INFLUENCE OF MATRIX-SUSPENDED DEMINERALIZED BONE ON OSSEOUS REPAIR USING A CRITICAL-SIZED DEFECT IN THE RAT (RATTUS NORVEGICUS) CALVARIUM

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Demineralized freeze-dried bone (DFDB) in matrix form must be rehydrated with a carrier medium which allows for easy manipulation during periodontal surgery. The purpose of this study was to evaluate how human DFDB suspended in a polyol matrix affects new bone formation in the rat calvarium critical-sized defect (CSD) model. Fifty-five adult male Harlan Sprague-Dawley rats were assigned to 1 of 5 treatment groups: polyol, 100% DFDB, 47% DFDB/polyol, 47% DFDB, or an unfilled control. They were then placed into 8-m calvarial CSDs. The bone donor source company for the DFDB and DFDB/polyol groups was the same. Calvaria were harvested 10 weeks after surgery and evaluated histomorphometrically. The diameter of bone particles from the 3 groups containing DFDB was measured by scanning electron microscopy. There was no statistically significant difference in the percentage of bone fill between any of the groups, although the 100% DFDB group exhibited the most bone fill. The 47% DFDB/polyol and 47% DFDB groups had similar amounts of bone formation. The average size of the demineralized bone particles from the 100% DFDB group was significantly smaller than that of the other 2 groups containing DFDB. Adding a polyol to DFDB produced similar osseous regeneration in the rat calvarium defect model vs DFDB alone. Yet from a clinical standpoint, the polyol enhanced graft handling and stability. Graft particle size may have an effect on bone fill.

Key Words: grafts, demineralized, freeze-dried, bone, polyols

INTRODUCTION

Clinical manifestations of attachment loss and coincident alveolar bone loss associated with periodontitis necessitate therapeutic intervention by a dental care provider. Many patients present with intrabony defects that could benefit from bone grafting. The exceptional regenerative capabilities of bone make it one of the most transplanted of human tissues. Although autogenous bone is considered the ideal regenerative material for these defects, patients may not tolerate an operating room procedure to harvest large amounts of bone from the iliac crest or ribs. Additionally, many patients may not have enough intraoral sites to harvest an
adequate amount of bone. The complications and very nature of harvesting autografts gave rise to development of allografts, xenografts, and alloplasts. Until an ideal bone-grafting material is developed, the regeneration of bone will remain a major focus of periodontal research.

Most grafts used to fill osseous defects come from human cadavers. However, animal- and synthetic-derived grafts are increasing in use. All of these treatment modalities can be performed without creating a second surgical site. Demineralized freeze-dried bone (DFDB) allografts have been used in dentistry for more than decades and are the most widely used allografts in treating intrabony defects.\(^1\)–\(^4\)

This allograft is prepared by demineralizing bone in hydrochloric acid to expose bone morphogenic proteins (BMPs). The demineralized bone matrix (DBM) is then freeze-dried to provide an indefinite shelf life and to decrease allograft antigenicity.\(^5\)–\(^6\)

Demineralized freeze-dried bone allografts are readily available, safe, osteoinductive, and osteoconductive.\(^7\)–\(^9\)

Ultimately, the defect size, amount of donor bone available, patient preference, and clinician experience may all influence which materials are used to treat the defect.

The ideal regenerative material would have excellent handling properties and would produce a significant amount of new bone faster than other grafting materials. Although it is most commonly hydrated with sterile water to improve handling, DFDB can also be combined with other suitable carriers to form a gel, putty, or sheet configuration.\(^10\)–\(^12\)

Poloxamer 407, a polyol, has been shown to be an effective carrier for the BMPs.\(^13\)

This poloxamer is classified as a triblock copolymer that consists of 3 layers. The outer layers are made of polyethylene, and the inner layer is polypropylene. Poloxamer 407 is a reverse-phase copolymer and reacts to temperature changes unlike conventional copolymers. At increased temperatures (eg, body temperature), the viscosity increases and the texture of the medium becomes thicker and more stable. The medium is 70% resorbed within 30 hours and excreted unchanged through the kidneys.

