considered significant if $p < 0.05$.

RESULTS

All rabbits tolerated the surgical procedures well. No significant reductions in body weights were noted, and no post-operative infections were observed.

3D-CT scans revealed that the autografts were immediately incorporated into the donor defect sites, and the defect margins were not visible by 6 wks post-operatively (Fig. 2). The defects in the other three experimental groups showed various amounts of bony ingrowth from the margins. The control defects and the defects repaired with seeded Caprotite® disks showed the most bony ingrowth and irregular margins compared with the defects repaired with unseeded Caprotite® disks by 6 wks post-operatively.

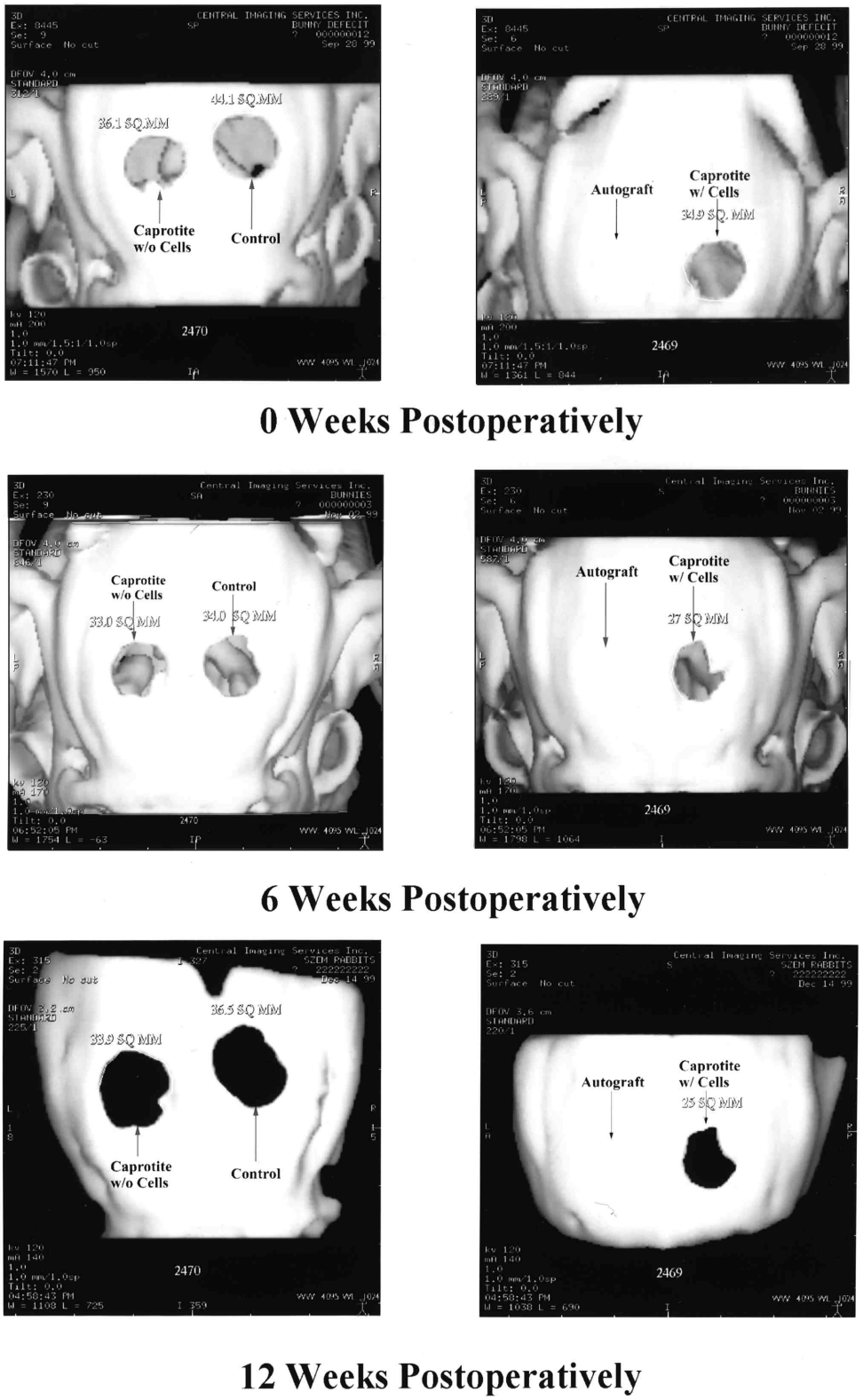

Figure 2. Serial 3D-CT reconstructions of calvarial defects at 0, 6, and 12 wks post-operatively. Note the complete obliteration of the defect filled with the autograft by 6 wks and the limited bony ingrowth along the margins of the defects in the other three groups.

