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Effect of Age on Biomaterial-mediated *in situ* Bone Tissue Regeneration

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Abstract

Emerging studies show the potential application of synthetic biomaterials that are intrinsically osteoconductive and osteoinductive as bone grafts to treat critical bone defects. Here, the biomaterial not only assists recruitment of endogenous cells, but also supports cellular activities relevant to bone tissue formation and function. While such biomaterial-mediated *in situ* tissue engineering is highly attractive, success of such an approach relies largely on the regenerative potential of the recruited cells, which is anticipated to vary with age. In this study, we investigated the effect of the age of the host on mineralized biomaterial-mediated bone tissue repair using critical-sized cranial defects as a model system. Mice of varying ages, 1-month-old (juvenile), 2-month-old (young-adult), 6-month-old (middle-aged), and 14-month-old (elderly), were used as recipients. Our results show that the bio-mineralized scaffolds support bone tissue formation by recruiting endogenous cells for all groups albeit with differences in an age-related manner. Analyses of bone tissue formation after 2 and 8 weeks post-treatment show low mineral deposition and reduced number of osteocalcin and tartrate-resistant acid phosphatase (TRAP)-expressing cells in elderly mice.

Graphical Abstract

Tissue engineering strategies that promote tissue repair through recruitment of endogenous cells will have a significant impact in regenerative medicine. Previous studies from our group has shown that biomineralized materials containing calcium phosphate minerals can contribute to neo-bone tissue through recruitment and activation of endogenous cells. In this study, we investigated the effect of age of the recipient on these biomaterial-mediated bone tissue repair. Our results show that the age of the recipient mouse had a significant impact on the quality and quantity of the

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engineered neo-bone tissues, in which delayed/compromised bone tissue formation was observed in older mice. These findings are in agreement with the clinical findings that the age of the patient is a key factor in bone repair.

Orthotopic bone repair by osteoinductive mineralized scaffolds

Effect of age

Scaffold mediated *in situ* bone tissue engineering

Keywords

mineralized biomaterial; cranial defect; bone repair; osteoinductive; endogenous cells; *in situ* tissue engineering; age effect

1. Introduction

Bone grafting is one of the most common procedures used to treat complex bone fractures, which are hard to heal otherwise [1]. Current grafts include autografts and allografts. These methods, however, suffer from various drawbacks such as limited sources, donor site complications, and potential for disease transmission [2–4]. To overcome these limitations, tissue engineering strategies of using biomaterials in combination with osteoinductive molecules and/or exogenous cells, have been widely studied [5, 6]. Although conventional tissue engineering strategies that use biomaterials and exogenous cells are promising, activating endogenous cells to regenerate the compromised bone tissue or assist bone tissue repair are more clinically attractive. Towards this, potent osteoinductive biomolecules, such as bone morphogenetic proteins (BMPs), have been extensively used. In fact, BMP-2 and BMP-7 (or osteogenic protein-1, BMP-7) are approved for clinical use in treating non-union bone fractures and spinal fusion [7]. While BMPs are very effective in promoting bone tissue regeneration, their widespread clinical use is hampered by various side effects such as inflammatory complications, osteolysis and unwanted ectopic bone formation[8–10].

Another approach is the use of osteoinductive biomaterials such as bioglasses, which emulate certain aspects of the mineral phase of native bone. *In vitro*, these materials have demonstrated the abilities to direct osteogenic differentiation of progenitor cells [11–14]. Bioactive ceramics, including bioglasses and calcium phosphate-based materials, were able to form a surface layer of apatite-like mineral in simulated body fluid, a solution with pH

and ionic composition close to human plasma [15, 16]. These materials, when implanted *in vivo*, were able to assist repair of bone defects [17–19] through recruiting endogenous osteoprogenitor cells and directing their osteogenic differentiation [20]. However, a drawback of these systems is a weak adherent bond between the newly-formed apatite-like mineral layer and the ceramic, which usually leads to ultimate delamination of these two layers [21]. This could cause serious problems and result in implant failures. Biomaterialized materials comprised of both the organic and inorganic phases could be an alternative solution for promoting bone tissue repair *in vivo*. The crystalline phase of the mineral component could be a key parameter in determining the osteoinductive function of calcium phosphate-based materials or ceramics [22]. Development of mineralized materials through biomaterialization can be used to control the crystalline nature of the mineral phase and thereby their osteoinductivity [23–26].

