

Uncultured Marrow Mononuclear Cells Delivered Within Fibrin Glue Hydrogels to Porous Scaffolds Enhance Bone Regeneration Within Critical-Sized Rat Cranial Defects

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For bone tissue engineering, the benefits of incorporating mesenchymal stem cells (MSCs) into porous scaffolds are well established. There is, however, little consensus on the effects of or need for MSC handling *ex vivo*. Culture and expansion of MSCs adds length and cost, and likely increases risk associated with treatment. We evaluated the effect of using uncultured bone marrow mononuclear cells (bmMNCs) encapsulated within fibrin glue hydrogels and seeded into porous scaffolds to regenerate bone over 12 weeks in an 8-mm-diameter, critical-sized rat cranial defect. A full factorial experimental design was used to evaluate bone formation within model poly(L-lactic acid) and coralline hydroxyapatite scaffolds with or without platelet-rich plasma (PRP) and bmMNCs. Mechanical push-out testing, microcomputed tomographical analyses, and histology were performed. PRP showed no benefit for bone formation. Cell-laden poly(L-lactic acid) scaffolds without PRP required significantly greater force to displace from surrounding tissues than control (cell-free) scaffolds, but no differences were observed during push-out testing of coral scaffolds. For bone volume formation as analyzed by microcomputed tomography, significant positive overall effects were observed with bmMNC incorporation. These data suggest that bmMNCs may provide therapeutic advantages in bone tissue engineering applications without the need for culture, expansion, and purification.

Introduction

TISSUE ENGINEERING APPROACHES to regenerating lost or damaged tissues often focus on the use of adult stem cells. Among many available sources, mesenchymal stem cells (MSCs) derived from bone marrow or adipose tissue are commonly used as multipotent autologous cell sources and can be collected through relatively noninvasive methods.¹ A number of studies have demonstrated the advantages of using MSCs for the repair or regeneration of multiple tissue types,^{2–9} and multiple clinical trials are underway investigating the use of MSCs for the treatment of both acute and chronic conditions.¹⁰

Despite many studies establishing the benefits of MSCs, a number of factors remain unaddressed regarding their widespread use. First, there is little to no standardization of MSC harvest, expansion, and characterization,^{11–14} and studies suggest that these factors may play a significant role

in the success or failure of applications employing these cells.^{15–21}

In addition to raising questions about the standardization and optimization of culture techniques, *ex vivo* expansion of autologous MSCs requires a delay between harvest and treatment, a likely increased cost of treatment to the patient, and introduces the risk for contamination or adverse cellular changes related to the expansion.^{22–24} Limited long-term data are available to adequately understand the risks of using culture-expanded MSC in clinical therapy, and, although early reports are promising,²⁵ a number of concerns, including those of genomic instability and resultant tumorigenesis, remain.^{26–30} Additionally, it is unclear how patient-to-patient variability and changes in cellular function related to age or other demographic factors may impact the use and success of such cell-based therapies.^{31–37}

One potential method to avoid many of the problems associated with harvest and subsequent culture of MSCs is to

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use uncultured cell sources that may contain MSCs. Such an approach greatly diminishes the number and purity of the available multipotent cell population and, in certain cases of cardiovascular repair and regeneration, has been shown to limit the efficacy of cell-based therapies.^{38,39} In contrast, Samdani *et al.* directly compared bone marrow mononuclear cells (bmMNCs) with culture-expanded MSCs in a spinal cord injury model and found that bmMNCs offered a greater protective benefit and reduced scar formation relative to MSC-treated injuries.⁴⁰

Previous studies in bone and cartilage tissue engineering have shown significant therapeutic benefits associated with the use of uncultured whole bone marrow or bmMNCs.^{41–44} The ability to perform the necessary cell harvest and any purification steps perioperatively and the relative ease of clinical translation for these one-step processes were cited as advantages to more commonly investigated techniques involving culture-expanded MSCs.^{40,45} Gan *et al.* found that marrow MNCs enriched perioperatively using a cell processor performed as well as bone grafts in spinal fusion.⁴⁵ Muschler *et al.* found similar results for spinal fusion using a matrix enrichment procedure to increase bmMNC engraftment.⁴⁶ Further supplementing uncultured whole marrow with growth factors such as transforming growth factor- β 1 has also been shown to have a beneficial effect on healing of critical-sized bone defects.⁴⁷

Due to the decreased number of multipotent cells available without *in vitro* expansion and purification and the decreased amount of time available for seeding cell-scaffold constructs during perioperative construct fabrication, cell delivery vehicles have been used to ensure MSC localization to a defect site or within a scaffold. The presence and properties of these delivery vehicles, which are primarily hydrogels such as fibrin glue,^{48–50} alginate,⁵¹ collagen,⁵² hydroxypropylmethylcellulose,⁵³ and platelet-rich gels,⁵⁴ can greatly influence cell survival and differentiation. Catelas *et al.* and Ho *et al.* previously demonstrated the balance between MSC proliferation and differentiation achieved by modulating the fibrinogen concentration within fibrin gels.^{55,56} Similar effects have also been seen in collagen gels.⁵⁷

In the present study, we hypothesized that bone formation within a critical-sized defect would be enhanced using uncultured bmMNCs encapsulated in fibrin gels for delivery of the cells within model scaffolds made of poly(L-lactic acid) (PLLA) or coralline hydroxyapatite (HA). Scaffold properties were also expected to play a significant role on bone formation. Platelet-rich plasma (PRP) was included for potential growth factor delivery to the encapsulated cells and hypothesized to further enhance bone formation. All materials used were U.S. Food and Drug Administration regulated, and an attempt to minimize processing procedures was made to simulate an entire harvest and construct fabrication protocol that could be reasonably completed over the course of a single operation.

Materials and Methods

Experimental design

This study used a full factorial design with three factors tested at two levels each (scaffold material: coral or PLLA; PRP: presence or absence; uncultured marrow MNCs: pres-

ence or absence), resulting in a total of eight experimental groups. The groups are described in Table 1. For each group, a total sample size of 14 was used, with 6 samples per group randomly assigned for mechanical (push-out) testing and the remaining 8 used for microcomputed tomography (μ CT) and histology.