As research in osseous grafting continues, there needs to be a reliable animal model that can be used to evaluate graft materials before human use. The critical-sized defect (CSD) in the rat calvarium has been recommended before subjecting higher phylogenetic species to testing.\(^14\)

The CSD model, using the cranium, is relevant in periodontal research because its embryonic origin is similar to that of the mandible. In dentistry, synthetic membranes are used to inhibit epithelium and connective tissue from proliferating into osseous defects and/or to maintain grafting materials in place. When combined with the CSD model, these membranes allow the slower growing mesenchymal (bone) cells time to repopulate a defect while preventing the faster growing epithelial and connective tissue cells from propagating.\(^15\)

The aim of this study was to evaluate the osseous repair potential of a matrix-suspended human demineralized bone graft in the rat calvarium model with and without a poloy carrier.

**Materials and Methods**

Fifty-five adult male Harlan Sprague-Dawley rats (Rattus norvegicus), approximately 92 to 105 days old and weighing between 325 and 375 g, were randomly assigned to 1 of 5 experimental groups of 11 rats each based on a blind drawing. The rats were housed on a 12-hour light and dark cycle. Food (Rodent Blocks, Harlan Teklad, Indianapolis, Ind) and water were provided ad libitum. Bedding was changed at least twice weekly. The Institutional Animal Care and Use Committee, Dwight David Eisenhower Army Medical Center, Fort Gordon, Georgia, approved and provided oversight for this research protocol (DDEAMC 01-17A).

**Preparation of materials**

The 47% DFDB/polyol gel formulation (DynaGraft DBM, GenSci OrthoBiologics, Irvine, Calif) was supplied in a 10-mL syringe. The material was expelled into a preweighted sterile container and the weight recorded. The material was placed into sterile 3-mL centrifuge tubes and spun at 9500g for 10 minutes at 2°C in a centrifuge (Model J-6M using a TY JS-4.2 swinging bucket rotor, Beckman Coulter, Palo Alto, Calif). The supernatant representing the poloxamer 407 was removed and stored refrigerated in capped sterile vials until use. The pellet, representing the DFDB, was washed 10 times by resuspending the pellet in 10 mL of sterile water, mixing by vortex, and recentrifuging. After each centrifugation, the DFDB was dried to a constant weight to ensure complete removal of the poloxamer 407. The DFDB was stored at room temperature until use. The demineralized bone was hydrated with saline before use.

**Surgical procedures**

All surgeries were performed with aseptic techniques. After a 7-day quarantine, the rats were anesthetized intraperitoneally with a combination of 150 mg of ketamine HCl (65 mg/kg, Bristol Labs, Syracuse, NY) and 30 mg of xylazine HCl (7 mg/kg, Miles Labs, Indianapolis, Ind). The 47% DFDB/polyol gel formulation (DynaGraft DBM, GenSci OrthoBiologics, Irvine, Calif) was supplied in a 10-mL syringe. The material was expelled into a preweighted sterile container and the weight recorded. The material was placed into sterile 3-mL centrifuge tubes and spun at 9500g for 10 minutes at 2°C in a centrifuge (Model J-6M using a TY JS-4.2 swinging bucket rotor, Beckman Coulter, Palo Alto, Calif). The supernatant representing the poloxamer 407 was removed and stored refrigerated in capped sterile vials until use. The pellet, representing the DFDB, was washed 10 times by resuspending the pellet in 10 mL of sterile water, mixing by vortex, and recentrifuging. After each centrifugation, the DFDB was dried to a constant weight to ensure complete removal of the poloxamer 407. The DFDB was stored at room temperature until use. The demineralized bone was hydrated with saline before use.

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Shawnee, Kan) and sustained with 0.25% to 0.50% isoflurane with 1 L/min oxygen as needed for appropriate anesthesia. The anesthetized rat had sterile eye lubricant ointment placed in each eye to protect it during shaving and the surgical procedure. The rats were shaved and prepared for surgery in a sterile animal operatory.