Recently, we have shown that biomaterialized macroporous materials containing calcium phosphate minerals induce osteogenic differentiation of stem cells, including pluripotent stem cells (hESCs and hiPSCs) [27, 28] and human mesenchymal stem cells (hMSCs) [29, 30]. These materials, when implanted *in vivo*, contribute to ectopic [30] and orthotopic [31, 32] bone tissue formation through recruitment of endogenous cells. While physical (e.g. pore size) and chemical (e.g. osteoinductivity) properties of the scaffolds play a key role in the recruitment of endogenous cells and their function (including osteogenic differentiation), a number of host-specific parameters such as the age of the recipients could be an important factor in determining the treatment outcome. It is very well documented that tissue repair abilities decline with age and bone healing is no exception. Studies have shown a decline in bone healing capacity in the elderly [33–36]. Such a decline in tissue repair has been touted to be associated with multiple age-related alterations of cellular activities and microenvironment in the body, including decreased number and/or function of stem cells [37, 38], structural and cellular changes in periosteum [39, 40], changes in the local signaling at injury site [41], and rate of vascularization [34]. Thus, it is crucial to gain a better understanding of the effect of physiological age of the host tissue on biomaterialized material-mediated bone tissue repair *in vivo*.

In this study, we investigated the effect of the host age on biomaterialized material-mediated bone tissue repair by employing mice of varying ages (1, 2, 6, and 14-month-old). We used critical-sized cranial defects as a model system and assessed biomaterial-mediated recruitment of host cells and bone tissue formation as a function of post-implantation time. While almost complete closure of defects was achieved in all ages by 8 weeks post-implantation, significant differences, in terms of pace of healing and extent of neo-bone tissues and mineral depositions, were observed.

2. Materials and Methods

2.1 Graft synthesis and mineralization

Synthesis of poly (ethylene glycol) diacrylate (PEGDA) [Mn (number-average molecular weight) = 3.4 kDa] and N-acryloyl 6-aminocaproic acid (A6ACA) was performed as previously reported [42, 43]. Macroporous copolymer hydrogels of PEGDA and A6ACA (PEGDA-co-A6ACA) were prepared through cryogelation as described elsewhere [30].

Briefly, a precursor solution containing 20% (w/v) PEGDA and 0.5 M A6ACA, 0.5% (w/v) ammonium persulfate (APS) and 0.2% (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED) were prepared in 0.5 M NaOH. A drop of 75 μ l precursor solution was dispensed into a pre-chilled polystyrene petri-dish. A pre-chilled circular glass coverslip of 15 mm in diameter was then put onto the precursor solution, and the solution was allowed to polymerize at -20°C for 24 hours. After gelation, PBS at room temperature was added into the petri-dish to facilitate thawing of the ice crystals, thus yielding an interconnected macroporous structure. The resultant macroporous hydrogels (i.e. cryogel) were subsequently punched into disks of 4 mm in diameter and 0.7 mm in height. Mineralization of the macroporous scaffold was achieved by incubating the cryogel structures in simulated body fluid (m-SBF) for 12 h and, subsequently, in 40 mM Ca^{2+} and 24 mM PO_4^{3-} (pH 5.2) for 1 h [44]. m-SBF was prepared as detailed previously by Oyane et al. [15]. Briefly, 1 L of a HEPES-NaOH buffered solution (pH 7.4) was prepared containing 142 mM Na^{+} , 103 mM Cl^{-} , 10 mM HCO_3^{-} , 5 mM K^{+} , 1.5 mM Mg^{2+} , 1.0 mM HPO_4^{2-} , and 0.5 mM SO_4^{2-} , 2.5 mM Ca^{2+} . Prior to usage, the solution was warmed to 37°C . The scaffolds were briefly rinsed in ultrapure water, followed by incubation in m-SBF for 48 h at 37°C . After the complete biomineralization, the mineralized scaffolds were sterilized by incubating in 70% ethanol for 3 hours, followed by rinsing with sterile PBS for 3 days.

2.2 Calcium and phosphate assays

Calcium and phosphate assays were performed to determine the amount of Ca^{2+} and PO_4^{3-} in the biomineralized scaffolds and their release to the surrounding medium. The scaffolds were rinsed and homogenized in ultrapure water. The homogenized scaffolds were freeze-dried, and their dry-weights were recorded. Dried scaffolds were incubated in 0.5 M HCl at 25°C . Incubation medium was collected after 3 days, which was sufficient to dissolve all the minerals from the scaffolds, and the amount of Ca^{2+} and PO_4^{3-} was measured.

To assess dissolution of Ca^{2+} and PO_4^{3-} from the mineralized scaffolds and their release to the surrounding medium, dried scaffolds were rehydrated and equilibrated in ultrapure water. After reaching the equilibrium swollen state, the scaffolds were incubated in 1 M Tris-HCl (pH = 7.4), free of these ions, at 37°C for 7 days. Incubation solution was collected and replaced with fresh medium daily. The collected incubation solution was used to determine the release of Ca^{2+} and PO_4^{3-} ions from the scaffolds.