Materials

Coralline HA scaffolds (Pro Osteon 200, Interpore Cross International) measuring 8 mm in diameter \times 2 mm in height and sterilized by gamma irradiation were used as supplied by the manufacturer. Nonwoven PLLA fiber mesh scaffolds (Concordia Medical) measuring 8 mm in diameter \times 1 mm in height with a volumetric porosity of 90%, 300–500 μ m pore sizes, and a 40 μ m fiber diameter were sterilized using ethylene oxide and placed in a sterile biosafety cabinet to ventilate for a minimum of 24 h before use. Scanning electron micrographs of the surface of the coral and PLLA scaffolds are shown in Figure 1. Fibrin glue (TisseelTM, Baxter Healthcare Corp.) consisting of a human fibrinogen complex (or sealer protein) and human thrombin was used as described below. The fibrinogen complex possibly contains osteogenic growth factors such as transforming growth factor β and basic fibroblast growth factor.⁵⁵

Animal use

All animal work was performed in accordance with protocols approved by the Rice University Institutional Animal Care and Use Committee. A total of 134 healthy, male syngeneic Fisher 344 rats (Harlon Bioproducts) aged 12 weeks old and weighing 225–249 g were used in this study. Twenty-two rats were used as bone marrow and/or blood donors, and the remaining 112 rats were used in surgery.

PRP harvest and processing

PRP was freshly harvested the day of surgery from donor rats. Rats were euthanized under general anesthesia via CO₂ asphyxiation; upon confirmation of death, a cardiac puncture was performed to withdraw \sim 5 mL of blood into a sterile syringe containing 500 μ L sterile filtered 3.8% sodium citrate (Sigma-Aldrich). Blood from at least two donors was pooled into sterile 15 mL centrifuge tubes (BD Biosciences) and placed on ice. PRP was isolated using a standard laboratory centrifuge (Precision Durafuge 100; Thermo Fisher Scientific) according to a previously established method.⁵⁸ Briefly, after

TABLE 1. EXPERIMENTAL GROUPS TESTED

Designation	Scaffold material	PRP	bmMNC
P-	PLLA	-	-
PP-	PLLA	+	-
P-M	PLLA	-	+
PPM	PLLA	+	+
C-	Coral	-	-
CP-	Coral	+	-
C-M	Coral	-	+
CPM	Coral	+	+

PRP, platelet-rich plasma; bmMNC, bone marrow mononuclear cell; PLLA, poly(L-lactic acid).

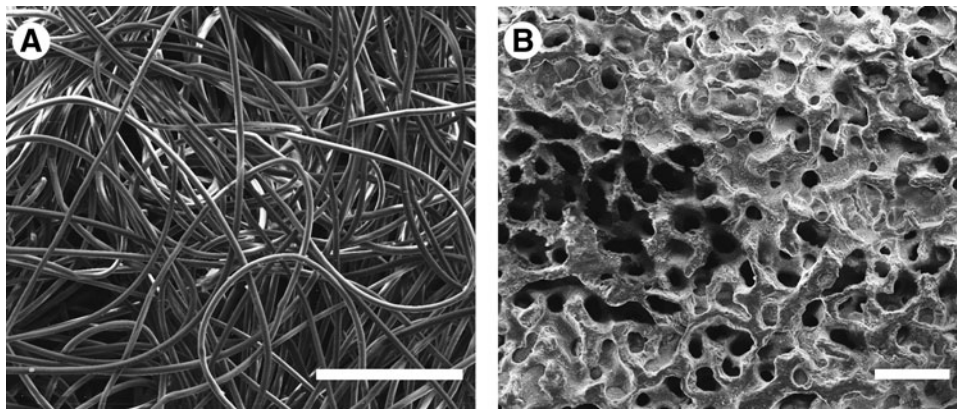


FIG. 1. Scanning electron micrographs of the PLLA (A) and coral (B) scaffolds. Scale bars represent 500 μm . PLLA, poly(L-lactic acid).

centrifugation at 400 g for 10 min with no brake applied, the supernatant was sterilely pipetted to a second sterile centrifuge tube, which was then centrifuged at 800 g for 10 min with a brake applied at the end of the cycle. The supernatant of platelet-poor plasma was then removed, and the remaining PRP was returned to ice. Platelet enrichment between peripheral blood and PRP from eight donor rats was confirmed during a preliminary study using a veterinary hematology analyzer (scil Vet abc, scil animal care company). Before use, PRP fibrinogen content was quantified using a spectrophotometric method.⁵⁹

Bone marrow harvest and construct preparation

On the day of surgery, bone marrow was harvested from the long bones of the hind limbs of euthanized donor rats. Under sterile conditions, the femora and tibiae were isolated, stripped of soft tissue, and placed into sterile minimum essential medium alpha (α -MEM, HyClone Laboratories Inc.). The epiphyses were then removed. Bone marrow was flushed by piercing the diaphyses with a 16-gauge syringe and delivering heparinized phosphate-buffered saline (HyClone Laboratories Inc.). Marrow was disaggregated by serially passing through 18 followed by 20-gauge syringes. A cell suspension was created by vacuum filtering the marrow through a 100- μm nylon filter (Millipore Corporation). MNCs were then isolated by Ficoll density gradient centrifugation (Accuspin™ System Histopaque®-1077, $\rho = 1.077 \text{ g/mL}$; Sigma-Aldrich) performed according to the manufacturer's instructions. Cells were washed three times and resuspended in sterile Tris-buffered saline (TBS; Sigma-Aldrich) or PRP to a final concentration of 6×10^7 cells/mL.

PLLA and coral scaffolds were prewet in gradient ethanol beginning 24 h before seeding and/or implantation. Both the fibrinogen complex and thrombin components of the fibrin glue were freshly prepared each morning according to the manufacturer's instructions, and the fibrinogen concentration was verified using the Ellis method.⁵⁹ The fibrinogen concentration was diluted with TBS to 102 mg/mL, and the thrombin solution was diluted with 30 mM sterile CaCl_2 in TBS to a concentration of 200 IU/mL.