The rats were placed in a rodent stereotaxic device (Stoelting Company, Wood Dale, Ill) and draped. Anesthesia was maintained with isoflurane while using a scavenger. An anesthesia nose cone was fitted to the stereotaxic device and attached to the rat. Volatile anesthesia was administered and adjusted to maintain an appropriate anesthetic plane. A 3-cm midline incision was made through the skin along the sagittal suture of the skull. The soft tissue and periosteum were elevated and reflected. Under constant saline irrigation, an 8-mm craniotomy was created with a dental handpiece at slow speed using a sterilized 8-mm diamond grit trephine bur (Continental Diamond Tool Corporation, New Haven, Ind) and copious saline irrigation. Care was taken to leave the dura intact. The edges of the bony defect were smoothed as needed using a No. 2 round bur with an interrupted 4–0 polyglactin 910 suture (Vicryl, Ethicon, Johnson & Johnson Co, Somerville, NJ), and the periosteum was sutured over the membrane. A sterile, precut 9-mm polytetrafluoroethylene (PTFE) membrane (Millipore Corporation, Bedford, Mass) with a pore size of 0.5 μm was placed into the CSD to prevent contact between the dura and the graft material. This membrane was slightly larger than the defect to isolate the material and ensure that all bony margins were protected. Defects were filled with the appropriate material and a second sterile, precut 9-mm PTFE membrane was placed over the defect.

The periosteum was sutured over the membrane with an interrupted 4–0 polyglactin 910 suture (Vicryl, Ethicon, Johnson & Johnson Co, Somerville, NJ), and the overlying epidermis was closed with multiple, horizontal mattress and interrupted 4–0 polyglactin 910 suture. After the surgical procedures, the rats were observed for signs of pain or distress by the attending veterinarian or veterinary staff. Buprenorphine (Reckitt & Colman Products Ltd, Richmond, Va) (0.5mg/kg) was administered subcutaneously to each rat immediately after the surgery and for 3 days after surgery by the Laboratory Animal Support Staff (LASS). The LASS continued to monitor the animals and administer additional analgesics as needed. All rats were killed by carbon dioxide asphyxiation at 10 weeks after surgery. The cranium, containing the defect, was harvested and cleaned of soft tissue. Specimens were fixed in 10% formalin solution (Fisher Chemicals, Fair Lawn, NJ) until ready for evaluation.

Histology

Histomorphometric analysis was performed to quantify bone fill present in the defects. The calvarial specimens fixed in formalin were sectioned sagitally to yield 2 samples of approximately equal size. The samples were slowly demineralized (Cal-Ex Decalcifying Solution, Fischer Chemicals) for 10 days. Half of the demineralized samples were then placed in cassettes, embedded in paraffin wax, sectioned at 3-μm thickness on a 2030 microtome (Biocut, Leica, Reichert-Jung, Nussloch, Germany), and stained with hematoxylin and eosin. Data obtained included the bone fill between the membranes and the total area between the membranes. Histologic slides were scanned using an imaging system software program (PCI Imaging Systems, Compix, Cranberry Township, Pa), and a montage of each specimen (Figure 1) was created using a camera (Photometrics CoolSnapFx, Roper Scientific, Tucson, Ariz) attached to an inverted microscope (Nikon Diaphot 300, Southern Micro Instruments, Atlanta, Ga) at 4 power. Regions of interest (ROIs) were outlined and calculated in square millimeters.

Ten demineralized bone particles per group were measured from the 100% DFDB group, 47% DFDB group, and 47% DFDB/polyol group using a scanning electron microscope (JEOL, Model JSM-6400V, Peabody, Mass) (Figure 2). The size of each bone particle was measured at the largest portion of the bone particle.

Statistical methods

The histomorphometric and scanning electron microscopic analysis distribution of data was determined using the Kruskal-Wallis 1-way analysis of variance by ranks for normality of the mean. If a significant difference between groups was observed, pairwise multiple comparison procedures (Duncan’s method) test was used to compare all pairs of treatments. Statistical significance was defined as $P \leq .05$.