A calcium assay was conducted based on the manufacturer's protocol (Calcium reagent set, Pointe Scientific, catalog number: C7503) as described previously [45]. Briefly, 20 μ l of the sample solution was mixed with 1 ml of assay solution containing o-Cresolphthalein complexone (CPC). The absorbance of the mixture solution was measured at 570 nm by a UV/Vis spectrophotometer (Beckman Coulter, DU 730). The Ca^{2+} concentration was computed from a standard curve generated for a concentration range of 0–4 mM Ca^{2+} .

A phosphate assay was conducted as detailed elsewhere [46]. Briefly, an assay solution was prepared by mixing 1 volume of 10 mM ammonium molybdate, 2 volumes of acetone, and 1 volume of 5 N H_2SO_4 . One ml of this assay solution was mixed with 125 μ l of the sample solution. To this, 100 μ l of 1M citric acid was added. The absorbance at 380 nm of the

resultant product was determined by a UV/Vis spectrophotometer. The PO_4^{3-} concentration was computed from a standard curve generated for a concentration range of 0–4 mM PO_4^{3-} .

2.3 Scanning electron microscopy (SEM) and energy dispersive spectra (EDS)

Scanning electron microscopy (SEM) was used to examine the pore structures of the mineralized scaffolds. The scaffolds were cut into thin slices, followed by freeze-drying for 24 h. These samples were sputter coated (Emitech, K575X) with iridium for 7 s and imaged using SEM (Philips XL30 ESEM). The mineral composition of the scaffolds was determined through EDS analysis. ICA software was used to quantify the calcium to phosphate atomic ratio (Ca/P) from the resulting elemental spectra.

2.4 Surgical procedures and biomaterial implantation

For the *in vivo* studies, female C57BL6/J mice (1, 2, 6 and 14-month-old) were used. All animal studies were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego and National Institutes of Health (NIH). In preparation for surgery, mice were anesthetized using ketamine hydrochloride (Ketaset, 100 mg/kg), xylazine (AnaSed, 10 mg/kg) and buprenorphine (0.05 mg/kg) via intraperitoneal injection.

A skin incision was made along the length of the calvaria to expose the parietal region and a 4 mm-diameter defect was made in both the left and right parietal bones with a 4-mm biopsy punch [47]. For the experimental group, sterile mineralized scaffolds were transplanted into each defect site. Groups with untreated defects were used as sham groups. Following implantation, the skin was sutured and mice were kept on a warm heating pad until waking. After the surgery, all the mice were kept in separate housing cages. The ability of the mineralized biomaterials to support neo-bone tissue formation were compared against groups with untreated defects. The bone tissue formation within the bone defects were analyzed at 2 and 8 weeks post-procedure.

2.5 Micro-computed tomography (μCT)

Micro-computed tomography (μCT) was performed to assess hard tissue formation within the cranial defect at 2 and 8 weeks post-implantation. The entire skull of each mouse was harvested and fixed in paraformaldehyde (PFA) by immersing the specimens in 4% PFA for 4 days at 4°C. The retrieved samples were rinsed with PBS and secured tightly between styrofoam disks within a conical tube. Scanning was performed using SkyScan 1076 (Bruker: 9 μm pixel, 50 kV, 0.5 mm Al filter). Scan reconstruction was performed using the NRecon software (SkyScan, Bruker). Using CT Analyzer software (SkyScan, Bruker), cranial bone tissues were segmented using a threshold of 90–255. The reconstructed images were used to generate 3-D models by using CT Analyzer software (SkyScan, Bruker). Bone mineral density (Bone volume/Total volume, BV/TV) was quantified from the reconstructed images.

2.6 Histology and immunohistological staining

The PFA fixed samples were decalcified by using 10% ethylenediaminetetraacetic acid (EDTA, pH 7.4), where the samples were incubated in the solution for 2 weeks at 4°C. The

decalcified samples were gradually dehydrated with increasing concentration of ethanol and equilibrated in CitriSolv. Following dehydration, samples were incubated in a mixture of 50% (v/v) CitriSolv, 50% (v/v) paraffin for 30 min at 70°C. The samples were then embedded in paraffin and sliced into sections of 10- μ m thickness using a rotary microtome (Leica, RM2255). Before staining, the sections were deparaffinized using CitriSolv and subsequently rehydrated with decreasing concentration of ethanol until the samples were equilibrated with deionized (DI) water.

Hematoxylin and eosin (H&E) staining was performed by incubating the rehydrated samples in hematoxylin solution for 2 minutes (Ricca, catalog no. 3536-16), followed by eosin-Y solution (Richard-Allan Scientific, catalog no. 7111) for 20 seconds. The stained sections were dehydrated, mounted and imaged using a Keyence microscope (BZ-X700).