Defects repaired with the autografts showed no change in mean defect area at any post-operative interval (Fig. 3). Control defects

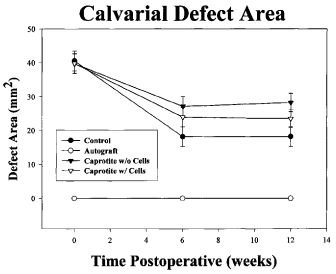

Figure 3. Mean (± SE) defect area by group and post-operative interval (n = 10/group/post-op interval). Note the significantly ($p < 0.05$) reduced defect area in the control group compared with the unseeded Caprotite® disk at 6 and 12 wks post-operatively. No significant ($p > 0.05$) differences were noted between the control group and the seeded Caprotite® group at 6 and 12 wks post-operatively.

had approximately 55% of the defect filled in with bone by 12 wks post-operatively, followed by defects repaired with seeded Caprotite® disks (41%) and defects repaired with unseeded Caprotite®2 disks (29%). Two-way analysis of variance revealed significant Group ($F = 79.07$; $p < 0.001$) and Time (24.23; $p < 0.001$) main effects and a significant Group x Time interaction ($F = 3.44$; $p < 0.01$). Multiple-comparison tests revealed that the autograft group had significantly ($p < 0.05$) smaller mean defect areas than the other three groups at all post-operative time intervals. No significant differences ($p > 0.05$) were noted in mean defect area between the control group and the seeded Caprotite® disk group at any post-operative time interval, while both of these groups had significantly ($p < 0.05$) smaller mean defect areas than the group with unseeded Caprotite® disks.

Histological examination at 12 wks post-operatively showed that defects repaired with autografts had the greatest ossification and graft incorporation compared with the other groups (Fig. 4). Control defects showed extensive fibrous tissue ingrowth and poor ossification, mainly along the margins. In contrast, defects repaired with seeded Caprotite® disks still showed the unresorbed disks with new bone growth extending from the lateral margins as well as from islands in the middle of the disks. Defects repaired with unseeded Caprotite® disks also showed the unresorbed disks but very little new bone regeneration. There were no signs of inflammatory reactions or mast or giant cells in the soft tissue surrounding the seeded or unseeded Caprotite® disks.

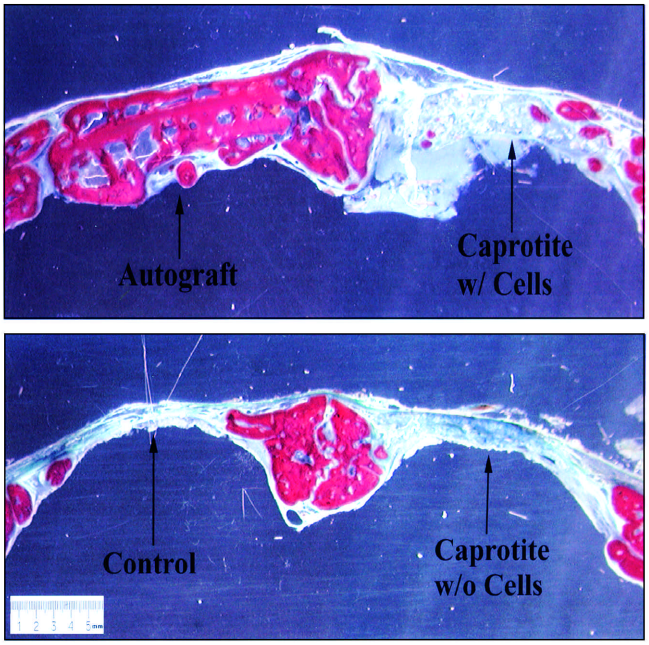

Figure 4. Coronal histological sections through the calvaria showing defects from the four groups. Note the complete bony incorporation of the autograft into the surrounding host site, the bony ingrowth from the margins in the control defect, the bony islands in the seeded Caprotite® disk, and the relative lack of ossification in the unseeded Caprotite® disk. Specimens stained with Masson's trichrome; original magnification, 1.25x.