For all experimental groups, 50 μL each of fibrinogen complex and thrombin were delivered to the scaffold, resulting in 100 μL of fibrin glue with final concentrations of 17 mg fibrinogen/mL and 100 IU thrombin/mL. For scaffolds loaded with bmMNCs without PRP, volumes of cells suspended in TBS and fibrinogen complex were added in a

2:1 ratio to a sterile centrifuge tube and vortexed. For scaffolds loaded with PRP, the bmMNC/PRP suspension or PRP alone was added to an equal volume of fibrinogen complex diluted with TBS and vortexed such that the final fibrinogen concentration within the PRP/fibrinogen complex solution was 34 mg/mL. For all experimental groups, after vortexing, 50 μL of the fibrinogen complex solution was then pipetted onto each prewet scaffold within separate wells of an ultra-low attachment 24-well plate (Corning Inc.) that was then placed within a 37°C incubator for 5 min to allow the fibrinogen complex to soak the scaffold. After this, 50 μL of thrombin solution was pipetted onto the scaffolds, which were then returned to the incubator for 15 min. After 15 min, a thin layer of α -MEM was added to the well to cover the scaffold. Scaffolds were returned to the incubator until implantation.

Animal surgery, euthanasia, and implant harvest

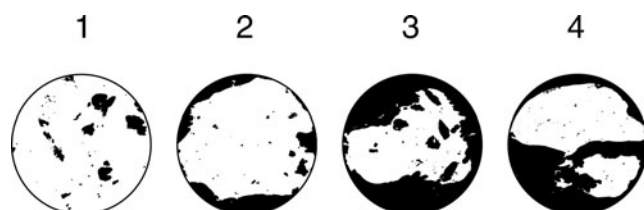
Critical-sized, 8-mm-diameter defects within the rat calvarium were created under general inhalational anesthesia, filled with freshly prepared implants, closed, and cared for postoperatively as previously described.⁶⁰ After 12 postoperative weeks, animals were euthanized according to established and approved methods,⁶⁰ and the implants and surrounding tissues were harvested with a high-speed surgical drill (TPS®; Stryker) and 701 cutting bur. Before harvest, samples were randomly designated for mechanical testing or μCT /histology. After harvest, samples to be mechanically tested were stripped of any extraneous soft tissues superficial or deep to the implant, immediately placed in ice-cold phosphate-buffered saline, and mechanically tested within 20 min. Samples designated for μCT /histology were individually placed in 10% neutral buffered formalin and stored on a shaker table at 4°C for 72 h.

Mechanical push-out testing

Push-out testing was performed to determine the interfacial shear strength of the implanted constructs after 12 weeks. Test were performed on a mechanical testing bench (858 Mini Bionix II; MTS Systems Corp.) using a custom-made push-out and support jigs allowing 1 mm of clearance between the construct and the support jig around the entire circumference of the construct to minimize the effect of the testing jig on the interfacial stress distribution.⁶¹ Tissue samples were oriented with the construct centered over the

TABLE 2. SCORING GUIDE FOR BONY BRIDGING AND UNION USING MAXIMUM INTENSITY PROJECTIONS FROM MICROCOMPUTED TOMOGRAPHY

Description	Score
Bony bridging over entire span of defect at longest point (8 mm)	4
Bony bridging over partial length of defect	3
Bony bridging only at defect borders	2
Few bony spicules dispersed throughout defect	1
No bone formation within defect	0



support jig, and the push-out jig applied a vertical force on the constructs at a constant rate of 0.5 mm/min. Testing was stopped after a peak force was reached.

μ CT imaging and analysis

Bone volume and extent of bridging within the explanted samples were analyzed by μ CT (SkyScan 1172 high-resolution micro-CT; SkyScan) as previously described.⁶² For PLLA constructs, lower and upper threshold indices were 70 and 255, respectively. For coral constructs, threshold indices of 90 and 255 were used. For PLLA samples, axial maximum intensity projections (MIPs) were generated, and bony bridging was evaluated by three observers (J.D.K., P.P.S., and F.K.K.) according to the scoring system described in Table 2.

Histological processing

Fixed samples were serially dehydrated in graded ethanol (70%–100%). Samples with PLLA constructs were left un-

decalcified and embedded in methylmethacrylate. Samples with coral constructs were mildly decalcified with EDTA and embedded in methyl methacrylate. Serial 5- μ m coronal sections through the center of the construct were then cut using a rotation microtome (Leica) and stained with hematoxylin and eosin, von Kossa/van Gieson (PLLA constructs only), or Goldner's trichrome according to established methods.⁶³

Light microscopy and histological scoring

Stained sections were analyzed and imaged using a standard light microscope with attached digital camera (Eclipse E600; Nikon Instruments Inc.). Three observers (J.D.K., P.P.S., and F.K.K.) evaluated each of the sections according to a previously used scoring system, shown in Table 3, for assessing the construct–bone interface and the bone growth within the construct pores.⁶²

Statistical analyses

Continuous variable data are represented as means \pm standard deviation. Ordinal variables are presented as frequencies. All analyses were performed using R version 2.10.0 (R Foundation for Statistical Computing). The *a priori* defined significance level for all statistical tests was 0.05.

Peak loads obtained from push-out testing and percentage bone volumes were analyzed using two-way analyses of variance (bmMNC \times PRP). *Post hoc* multiple comparisons were made using the step-down Holm-Sidak test.⁶⁴

MIP and histology scoring were analyzed using a non-parametric method for analyzing two-way ordinal data from factorial experiments.^{65,66} Statistical significance for main effects and interaction were determined using analyses of variance-type statistics, and multiple comparisons were made based on estimated 95% confidence intervals.

Results

PRP processing

Approximately 250 μ L of PRP was obtained from 4.5 mL peripheral blood per donor rat. Preliminary studies showed an 8.6 ± 3.2 -fold platelet enrichment in the PRP compared to

TABLE 3. QUANTITATIVE HISTOLOGICAL SCORING PARAMETERS

Description	Score
<i>Hard tissue response at scaffold–bone interface</i>	
Direct bone to implant contact without soft interlayer	4
Remodeling lacuna with osteoblasts and/or osteoclasts at surface	3
Majority of implant is surrounded by fibrous tissue capsule	2
Unorganized fibrous tissue (majority of tissue is not arranged as capsule)	1
Inflammation marked by an abundance of inflammatory cells and poorly organized tissue	0
<i>Hard tissue response within the pores of the scaffold</i>	
Tissue in pores is mostly bone	4
Tissue in pores consists of some bone within mature, dense fibrous tissue, and/or a few inflammatory response elements	3
Tissue in pores is mostly immature fibrous tissue (with or without bone) with blood vessels and young fibroblasts invading the space with few macrophages present	2
Tissue in pores consists mostly of inflammatory cells and connective tissue components in between (with or without bone) OR the majority of the pores are empty or filled with fluid	1
Tissue in pores is dense and exclusively of inflammatory type (no bone present)	0

peripheral blood. Fibrinogen concentrations in the PRP were 14.2 ± 4.5 mg/mL. To keep the fibrinogen concentration constant in the gel fraction of all constructs, the fibrinogen complex concentration in constructs with PRP was slightly lower than in those without.