Histomorphometric analysis was conducted to evaluate the quantity of the neo-bone tissue. Six H&E images of each defect were randomly selected and areas of the newly formed bone tissues resembling the morphology of native bone were identified. The total area of the newly formed bone tissue, as well as the total defect area, were analyzed using ImageJ. The mean areal density of the newly formed bone was presented as the fraction of bone area per defect area.

Osteoclasts were detected by the tartrate-resistant acidic phosphatase (TRAP) staining. Briefly, The TRAP incubating solution was prepared by first mixing 50 μ L of Fast Garnet GBC base solution with 50 μ L of sodium nitrite solution. This mixture was added to 4.5 mL of pre-warmed DI water at 37°C. After mixing, 50 μ L of Naphthol AS-BI phosphate solution, 200 μ L of acetate solution, and 100 μ L of tartrate solution were added and mixed to generate a working solution. Rehydrated sections were immersed in the working solution, incubated at 37°C for 1 h in dark, and rinsed with DI water. After staining, the sections were dehydrated with increasing concentration of ethanol and finally with CitriSolv until equilibrium was reached. Slides were mounted with glycerol and imaged immediately. Quantification of TRAP-positive staining was performed using ImageJ. The mean areal density of TRAP-positive area was presented as the percentage of total TRAP-positive area over total tissue area.

For immunohistochemical and immunofluorescent staining, rehydrated sections were immersed in a solution of proteinase K (20 μ g/mL; Invitrogen, catalog #1000005393) in 95% (v/v) TE buffer (50 mM Tris-HCl, 1mM EDTA, and 0.5% [v/v] Triton X-100; pH 8) with 5% (v/v) glycerol and incubated for 15 min at 37°C. For immunohistochemical staining, sections were incubated in a blocking buffer containing 3% (w/v) bovine serum albumin (BSA) for 1 h at 25°C. Samples were incubated with primary antibody against osteocalcin (OCN) (1:100, rabbit polyclonal, Abcam, catalog#ab93876), or BMP-2 (1:100, rabbit polyclonal, Abcam, catalog #ab1493) in blocking solution for 9 h at 4°C. Sections were rinsed with PBS and treated with 3% (v/v) hydrogen peroxide for 7 min at 25°C. The samples were then incubated with secondary antibody (1:100, horseradish peroxidase-conjugated donkey anti-rabbit, Jackson ImmunoResearch, catalog #711-035-152) in a blocking solution for 1 h at 25°C. The treated sections were rinsed with PBS and developed by immersion in 3,3'-diaminobenzidine (DAB) substrate solution (Vector Laboratories,

catalog #SK-4100) for 1 min at 25°C. The sections were washed with PBS and gradually dehydrated using increasing concentrations of ethanol and incubated in CitriSolv until equilibrium was reached. Slides were mounted and imaged immediately. For immunofluorescent staining, sections were washed with PBS and permeabilized using 0.1% Triton X-100 for 10 min at 25°C, which were then treated with sodium borohydride solution (2.5 mg/mL in 50% [v/v] ethanol) for 30 min at room temperature. The sections were immersed in a blocking solution (3% [w/v] BSA and 0.1% [v/v] Triton X-100) and incubated for 1 h at 25°C. Sections were then incubated with primary antibody against CD31 (Platelet endothelial cell adhesion molecule [PECAM-1], 1:100, goat Santa Cruz Biotechnology, catalog #sc-1506) in a blocking buffer for 12 h at 4°C and rinsed with PBS.

The immunohistochemical (IHC) and immunofluorescent staining were analyzed using ImageJ. For IHC staining, the expression of the markers was represented using mean histogram intensity as described earlier [48]. Histogram intensity ranges from 0–255, with 255 representing white (lightest) and 0 representing black (darkest), indicating that the higher the histogram intensity, the lower the expression of the marker. For immunofluorescent staining, the mean areal percentage of the CD31-positive region was presented as percentage of total CD31-positive area over total defect area.

2.7 Statistical Methods

Each experiment used 5 biological replicates ($n=5$) and repeated at least twice to independently verify the findings. GraphPad Prism 7 software was used to perform statistical analysis of the data. Two-tailed Student's *t*-test was applied when comparing two groups at the same time point. One-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test was used for comparisons between multiple groups within the same time point.