DISCUSSION

Repair of large bony defects requires the surgical transfer of bone from donor to wound site, which is considered to be the clinical "gold standard". However, such procedures can increase patient morbidity, and donor sources are finite. Ideal bone substitutes should be strong, malleable, osteoconductive, osteoinductive, resorbable, inexpensive, and easy to use intra-operatively, while promoting cell adhesion, proliferation, and differentiation (Whang *et al.*, 1999; Calvert *et al.*, 2000; Hutmacher, 2001). This substitute should also be an effective carrier that predictably releases cues and cells, protects the delivered substances, facilitates tissue ingrowth, and establishes an environment that supports bone regeneration (Einhorn and Lee, 2001; Hutmacher, 2001).

Caprotite®, in theory, has many of the properties of an ideal bone substitute. It can be manufactured to any shape with a pore size of 150-250 microns and a porosity of about 80%. Caprotite® has a relatively slow degradation rate (36% weight loss after 8 wks) and a tensile strength and Young's modulus about half of those of trabecular bone (Marra *et al.*, 1999a,b). Caprotite® disks seeded with cultured osteoblasts or fresh bone marrow showed cellular activity and collagen formation on the surface and at 0.1 to 0.5 mm deep into the scaffold. Positive Von Kossa and alkaline phosphatase staining, as well as scaffold mineralization, were also noted after 8 wks in culture, which suggests that Caprotite® can support and maintain seeded osteoblasts and has adequate pore size and porosity of the extracellular matrix, necessary for cellular respiration and nutrient transport (Calvert *et al.*, 2000).

These preliminary findings led us to utilize seeded Caprotite® disks to repair calvarial defects in the present study. Results revealed that defects repaired with seeded Caprotite® disks did not show significantly different bone area regeneration compared with unrepaired control defects and unseeded Caprotite® disks. However, defects repaired with seeded disks showed small bony islands throughout the scaffold and no fibrous non-unions, while unrepaired defects and defects repaired with unseeded disks showed bony ingrowth only from the defect margins and fibrous non-union in the center. These findings suggest that scaffold seeding promoted osteogenesis in the defects, but at a very slow rate.

Several factors related to manufacturing and study design may be responsible for these results:

(1) There may have been negative effects of increased salinity from residual NaCl sieving on the bone cells or surrounding wound site. However, examination of disks revealed no residual NaCl, while seeded disks promoted substantial cell growth in culture (Marra *et al.*, 1999a,b).

(2) The design of the study in which different experimental conditions were used in the same rabbit may have influenced the results through molecular "cross-talk" between conditions.

(3) The degradation time of these constructs may be too slow or may have produced inflammatory reactions. Although the constructs were still visible by 12 wks post-operatively, there were no signs of inflammation, giant or mast cells, or extensive bone resorption in any defect.

(4) The concentration of autologous bone marrow stromal cells in the seeded disks may have been low. While the number of nucleated cells in the cell suspension was controlled to more than 1 x 10^8/mL, the actual number of cells adhering to the disks following soaking may have been less. Preliminary studies have showed that seeded Caprotite® disks, implanted into the rabbit rectus abdominis muscle, showed viable cells and enhanced mineralization compared with unseeded implants (Marra *et al*., 1999a,b; Calvert *et al*., 2000), and thus may not be a factor. However, the source and number of these cells still need to be verified (*i.e.*, whether from the initial cell suspension, adjacent bone and connective tissue, or neovascularization) and further characterized before this confounding factor can be ruled out.

(5) The last factor may be related to the porosity and pore size of the disks, which may have been too small to promote or maintain cellular ingrowth and viability. However, preliminary *in vitro* data showed viable osteoblasts both on the surface of the construct and deep to the cortical surface 8 wks later (Marra *et al*., 1999a,b; Calvert *et al*., 2000). The poor wound healing was probably a result of an interaction of several factors described above which needs to be examined further.

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