Construct fabrication and animal surgeries

Constructs were successfully fabricated, with each construct containing 100 μ L of gel with, where appropriate, 1.5×10^6 bmMNCs/scaffold, 17 mg/mL fibrinogen and 100 IU/mL thrombin within the gel, and 25 μ L PRP. Gel was well formed within the pores of the scaffolds \sim 1 min after the addition of thrombin.

One animal died intraoperatively and was replaced before the completion of the study. All other animals survived the surgery and postoperative period without complications. At the time of euthanasia and sample harvest, there were no signs of gross infection or adverse tissue response surrounding the implants.

Mechanical push-out testing

Push-out testing results in the form of peak loads are shown in Figure 2. Due to differences in the Young's moduli

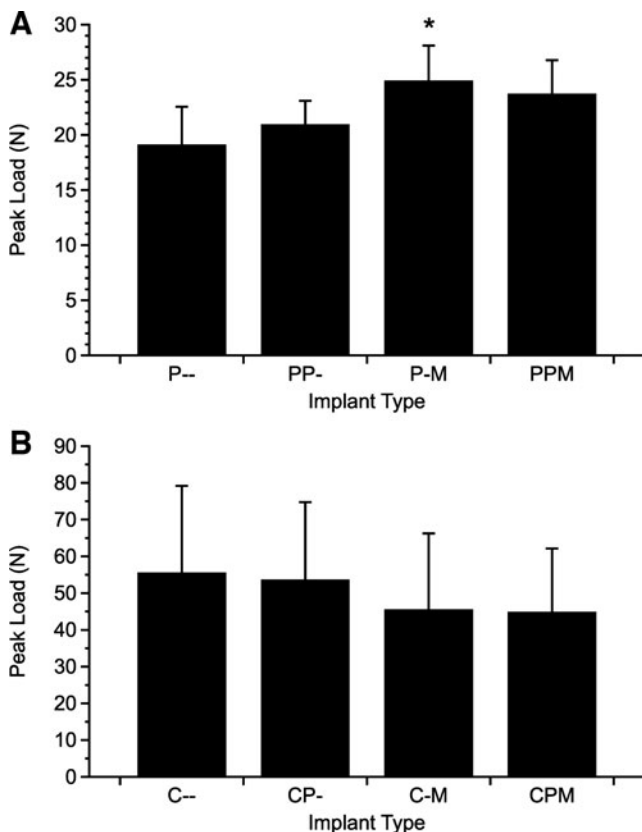


FIG. 2. Results of mechanical push-out testing. Immediately after harvest, constructs were placed on a custom testing jig and a vertical force was applied. The peak load required to displace the implants from the surrounding tissues was recorded for both PLLA constructs (A) and coral constructs (B). Data are reported as mean \pm standard deviation ($n = 6$). Statistical significance between groups and the corresponding control is denoted by *.

of the scaffold materials, direct comparisons between coral and PLLA constructs cannot be made.⁶¹ For the PLLA constructs, the group containing only bmMNCs (P-M) required significantly more force to displace the construct than the control (P-) constructs. Among the PLLA constructs, no other significant differences were found. Overall, the presence of bmMNCs had a significant effect on the push-out force ($p < 0.005$), whereas PRP and the interaction of PRP and bmMNCs were not significant.

No significant differences in push-out strength were found for the coral constructs. The overall effect of bmMNCs seeded onto the scaffolds, the presence of PRP, and any interaction between these factors were also insignificant.

μ CT analysis

Results from μ CT imaging were analyzed to determine the volume percentage of regenerated bone within the constructs (Fig. 3). For PLLA constructs, significant differences in bone formation again existed only between the control constructs and the group containing only bmMNCs (P-M). For coral constructs, no significant differences were present in

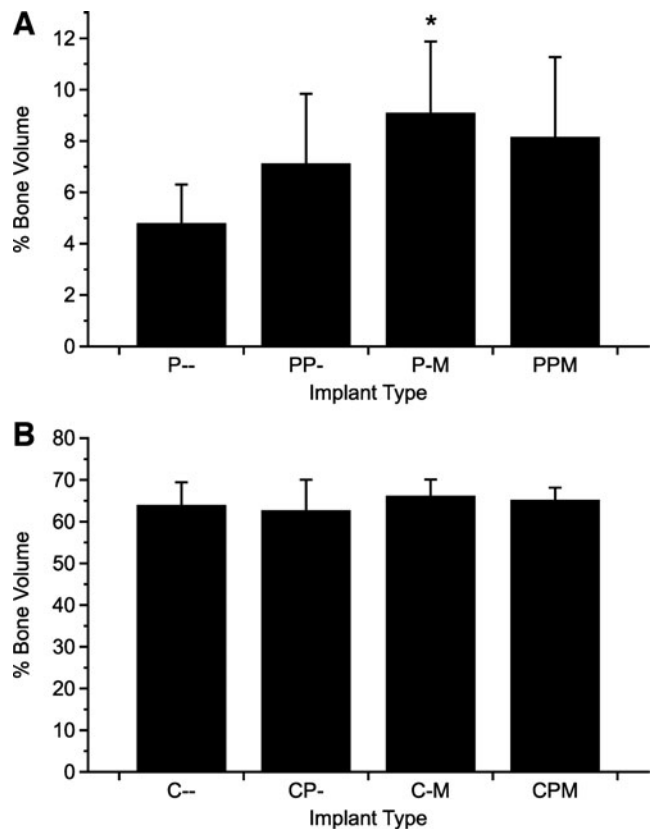


FIG. 3. Percent object volume of bone formation within the cranial defect. Explanted constructs and surrounding tissues were scanned by microcomputed tomography and analyzed to determine the percentage of volume within the defect space occupied by mineralized tissue for both PLLA constructs (A) and coral constructs (B). The reported values for the coral constructs include the volume of the scaffold. Data are reported as mean \pm standard deviation ($n = 8$). Statistical significance between groups and the corresponding control is denoted by *.

pairwise comparisons; however, for both PLLA and coral constructs, the addition of bmMNCs had a significant overall effect on bone formation. The effect of PRP and the interaction of PRP and bmMNCs were not significant for both types of constructs.