3. Results

3.1 Fabrication and Characterization of mineralized biomaterials

Macroporous cryogels made of PEGDA-co-A6ACA were used for the studies. The SEM images showed macroporous architecture of the mineralized scaffold networks composed of randomly oriented, interconnected macroporous structures measuring approximately 80–200 μm (Figure 1A). The cryogels were mineralized to incorporate calcium phosphate moieties, where the carboxyl functional groups of the A6ACA moieties bind to Ca^{2+} and initiate nucleation and growth of mineral crystals. The bound minerals of the scaffold showed a flat, plate-shaped morphology (Figure 1B). EDS analysis further confirmed the presence of CaP minerals within the mineralized scaffolds and the calcium (Ca^{2+}) to phosphate (PO_4^{3-}), Ca/P ratio was estimated to be 1.5 (Figure 1C). As anticipated, both Ca^{2+} and PO_4^{3-} were released from the mineralized scaffolds and showed similar release profiles (Figure 1D-E). Each biomineralized scaffold contained a total amount of 102.65 ± 2.91 mg/g of Ca^{2+} , and 155.32 ± 8.90 mg/g of PO_4^{3-} (Figure 1F-G). The molar ratio of the released Ca^{2+} to PO_4^{3-} was estimated to be $\sim 1.6 \pm 0.2$ (averaged over 7 days). This is very similar to the stoichiometric Ca/P ratio in hydroxyapatite (1.67), implying that the Ca^{2+} and PO_4^{3-} release from the scaffolds could be a result of dissolution of an apatite-like CaP phase from the scaffolds.

3.2 Evaluation of hard tissue formation

Hard tissue formation within the cranial defects treated with the mineralized scaffolds, as well as the sham (untreated) groups, was examined by μ CT at 2 and 8 weeks post-implantation (Figure 2). μ CT images after 2 weeks showed some form of hard tissue formation in all groups treated with mineralized scaffolds albeit with varying levels of differences (Figure 2A). The extent of hard tissue formation within the defect decreased with increasing age with 6-month and 14-month-old mice having the lowest levels of calcified tissue within the defect at 2 weeks post-implantation (Figure 2A). At 8 weeks post-implantation, almost complete closure of the defects with calcified tissues of varying degree of calcification was observed in all groups (Figure 2A). In contrast, sham groups had minimal hard tissue formation within the defect site throughout the extent of the study.

Quantification of hard tissue formation by bone volume over total volume (BV/TV) within the defect site corroborated the observations made from the μ CT images (Figure 2B-D). At 2 weeks post-implantation, 1-month and 2-month-old mice had higher hard tissue formation compared to the older cohorts (6-month and 14-month-old mice). Between the 6-month and 14-month-old mice, no statistical difference was observed in hard tissue formation (Figure 2C). Quantification of hard tissue formation at 8 weeks after implantation demonstrated similar trends as that of 2-week, where decreased bone formation was observed in older cohorts (Figure 2D). At 8 weeks post-implantation, 6-month-old cohorts showed similar calcification as to 2-month-old cohorts. While post-implantation time-dependent increase in calcification was observed in 1, 2, and 6-month-old cohorts, no significant change in calcification was observed in 14-month-old mice between 2 and 8-weeks post-implantation. At 8 weeks post-implantation, the μ CT analyses showed similar BV/TV percentage between the newly formed and the surrounding native bone tissue for 1-month-old mice. For all other age groups, the BV/TV percentage of the native tissue was significantly higher compared to the neo-bone tissue within the implant (Figure 2E).

3.3 Bone tissue formation

The H&E staining of the excised implants showed neo-bone tissue formation within the defects. Despite significant host cell infiltration, only a limited amount of detectable bone tissue was observed in the case of 6-month and 14-month-old groups at 2 weeks. In contrast, 1-month-old mice showed some level of neo-bone tissue formation. The extent of bone formation increased with post-implantation time. By 8 weeks, neo-bone tissue formation was observed among all age groups with 1-month old group had the lower bone formation. Furthermore, in the 14-month-old group, a thin layer of fibrous-like tissue was found to present and 2-month-old mice showing a significantly higher level of bone formation compared to 6-month and 14-month-old mice. Between 6-month and 14-month-old cohorts, the 14-month-old group had the lower bone formation. Furthermore, in the 14-month-old group, a thin layer of fibrous-like tissue was found to present at the periphery of the defect sites (Figure 3A). Histomorphometric quantification of areal fraction of bone tissue based on the H&E staining confirmed that bone tissue formation decreased as mouse age increased (Figure 3B). In the case of sham groups, no bone formation was observed and the defects were bridged by a thin layer of fibrous tissue, which is consistent with that normally found in non-healing bone defects (Figure S1A) [49].

To further confirm the neo-bone tissue formation, the tissue sections were stained for OCN, a bone-specific matrix protein that is secreted by osteoblasts. At 2 weeks, all groups treated with mineralized scaffold showed the presence of extracellular matrix enriched with OCN (Figure S2A). However, the expression of OCN decreased as the age of the mice increased. A similar trend was observed at 8 weeks of post-implantation (Figure 4A). The mean histogram intensity of the images confirmed the aforementioned observations in which lower histogram values indicate higher OCN expression (Figure 4B). In the case of 14-month-old mice, no significant change in OCN expression between 2 and 8 weeks was observed. The fibrous tissue bridging the defect site of sham groups was found to show minimal OCN expression at 8 weeks post-implantation. (Figure S1B).