Results

Thirty-five calvarium samples were analyzed by histomorphometry. Only bone within the boundaries of the PTFE membranes and within the defect was included in these calculations. Both experimental and control groups had evidence of new bone formation within the ROI located adjacent to the periosteum and dura side membranes. With the exception of the 100% DFDB group, the PTFE membranes collapsed into the defect, thus not maintaining the space needed to
maximize new bone formation. The percentage of bone fill compared with the area between the membranes was not significantly different between any of the groups (see Table 1). This can be attributed to the varying degrees of collapse of the membrane into the defect site. The more the membranes collapsed, the less amount of bone would be needed to fill the area. The control group and polyol group had similar amounts of membrane collapse and subsequent bone formation as neither defect had anything maintaining space (Figure 1a and b). The 47% DFDB group and the 47% DFDB/polyol group had similar amounts of bone formation as each was filled with equal amounts of space maintaining bone graft material (Figure 1c and d). The 100% DFDB group had the most bone formation and had the most space.
maintained due to the defect being completely filled with bone graft material (Figure 1e).

Thirty calvarium samples were also analyzed by scanning electron microscopy (SEM) (Figure 2). The 3 bone-fill materials demonstrated that the average size of the demineralized bone particles from the 100% DFDB group was 755 μm, which was significantly (P ≤ .05) smaller than the average size of the extracted bone from the 47% DFDB group (1066 μm) or the 47%

DISCUSSION

Currently, DFDB is one of the most common graft materials being used clinically. To improve its handling characteristics, DFDB can be hydrated with sterile water to a pastelike consistency.10–12 Suitable biodegradable carriers, including pluronic polyols, which promote stability during wound healing, could also be used to improve the handling characteristics of graft materials. Pluronic polyols, composed of the condensation of polymeric oxypropylene and oxyethylene, are a family of nonionic surfactants.17 Poloxamer 407 is known by the trade name Pluronic F-127 (BASF Corporation, Mount Oliver, NJ).18 Pluronic polyols such as F-68 and F-127, have been shown to significantly increase the attachment and early growth rate of human gingival fibroblasts on plastic and dentin surfaces in vitro.19 Even though the exact mechanism of action is unknown, polyols may have useful early wound healing properties that facilitate the growth, attachment, and spread of human gingival fibroblasts.19 The DFDB/polyl group's weight composition is 23% demineralized bone powder (DFDB) and 77% poloxamer 407 and water. This group's volume is 47% demineralized bone powder and 53% poloxamer 407 and water. Poloxamer 407 is flowable at refrigerated

TABLE 1
Histomorphometric analysis*

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>% Fill ± SEM</th>
<th>P ≤ .05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>53.79 ± 10.54</td>
<td>No</td>
</tr>
<tr>
<td>Polyl</td>
<td>8</td>
<td>49.18 ± 17.05</td>
<td>No</td>
</tr>
<tr>
<td>47% DFDB</td>
<td>6</td>
<td>42.62 ± 8.73</td>
<td>No</td>
</tr>
<tr>
<td>47% DFDB/polyl</td>
<td>7</td>
<td>42.1 ± 13.7</td>
<td>No</td>
</tr>
<tr>
<td>100% DFDB</td>
<td>7</td>
<td>59.29 ± 9.8</td>
<td>No</td>
</tr>
</tbody>
</table>

*% Fill refers to the mean of the total amount of bone contained within the membranes. Complete fill would be 100%. DFDB indicates demineralized freeze-dried bone.