Bony bridging across defects filled with PLLA constructs was evaluated by scoring MIPs as shown in Figure 4. Again, the presence of bmMNCs had a significant effect on bony bridging, but the effect of PRP and the interaction of PRP with bmMNCs were not significant. Pairwise comparisons found the observed bridging between both groups containing bmMNCs (P-M, PPM) to be significantly different from both groups without bmMNCs (P-, PP-). Complete bridging through the midline of the defect was only observed in one sample from group PPM.

Histology

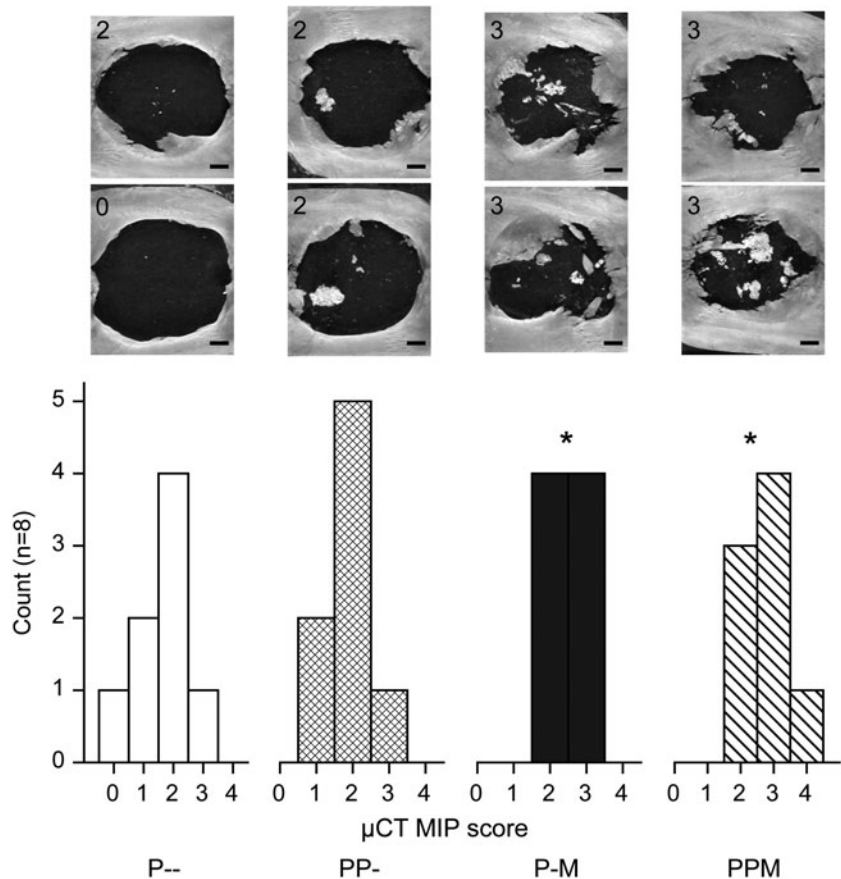
Histological sections were analyzed and scored to evaluate the tissue response at the bone–construct interface and the tissue growth within the construct pores. Representative histological samples are shown in Figure 5. For the defects filled with PLLA constructs, no significant differences between groups or significant effects were observed. A well-organized fibrous capsule surrounded each PLLA construct; thus, each sample received a score of 2 characterizing the interface. The scores reflecting the tissue response within the pores of the PLLA constructs are shown in Figure 6A.

The results of scoring the tissue response at the interface and within the pores of the coral constructs are shown in Figure 6B and C, respectively. No significant differences in the tissue response within the construct pores were found between groups; however, there was a statistically significant interaction between the bmMNCs and the PRP on the tissue response both at the interface and within the pores of the constructs. Neither factor had a significant effect when considered alone. Additionally, there was a statistically significant difference between the tissue response at the construct interface for the coral constructs with both bmMNCs and PRP (CPM) and the control (C-) constructs.

Discussion

To facilitate the translation of many tissue engineering technologies for clinical use, standardization or simplification of the approaches taken is necessary.⁶⁷ In the present study, we hypothesized that a process that could be completely performed over the course of a single surgical intervention could be developed incorporating uncultured bmMNCs within U.S. Food and Drug Administration–regulated materials for enhanced bone formation within a critical-sized rat cranial defect. For many of the analyses used, this was indeed the case. Uncultured bmMNCs encapsulated within a fibrin gel inside the pores of PLLA scaffolds regenerated significantly more bone within the defect, achieved greater bony bridging of the defect, and

FIG. 4. Scores of bony bridging across the cranial defects. Explanted PLLA constructs were scored by three observers according to the criteria in Table 2. Histograms of the scores are reported for each type of construct, and significant differences between construct scores and the control (P-) score are denoted by *. Images depict representative maximum intensity projections along with the corresponding score assigned to these samples (upper left corner of each image). Scale bars (lower right corners) indicate 1 mm.