At 2 and 8-week post-implantation, different age groups showed varying expressions of BMP-2, a protein commonly involved during osteogenesis (Figure 4A and Figure S2A). At 2 weeks post-implantation, all age groups showed the presence of BMP-2 within the defects treated with mineralized scaffolds. The expression of BMP-2 decreased as the age of the mice increased. Abundant expression of BMP-2 was observed in 1-month and 2-month-old groups, while moderate to slight expressions were found in the 6-month-old group and 14-month-old group, respectively (Figure S2A). At 8 weeks post-implantation (Figure 4A), the 6-month-old group had the highest expression of BMP-2 among all groups, while the 14-month-old group exhibited the lowest expression. Also, compared to the 1-month-old group, the 2-month-old group demonstrated higher expression of BMP-2. The mean histogram intensity of the images corroborated the findings in which lower histogram values indicate higher BMP-2 expression (Figure 4C and Figure S2C). Similar to OCN expression, the fibrous tissue bridging the defect site of sham groups was found to have a minimal BMP-2 expression at 8 weeks post-treatment (Figure S1B).

Tartrate-resistant acid phosphatase (TRAP) staining was performed to examine the presence of osteoclast-like cells within the newly formed bone tissue (Figure 4A and S2A). At 2 weeks post-implantation, TRAP-positive cells were detected in all defects treated with mineralized scaffolds except 14-month-old cohorts, where no TRAP-positive cells were detected (Figure S2A and Figure S2D). Following 8 weeks of implantation, TRAP-positive cells were detected in all age groups. Among the different age groups, more TRAP-positive cells were observed in younger mice (Figure 4A and Figure 4D). In 1, 2 and 6-month-old groups, TRAP-positive cells were located within the mineralized implants. However, in the 14-month-old group, TRAP-positive cells were observed only at the interface of the native tissue and the implant (Figure 4A). In the sham groups, no TRAP-positive cells were detected at 8 weeks post-implantation (Figure S1B).

3.4 Vascularization and Recruitment of cells

Hematoxylin and eosin (H&E) staining after 2 weeks of implantation showed significant host cell infiltration in all treated groups (defects covered by mineralized scaffolds) despite the differences in host age. The recruited cells were homogeneously distributed throughout the implant. Concomitant with the recruitment of cells, all the defects treated with the mineralized scaffolds were positive for vasculature throughout the defect, with the presence of red blood cells, indicating formation of anastomosed vessels with the host (Figure 3 and

Figure S4). The presence of vasculature within the implants persisted throughout the experimental time (8 weeks post-implantation). The presence of vasculature was further confirmed by staining for platelet endothelial cell adhesion molecule (PECAM-1; CD31) at 2 and 8 weeks (Figure S3A and Figure 5A, respectively). At 2 and 8 weeks post-implantation, all groups showed the presence of CD31-positive cells within the defects. At 2 weeks, there were more CD31 positive cells present in the 1 month and 2-month-old groups than 6-month and 14-month-old groups (Figure S3B). By the end of 8 weeks, amounts of vasculature decreased in 1, 2 and 14-month-old groups, whereas the 6-month-old group demonstrated more vasculature (Figure 5B).

4. Discussion

Previously, we have shown mineralized materials that can recruit endogenous cells and support their osteogenic differentiation to form bone tissue can be used to repair cranial defects [32]. We have also established that the CaP mineral component is necessary for recruiting and activating endogenous cells for bone repair [32]. Herein, we evaluated the effect of host age on mineralized biomaterial-mediated bone tissue formation using a critical-sized cranial defect as a model system. Consistent with our previous findings, results from this study show that the mineralized scaffolds support neo-bone tissue formation within the defect through recruiting endogenous cells [32].

Histological analyses of the excised tissues suggest that all implants were infiltrated with host cells despite the age differences among the recipients. The macroporous nature of the scaffold not only facilitated cell infiltration, but also aided homogeneous distribution of the host cells throughout the implant. Similar to cell infiltration, all implants were characterized by the presence of intraluminal red blood cells, suggesting that the neo-vessel within the implant had anastomosed with the host vasculature. A number of factors, such as microarchitecture of the scaffold and presence of calcium phosphate minerals, could contribute to implant vascularization [50, 51]. The mineral phase of the CaP-biomaterials also plays an important role in vascularization [51]. Besides scaffold properties, the infiltrated cells could also promote vascularization of the implants. Osteoblasts and mesenchymal progenitor cells are known to secrete pro-angiogenic growth factors such as vascular endothelial growth factor A (VEGF-A) [52, 53].