TABLE 2
Analysis of bone particle diameter*

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Diameter (μm) ± SEM</th>
<th>P ≤ .05</th>
</tr>
</thead>
<tbody>
<tr>
<td>47% DFDB</td>
<td>10</td>
<td>1065.93 ± 193.1</td>
<td>No</td>
</tr>
<tr>
<td>47% DFDB/polyl</td>
<td>10</td>
<td>1154.09 ± 176.81</td>
<td>No</td>
</tr>
<tr>
<td>100% DFDB</td>
<td>10</td>
<td>754.9 ± 206.16</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Bone particle diameter refers to the mean (μm) of the largest diameter on each DFDB particle. DFDB indicates demineralized freeze-dried bone.
temperatures but becomes increasingly solidified at elevated temperatures, including room and body temperature. This unusual reverse-phase property is believed to be due, at least in part, to the composition of the hydrophilic (oxyethylene) and hydrophobic (oxypropylene) subunits. Once the DFDB/polyol becomes solidified, the DFDB should theoretically not flow away from the surgical site, which facilitates structural support of the defect. Poloxamer 407 is designed to be resorbed in about 3 days, leaving the DFDB at the defect site. Once the poloxamer 407 disperses, enhanced ingrowth of vascular tissue at the DFDB implant site may occur.

One purpose of this study was to evaluate whether the 47% DFDB/polyol group affects new bone formation using the rat calvarium CSD model in vivo. The graft material in this case is a xenograft because it contains human demineralized bone. This study also used 2 PTFE membranes, not approved for human use, to allow the slower-migrating mesenchymal cells, only from the surrounding bone and bone marrow, to repopulate the defect. This type of PTFE membrane was used for the following reasons: (1) graft containment, (2) biocompatibility, (3) hemostasis, (4) isolating the graft from the dura and periosteum, (5) rigidity to protect the defects, (6) nonresorbability, and (7) low cost. Some of the groups in this study may have benefited from the collapsed membranes, as is evident from the new bone that was produced from the perimeter of the CSD without the influence of the dura or periosteum. Fowler et al did not use membranes in their CSD experiment and found more bone fill (39.75%) than in CSD studies using membranes. The present study supports the concept that osteoprogenitor cells are present along the periosteum and dura. Chesmel et al, who used 8-mm CSDs in athymic rats comparing human DFDB with glycerol to rat autogenous DFDB and glycerol, found decreased amounts of new bone formation with the human DFDB. Their study, which did not use membranes, found the presence of new bone centrally rather than at the perimeter. The PTFE membranes could have attributed to the peripheral bone growth observed in our study vs their central bone growth in the CSD. This study confirmed the findings of Fowler et al that demineralized bone alone resulted in better defect resolution than demineralized bone plus pluronic F-127, nongrafted, or pluronic F-127 alone sites.

There were significant differences in the DFDB particle sizes between the groups (see Table 2). The SEM (magnification ×20) analysis showed the 100% DFDB group’s average particle size was 754.9 μm (P ≤ .05) while the average particle size of the 47% DFDB/polyol gel formulation group and the 47% DFDB group was greater than 1000 μm. Zhang et al found DFDB particle sizes ranging from 550 to 710 μm to be optimally osteoinductive. The bone donor source company supplied DFDB with an expected particle size ranging from 125 to 800 μm for use in the DFDB/polyol gel formulation. However, our SEM analysis of the particles found them to be larger than 1000 μm. This increase in size could be attributed to hydration of the particles or larger bone particles were used in this particular 47% DFDB/polyol gel formulation. The bone particle size may have had an effect on bone fill. Shapoff et al found that freeze-dried bone allograft particles ranging from 100 to 300 μm enhanced osteogenesis in orthotopic sites in rhesus monkeys compared with larger particles ranging from 1000 to 2000 μm. However, Fucini et al found no statistical significant difference in bone fill between defects grafted with DFDB ranging from 250 to 500 μm or 850 to 1000 μm.

**Conclusions**

While handling characteristics were dramatically improved, the 47% DFDB/polyol group did not enhance the bone-filling characteristics of the DFDB. This could be attributable to the low amount of DFDB (23% by weight) in the gel configuration. As expected, the 47% DFDB/polyol group performed comparable to the 47% DFDB group. The 100% DFDB group showed more bone fill than the other groups. The space maintenance property of a graft material may play a vital role in facilitating the ingress of new bone formation. Future studies are needed to investigate ways to prevent the collapse of membranes (space-maintaining membranes) into the CSD and additional space maintaining materials.

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**Disclaimer**

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REFERENCES