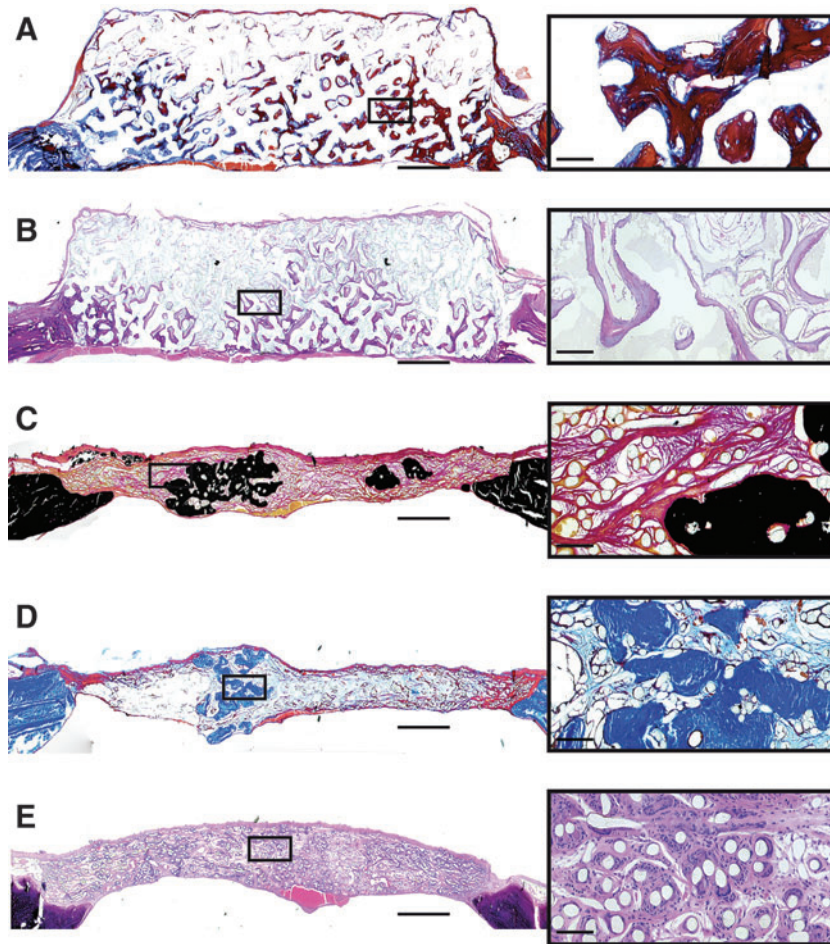


FIG. 5. Representative histological sections from the experimental groups. Group and stain are as follows: (A) CP-, Goldner's trichrome; (B) CPM, hematoxylin and eosin; (C) P-M, Von Kossa/Van Gieson; (D) PPM, Goldner's trichrome; (E) P-, hematoxylin and eosin. Scale bars in the full size images on the left represent 1 mm, while in the magnified micrographs to the right, the scale bars represent 100 μ m. In A, mineralized tissue is stained blue or deep red due to the partial demineralization required during processing. In (D), mineralized tissue is stained bright blue, whereas osteoid is stained deep red.

were better incorporated into the cranium as measured by mechanical testing than were cell-free controls.

When comparing cell-laden and cell-free coral constructs, the results were not as clear. The presence of bmMNCs did not significantly increase the bone formation within the scaffolds as detected by μ CT, and the peak force needed to display the cellular constructs was less than that needed to displace the cell-free scaffolds, although not significantly so. The presence of PRP had no effect on bone formation as determined by mechanical testing and μ CT regardless of scaffold material or whether bmMNCs were also present; however, a significant interaction was observed between PRP and bmMNCs when evaluating the tissue formation within the pores and at the interface of the coral constructs by histology.

Scaffold material played an obvious role in bone formation, mechanical integrity of the implanted constructs, and the effect of bmMNC incorporation. Differences in composition, pore morphology, and mechanical properties between the two scaffolds prevent direct comparison of results obtained using the different scaffolds; however, the studies demonstrate the importance of scaffold physicochemical properties on bone formation. PLLA was chosen as a polymeric scaffold due to its regulatory history, long-term maintenance of mechanical properties, and because MSCs have previously been demonstrated to differentiate well on PLLA fiber meshes.⁶⁸ Polymeric fiber meshes with similar

fiber diameters and densities have previously been used to characterize the effect of MSC culture and differentiation in the rat cranial defect.⁶⁹

Coralline HA bone grafts have long been used both in research and clinical applications.⁶⁹ In this study, bone formation in coral scaffolds was not significantly different across groups; however, for μ CT analysis of bone formation there was an overall significant effect of bmMNC incorporation. This result was somewhat surprising, as previous work has demonstrated MSC osteogenic differentiation and ingrowth on similar scaffolds.⁷⁰⁻⁷² Although reports showing little to no osteogenesis on coralline HA scaffolds do exist,⁷³ bony ingrowth did occur in all of the tested formulations using coral.

The use of PRP did not significantly affect bone formation in this study. This result is similar to those reported elsewhere in rats,⁷⁴ although there are multiple reports in humans⁷⁵ and animals,⁷⁶ including rats,^{77,78} of PRP enhancing bone formation. Still, evaluating the efficacy of PRP remains complicated. PRP in combination with uncultured bmMNCs has been found to increase bone regeneration in humans,⁷⁹ and rat PRP gels have similarly been shown to increase *in vitro* osteogenic differentiation of rat MSCs.^{80,81} Similarly, a significant interaction was found between PRP and uncultured bmMNCs by histological scoring of coral constructs, but evaluation by μ CT and mechanical testing did not reflect a significant interaction. Other studies have shown that PRP