OCN is an osteoblast-specific marker [54], whereas BMP-2 is mostly a pre-osteoblast marker [55]. While OCN expression showed an age-dependent decrease in expression throughout the study, BMP-2 expression in 6-month-old mice was more prominent than 1-month and 2-month-old mice at 8 weeks post-implantation. This high expression of BMP-2 could be associated with the delayed osteoblastogenesis in 6-month-old mice. Concurrent with these observations, μ CT analyses demonstrated that BV/TV percentage of 6-month-old mice, which was significantly lower than the 2-month-old group at 2 weeks post-implantation, resembled that of the 2-month-old group by 8 weeks post-implantation. Taken together, these observations imply delayed osteoblastogenesis/bone healing in the middle-age group compared to the younger groups. In contrast, the persistent low expression of these makers in the 14-month-old mice suggests impaired osteoblastic differentiation/ bone forming abilities in the elderly group. As for 1-month and 2-month-old mice, the low BMP-2

expression and high OCN expression at 8 weeks could indicate maturation of osteoblasts, suggesting more efficient bone reparative capabilities in the younger groups.

Coincided with high BMP-2 expression, 6-month-old mice also exhibited the highest vascularization at 8 weeks post-implantation among all the groups. This high vascularization could be associated with the high osteoblastogenesis in 6-month-old mice at 8 weeks post-implantation. Vascularization plays a key role in bone formation [56] and the crosstalk between osteoblasts and endothelial cells is necessary for bone formation and regeneration [57, 58]. In intramembranous osteogenesis, extensive vascularization was reported during the transition of preosteoblasts to osteoblasts [57]. In addition to promoting osteoblastogenesis, BMPs stimulate angiogenesis through the production of VEGF-A by osteoblasts[57].

Our findings showed that the number of TRAP-positive cells in the neo-bone tissue decreased as the age of mouse increased. Besides this decline, the TRAP-positive cells were only found at the interface of the implant and native bone in the case of the 14-month-old mice by 8 weeks. Osteoblasts not only play a central role in bone formation, but also regulate osteoclast maturation through soluble factors, which is necessary for bone resorption and remodeling[59, 60]. The role of cell-cell communication in terms of direct contact is necessary for osteoclastogenesis, and therefore the minimal osteoblast content within the implants could have contributed to the lack of TRAP-positive cells in the 14-month-old group. Osteoblasts have been shown to secrete monocyte chemoattractant protein-1 (MCP-1) to recruit osteoclastic precursors, and receptor activator of nuclear factor kappa-B ligand (RANKL) to induce osteoclast differentiation of these cells[59]. The lack of osteoblasts within the implant could have led to less precursor recruitment and osteoclast formation.

Osteoclasts not only play a key role in bone remodeling through bone resorption, but also contribute to bone formation through multiple processes [61, 62]. In addition to RANKL/RANK interactions, osteoclastic cells also communicate with osteoblastic cells through ephrinB2/EphB4 signaling (ephrinB2 expressed by osteoclast progenitor cells and EphB4 expressed by osteoblastic cells)[63]. Forward signaling through EphB4 in osteoblasts has been shown to enhance their differentiation resulting in increased bone mass[64]. Furthermore, resorption of matrix releases and activates a number of growth factors that are shown to promote osteoblast activity and bone formation[65].

Age of the recipients had a strong influence on the rate and the amount of bone formation. As evident by the radiographic and histochemical analyses, 6-month-old mice showed delayed bone regeneration, while the 14-month-old mice exhibited a compromised tissue repair ability with no significant increase in calcification observed between 2 to 8 weeks. These findings are in agreement with the clinical observations that the ability of the bone tissue repair declines with age [66–68]. Given that the physicochemical cues of the implant are consistent across the groups, the delayed bone regeneration in older groups could be associated with decreased osteogenic potential [69–72], lower clonogenic ability [38, 73, 74], decreased proliferation [73, 75], and diminished vascular support [76] of osteoprogenitor cells. Age-related decrease in growth hormone levels involved during bone

formation [77, 78], and decline in angiogenesis [34, 79, 80] may contribute to impaired bone repair. Since for C57BL/6/J mice, 14-month-old is not considered as an age of postmenopausal, the compromised bone repair observed in this study may not be entirely due to sex steroid deficiency associated with postmenopause [81]. Previous *in vitro* studies have shown that bone marrow-derived mesenchymal stem cells from aged mice exhibit lower VEGF expression, stromal cell-derived factor 1 chemokine (SDF-1), protein kinase B than those from young mice [82]. Both VEGF and SDF-1 are involved in neovascularization and angiogenesis. In addition, age-associated changes of inflammatory environments could also have a profound influence on bone regeneration through affecting the levels of inflammatory mediators that are pivotal for osteoblast differentiation and subsequent bone formation [83–85].

