Effects of Particle Size and Demineralization Time on BMP-2, -4, and –7 in Demineralized Bone Matrix

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INTRODUCTION:

Demineralized bone matrix (DBM) is a bone graft known for its clinical success in a variety of applications. DBM is a processed bone allograft composed of the collagen bone matrix and bound growth factors implicated in ectopic bone formation [1]. DBM is produced by acid extraction of bone which removes mineral to increase the bioavailability of the matrix bound bone morphogenetic protein (BMPs) pool. BMP-2, BMP-4, and BMP-7 are commonly considered the key components of DBM responsible for the osteoinductive response. DBM is also believed to retain the architecture of innate bone which may contribute to an osteoconductive response [2,3].

DBM is traditionally manufactured by grinding bone to a range of particle sizes or fibers. The geometry and structure alone may affect the biological components and in vivo responses. The effects of acid exposure time and DBM geometry and structure remains controversial. BMPs and the collagen matrix may become susceptible to diffusion or damage due to prolonged exposure in acid. Despite being mutually dependent variables regarding mineral content, particle size and demineralization time have not been rigorously analyzed in the same study, particularly extended acid exposure time points. This study isolated the role of particle size and demineralization time to further understand how these processes influence demineralization kinetics, BMP levels, and the architecture of DBM using rabbit cortical bone. The athymic rodent model is typically used to study the in vivo response of human DBM. Rabbit bone was chosen as the source material in our study. This allows for future preclinical work in the rabbit model with an intact immune system.

METHODS:

Material: Cortical bone from adult rabbits sourced from other ethically approved studies were milled and sieved to three particle size ranges: 150-250µm, 250-600µm, and 600-850µm. Particles were demineralized in 0.5N HCl (50mL/1g of bone) at: 0, 5, 10, 15, 20, 30, 40, 60, 90 minutes at room temperature with stirring in triplicate for each size range. The DBM was neutralized with PBS and DI water washes. Once neutralized (pH >6) the material was then lyophilized and aliquoted for analysis.

Analysis: Elemental composition of mineral (g/g) was obtained through inductively coupled plasma spectroscopy. Material structure was analyzed with scanning electron microscopy (SEM), X-Ray Diffraction (XRD), and stereozoom microscopy. Particle size was determined using ImageJ ParticleSizer plugin on light microscopy images (n=30 particles per group). Proteins were extracted with 4M GuHCl/0.05M Tris-HCl buffer as previously reported [4,5]. BMP quantities were measured using commercially available human BMP-2, -4, and -7 ELISA kits (RayBioTech).

Statistical Methods: Independent sample two-sided t-test assuming unequal variance was performed on particle sizes before and after demineralization. ELISA data was analysed using independent sample Kruskal Wallis non-parametric statistics. SPSS was used for all statistical analyses.

RESULTS:

Particle size was a significant factor for mineral kinetics with smaller particles demineralizing more quickly than larger particles. Peak demineralization (Ca^{2+}< 2% by weight) occurred at 5, 20 and 40 minutes for 150-250µm, 250-600µm, 600-850µm particles respectively. XRD signals display the loss of hydroxyapatite crystalline structure while the material retained high levels of mineral. Particle size analysis showed a significant difference in size between non-demineralized and demineralized (90-minute time point) particles. An area reduction of 36% for 150-250µm size group (p=0.001), 28% for 250-600µm size group (p=0.022), and 40% for 600-850µm size group (p<0.001) occurred. ELISA data revealed greater amounts of BMP-2 compared to BMP-7 and was more dependent on particle size for BMP-2 (Figure 1). BMP-4 was not detected for any of the samples.

DISCUSSION:

The study examined the role of particle size and demineralization time to further delve into their potential roles in DBM graft materials. Rabbit DBM was characterized in the current study, thus allowing future studies to examine the in vivo response of DBM in animals with intact immune systems. Alterations in the collagen matrix of DBM in the form of surface erosion and bulk changes were influenced by particle size and acid exposure time. SEM images support a change in native ultrastructure of DBM with particle size and time (Figure 2). ELISA data demonstrates that BMP-2 levels are also influenced by particle size more so than BMP-7 under the current conditions. This leads to insight into the storage and affinities of BMPs to the collagen matrix, which can be influenced by processing variables.

Limitations: This small sample size laboratory-based study is limited in that only particles of specific size ranges were studied using rabbit bone. In-vitro investigations provide useful information but fail to elucidate in vivo responses. Further in vivo studies on the influence of material structure and BMP levels are warranted to understand osteoinduction with this graft material in the presence of an intact immune system.

SIGNIFICANCE/CLINICAL RELEVANCE: DBM processing methods, including particle size/distribution as well as demineralization kinetics are important variables to consider in the development and clinical application of particulate DBM graft. Optimization in DBM processing has the potential to improve clinical efficacy or find new applications.

REFERENCES: [1] Urist M R, et al., *Journal of dental research*. **50,6**, 1971. [2] Liu M, et al., *Nanomaterials.* **8,12**: 999, 2018. [3] Gruskin E, et al., *Advanced drug delivery reviews*. **64,12**, 2012 [4] Pietrzak W S, et al., *The Journal of craniofacial surgery*. **26,1:** 296-9, 2015. [5] Song K, et al., *Cell and tissue banking.* **24,1**: 203-210, 2023.

IMAGES:

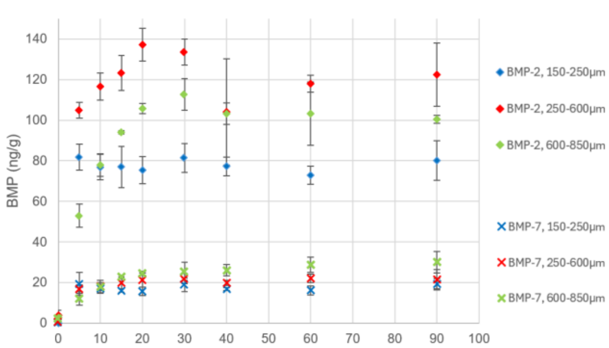

Figure 1. BMP-2 and BMP-7 throughout demineralization time.

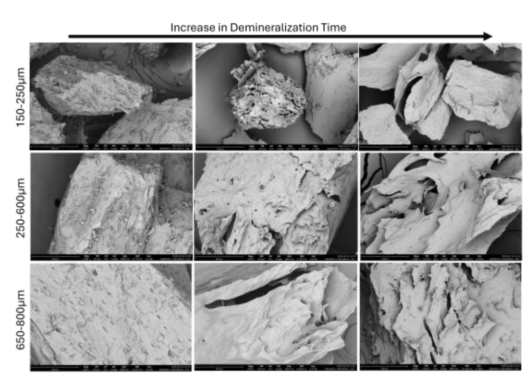

Figure 2. SEM images displaying increased morphological deterioration throughout demineralization (1000X magnification).