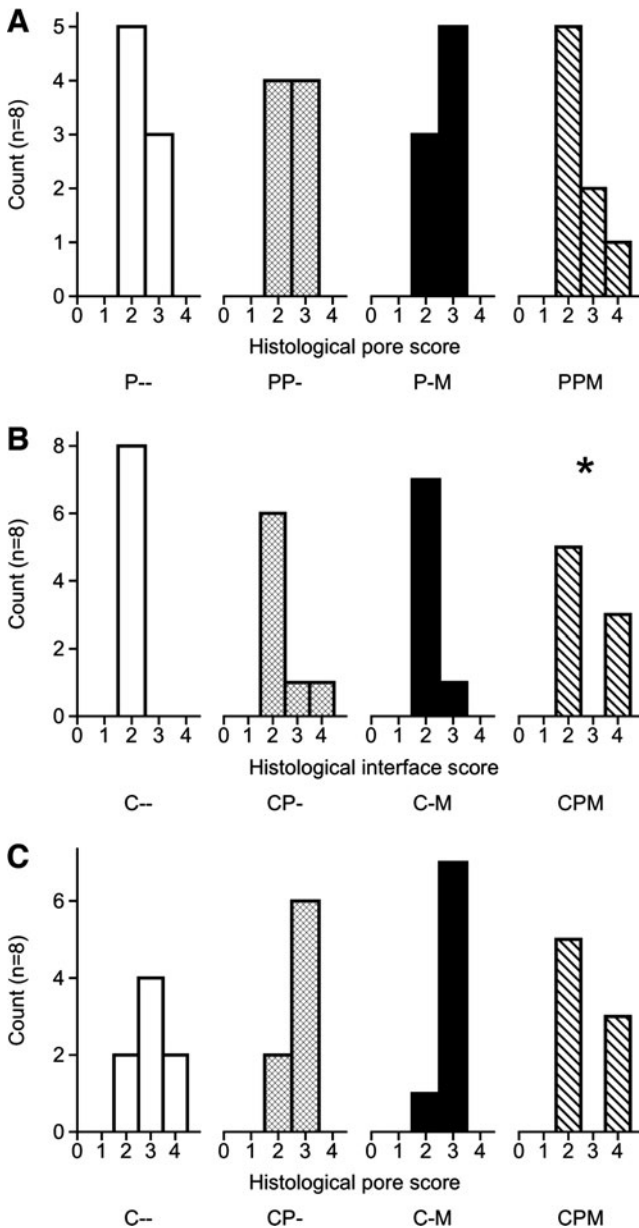


FIG. 6. Scores from histological analysis of tissue response within the construct pores (**A, C**) and at the implant–tissue interface (**B**). Histology samples were scored by 3 observers according to the criteria in Table 3. Histograms of the scores are reported for each type of construct, and significant differences between construct scores and the control (P- or C-) score are denoted by *. At the interface between the PLLA constructs and the surrounding tissue, all samples received a score of 2.

does not increase *in vitro* or *in vivo* differentiation of rat MSCs when compared with the effect of growth factors such as bone morphogenetic protein-7⁸² and was found to inhibit MSC differentiation in a study comparing PRP and bone morphogenetic protein-2.⁸³

A number of considerations regarding the variability and evaluation of PRP must be factored in when analyzing the discordant results for PRP use found in the literature. First, processing techniques, donor factors, and overall platelet

enrichment play a significant role in the determining the actual composition of any PRP.^{84–89} Further, there is an established interspecies variation in the growth factors contained in and osteogenic capability of PRP, with human PRP having higher growth factor concentrations and eliciting an increased osteogenic response compared to rat or goat PRP.^{81,90} Additionally, PRP effectiveness may in part be modulated by scaffold and cell differentiation state for applications involving cell-laden constructs.^{91,92} Kasten *et al.* found that PRP increased alkaline phosphatase activity in undifferentiated MSCs but not in those precultured in osteogenic media⁹¹; however, in the present study PRP was only found to significantly interact with uncultured bmMNCs when examining histological sections.

The present study had a number of strengths. First, it further establishes the utility of uncultured bmMNCs in bone formation, including demonstrating a mechanical benefit for cell-laden PLLA scaffolds. Additionally, the study uses only commercially available materials that have already been approved for clinical use, meaning other parameters, such as the explicit role of fibrin glue in cell delivery, the relative effect of culture and expansion of bmMNCs on bone formation, and optimal seeding densities can be explored with minimal variability in the substrate materials. This may also allow for more rapid clinical evaluation of the results. The effect of the scaffold on bone formation using PRP and bmMNCs was also demonstrated by the difference in results for PLLA and coral constructs. Finally, mechanical effects of substrate stiffness were carefully controlled by maintaining a constant fibrinogen concentration across samples; thus, any effect seen for PRP addition would have likely been based on bioactive factor delivery.

The primary weaknesses of the present study lie primarily in scope, and thus future studies are necessary to draw more definitive conclusions from the current study. As mentioned in the previous paragraph, future studies are needed to determine whether the benefits of using a simplified process that can be performed over the course of a single operation outweigh any benefits that may exist with MSC expansion and culture. Similarly, future studies should incorporate more time points to determine if there are any temporal effects of PRP addition. Finally, more sophisticated analyses should be done to determine the role of the delivered bmMNCs in bone formation, as the ability of a seeded cell population to recruit host osteoprogenitors may be more important for bone regeneration than the regenerative capability of the seeded cells.⁹³

A number of interesting results were found in this study. First, bmMNCs increased bone formation and construct incorporation for PLLA scaffolds, but the benefit in coral scaffolds was not clear. For mechanical testing of coral constructs, bmMNC incorporation within scaffolds did not provide a significant change in implant mechanical properties, although a significant overall effect was found for μ CT analysis. In a similar study by Le Nihouannen *et al.*, fibrin glue and granules of 60/40 HA/ β -tri-calcium phosphate (β -TCP) cement were mixed with and without freshly isolated bone marrow and placed within defects in rabbit femora.⁹⁴ After 12 weeks, there was no difference in bone formation within the defects as detected by μ CT. Ueno *et al.* did, however, demonstrate bone regeneration in rat calvarial defects filled with β -TCP granules and whole marrow.⁹⁵

Similarly, Becker *et al.* compared bone marrow and concentrated bmMNC seeding on β -TCP scaffolds and found that constructs seeded with bone marrow induced significantly more bone regeneration than those with bmMNCs, which were not statistically different from controls.⁹⁶

On the basis of these studies, whole marrow may be a better cell source for scaffolds where significant adsorption of potentially osteogenic proteins from the acellular marrow fraction may occur. Additionally, bone formation within the coral scaffolds was similar to that of PLLA scaffolds, but not significant when analyzed by pairs, when the object volume of control (unimplanted) coral scaffolds was not considered (Fig. 7). Since the coral scaffolds cannot be differentiated from newly regenerated bone by μ CT, variability in scaffold volume may have masked any effect of differences between the experimental groups. Further, we noticed bone resorption around some of the coral scaffolds during μ CT imaging. Although the resorption did not vary between groups as determined by imaging and quantifying the bone around the border of the scaffolds (Fig. 8) or histological scoring of the tissue response at the coral construct interface, this resorption likely affected the relatively high variability of the mechanical testing results. Interestingly, the group of coral constructs with the lowest overall interfacial shear strength (CPM) appeared to be significantly more integrated with the host bone compared to control (C-) groups by histology. These data may indicate that histological evaluation of the host-scaffold interface, because of its two-dimensional nature, does not accurately reflect the mechanical integration of the constructs as the failure of a majority of the constructs occurred at the bone-construct interface.