The results discussed in this study involves female mice, and similar experiments should be performed with male mice in order to fully elucidate effects of age on biomaterial-mediated bone regeneration. Furthermore, our current work only focuses on repairing of cranial defect, which involves intramembranous ossification. Hence, caution should be used while extrapolating results from this model to long bone defects which involve endochondral ossification[86].

5. Conclusion

In summary, this study demonstrates age-related host responses in the healing of critical-sized cranial defects treated with biomineralized materials. Our findings show that mineralized macroporous biomaterials are sufficient to induce hard tissue formation through recruitment of endogenous cells and achieve almost complete closure of critical-sized cranial defects in mice. Moreover, the age of the recipient mice had a significant influence on the quantity and quality of the neo-bone tissues characterized in terms of bone mineral deposition and bone tissue-specific markers. Specifically, as elucidated by radiographic and histological evidence, delayed bone formation and decreased quantity of neo-bone tissue formation were observed in older mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. SEM/EDS and calcium phosphate dissolution of mineralized PEGDA-co-A6ACA macroporous hydrogel.

(A-C) Scanning electron microscopy image of mineralized PEGDA-co-A6ACA macroporous hydrogel. Scale bar: 200 μm . (A) The macroporous network composed of randomly oriented, interconnected macroporous structures measuring 80–200 μm in diameter. Scale bar: 50 μm . (B) High magnification images revealed the presence of mineral crystals. (C) Elemental dispersive spectroscopy (EDS) spectrum showing the presence of calcium and phosphate in the mineralized phase with Ca/P ratio of 1.5. (D-G) Total mineral contents and dissolution of the mineralized phase into Ca^{2+} and PO_4^{3-} ions. The release of

(D) Ca^{2+} and (E) PO_4^{3-} as a function of time in ion-free Tris-HCl (1M, pH = 7.4) (F) Ca^{2+} content and (G) PO_4^{3-} content of the biomineralized scaffold prior to implantation.

Figure 2. Calcified hard tissue formation within critical-sized cranial defects.

(A) Micro-computed tomography (μ CT) images of cranial defects in 1, 2, 6 and 14-month-old mice treated with biom mineralized scaffolds (M) and untreated (sham) groups at 2 and 8 weeks post-implantation. Scale bar: 1 mm. (B) Quantification of BV/TV percentage of sham and mineralized groups at 2 and 8 weeks post-implantation. (C) Quantification of defect closure at 2 weeks post-implantation. (D) Quantification of defect closure at 8 weeks post-implantation. (E) Comparison of BV/TV percentage between the neo-bone tissue formed within the mineralized scaffold and the surrounding native bone tissue at 8 weeks post-

implantation. Asterisks denote p -values with statistical significance (***) indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

Figure 3. Morphological assessment of bone formation within critical-sized cranial defects at 8 weeks post-implantation.

(A) Hematoxylin and eosin (H&E) staining of cranial sections for 1, 2, 6 and 14-month-old mice. High magnification images and insets reveal neo-bone tissue within the defect site. Yellow asterisks denote bone formation. White arrows indicate intraluminal blood vessels. Scale bars: 500 μm (upper panel) and 100 μm (lower left panels), and 25 μm (lower right panels). (B) Histomorphometric quantification of bone tissues. Asterisks denote p -values with statistical significance. (**** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

Figure 4. Bone-specific markers in newly formed tissue within cranial defects of biomaterialized scaffold-treated groups at 8 weeks post-implantation.

(A) Left column: immunohistochemical staining for osteocalcin (OCN) of the cranial defect site; middle column: immunohistochemical staining for bone morphogenetic protein 2 (BMP-2) of the cranial defect site; right column: histochemical staining for tartrate-resistant acid phosphatase (TRAP) of the cranial defect site. Arrows indicate TRAP-positive cells present within the scaffolds. Scale bars: 100 μ m. (B) Mean histogram intensity of OCN expression in the left column of (A). (C) Mean histogram intensity of BMP-2 expression in the middle column of (A). (D) Quantification of areal percentage of TRAP-positive area per

defect area in the right column of (A) (**** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

Figure 5. Vascularization of cranial defects treated with biomineralized scaffolds at 8 weeks post-implantation.

(A) Immunofluorescent staining for platelet endothelial cell adhesion molecule (PECAM-1; CD31) and Hoechst 33342 staining of cell nuclei within the defect site. Scale bar: 100 μ m.

(B) Quantification of the areal percentage of CD31 positive area per defect area. (**** indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).