The use of fibrin glue may also have confounded the findings with respect to PRP use. Fibrin glue and modifications thereof have been shown to be an effective delivery medium for multipotent cells and growth factors in a number tissue engineering applications.⁹⁷⁻¹⁰⁰ Platelet-enriched fibrin glue has been shown to be an effective substrate for bone-tissue engineering applications both in the presence and absence of encapsulated MSCs.^{101,102} Ito *et al.* showed an

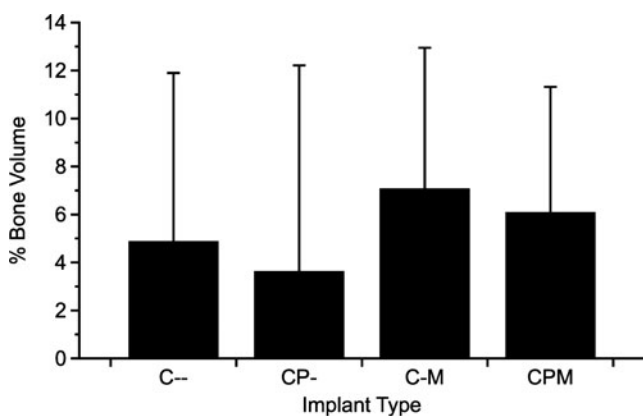


FIG. 7. Average volume of regenerated bone within coral scaffolds. The average volume of unimplanted coral scaffolds ($n = 10$, mean volume 70.89% \pm 4.79%) was subtracted from the volume of explanted samples ($n = 8$), leaving an estimate of the bone regeneration within each coral construct. Data shown are means \pm adjusted standard deviations.

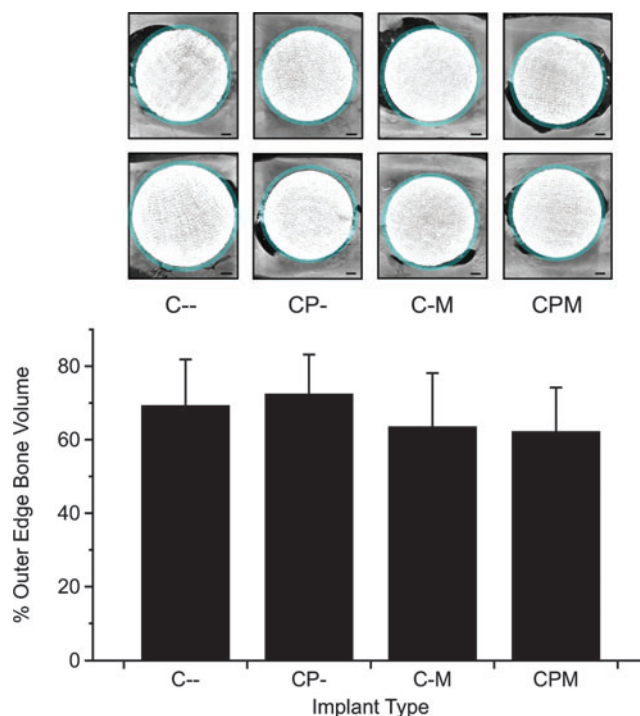


FIG. 8. Bone growth or resorption around the outer volume of the coral constructs was quantified. Representative images (top) denote with false coloring the border where bone was quantified around the defect. Scale bars represent 1 mm. The figure (bottom) shows the average percent bone volume present in the outer ring ($n = 8$, error bars = standard deviation). No significant differences were found.

increase in bone regeneration in a canine mandibular defect using MSC/PRP/fibrin glue hybrid constructs compared to MSC/fibrin glue constructs alone after 4 and 8 weeks¹⁰³; however, other studies have reported no benefit to similar hybrid constructs.¹⁰⁴ Fibrin glue encapsulation alone likely imparts a benefit in the delivery and retention of cells within the scaffold or defect.^{48,105,106} In previous studies demonstrating a benefit for PRP and MSCs in combination but not in comparison with other delivery methods or encapsulation substrates,^{107,108} the benefit may be due in large part to the role of the PRP as a delivery vehicle for the MSCs,¹⁰⁹ given the otherwise short-term survival of seeded MSCs.¹¹⁰

The need for a cell delivery vehicle may be dependent on the type of scaffold used; in a study by Haasper *et al.*, no benefit of cellular delivery by fibrin glue to scaffolds to which cells adhere more efficiently, such as bone matrix, has been demonstrated.¹¹¹ Fibrin glue may still play an important role in applications where bioactive factor delivery is appropriate and controllable and easily tuned release kinetics are desired.¹¹²⁻¹¹⁴

Because previous experiments have explicitly established that the fibrinogen concentration within the fibrin glue affects MSC differentiation and proliferation,^{55,56} an intermediate fibrinogen concentration (17 mg/mL), close to reported optimal values,¹¹⁵ was selected to allow cells to proliferate while still providing some substrate stiffness. In constructs with PRP, the amount of fibrinogen complex incorporated into the final glue was reduced to keep the fibrinogen

concentration constant. Because the fibrinogen complex may also contain growth factors, this reduction in concentration may have offset any benefits of growth factor delivery by PRP.

Conclusions

The many emerging technologies in tissue engineering provide a number of options and important considerations for researchers and clinicians. Efficacy, cost, and risk must be assessed, and researchers focused on quickly translating technologies must weigh regulatory pathways as well. The present study characterized scaffold, uncultured bmMNCs, and PRP combinations to determine their relative effectiveness in regenerating bone within a critical-sized rat cranial defect. PLLA scaffolds laden with bmMNCs delivered within fibrin glue regenerated significantly greater bone than control groups. Cells seeded on coral scaffolds had no significant effect on bone formation. By most methods of evaluation, PRP was not found to have a benefit in any formulation. Taken in the context of previous reports in the literature, these results provide further indication that a complex interplay occurs between scaffolds, cells, growth factors, and the host environment. A translational approach employing freshly harvested bmMNCs is likely beneficial compared to a strictly biomaterials-based approach in some cases; however, further work is needed to better understand when such an approach is warranted.

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Disclosure Statement

No competing financial interests exist.

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