

# Evaluation of the Natural Matrix Protein® (NMP®) bone allograft in vitro and in vivo

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## Introduction

The demineralization of bone increases the release of BMPs from the bone matrix making the demineralized bone matrix (DBM) osteoinductive<sup>1</sup>. It is often under appreciated that other growth factors are also present in bone matrix which can stimulate bone repair, including VEGF, PDGFbb and TGF- $\beta$ <sup>1,2</sup>. However, the clinical effectiveness of DBM at creating new osseous tissue as a bone void filler is limited. Pietrzak et al. demonstrated that both BMP-2 and BMP-7 exist within bone in both a tightly and a weakly bound state, and that only a fraction of these BMPs are released from the DBM upon incubation with buffer<sup>3,4</sup>. Histological investigation of DBM grafted sites indicates that significant amounts of the implanted matrix remain nine (9) months after clinical placement<sup>5</sup>. The result is that the majority of these growth factors remain trapped within in the bone matrix being unable to create any bone stimulatory effects.

This paper discusses the scientific results of an evaluation of a proprietary process that has been developed to increase the bioavailability of BMPs in bone matrix allografts, with the goal of increasing the effectiveness of these bone allografts. Allografts processed utilizing this propriety methodology are referred to as Natural Matrix Protein (NMP) grafts or more appropriately NMP bioimplants.

## Aims of this Investigation

1. Compare the bioavailability of BMP-2, BMP-7, VEGF, PDGFbb, and TGF $\beta$ 1 from human bone processed using either a traditional DBM or an NMP process,
2. Evaluate the activity of NMP bioimplants in an *in vitro* assay for osteoinduction (OI) and,
3. Compare the osteoinductive potential of commercial preparations of DBM and NMP bioimplants with rhBMP-2 on a collagen sponge (Infuse<sup>®</sup>) in a well-established *in vivo* OI assay.

## Materials

Human bone was obtained from AATB registered tissue banks where donors had provided consent for research use of their tissue. The bone was determined to be suitable for use based on the results of screening and testing as per FDA regulations and AATB standards. All bone was debrided of soft tissue, defatted and ground into particulates or fibers before undergoing demineralization or the NMP process.



NMP Fibers after hydration with water

## Methodology

### Bioavailability of Growth Factors

Test articles were prepared from donated allogeneic bone to create DBM and NMP bioimplants. 100mg samples of each test article (DBM and NMP) were extracted in 4mLs of Tris buffered saline (TBS) or 50mM acetic acid (AcOH) at room temperature on a rocker for 24 hours. The samples centrifuged and the supernatants stored at -20°C until assayed by ELISA (R&D Systems, MN) for BMP-2, BMP-7, PDGFbb, VEGF, TGF- $\beta$ 1.

### In Vitro Osteoinductive Activity

Samples of each test article were added to C2C12 cells. Controls included cells only, inactive DBM (extracted with guanidine HCl), and 50ng/mL rhBMP-2. After 14 days the cell layer was extracted with MPER (Sigma Aldrich, St Louis MO) the extract centrifuged and the supernatant stored at -20°C until assayed for alkaline phosphatase activity using para-nitrophenol phosphate<sup>6</sup>.

### In Vivo Osteoinductive Activity

Twenty male athymic rats were divided into 4 groups of 5 animals each containing bioimplants of either demineralized bone matrix (0.2 cc DBM), Infuse (0.105mg rhBMP-2/0.2cc ACS, Medtronic, Minneapolis, Minnesota), NMP Fibers (0.2cc Natural Matrix Protein bioimplant, Induce Biologics, Irvine, California) or NMP Microparticulate (0.2cc Natural Matrix Protein bioimplant, Induce Biologics, Irvine, California) in both hind legs, meaning 10 implants were evaluated in each group.

Young adult (6 to 9 weeks of age) male athymic rats were quarantined for seven (7) days. The animals were anesthetized with intraperitoneal ketamine (250 mg), xylazine (11 mg), and physiological saline (10 mL) using a dosage of 3.6 mL/kg body weight. A 1 cm skin incision was made through skin parallel to the femur. Approximately midway between the hip and the knee, the membrane between the muscle groups (gluteus superficialis and the biceps femoris) was punctured with the tips of the iris scissors, and then open the scissors to stretch the puncture opening. The forceps were then opened in the opposite direction of the stretch to form a pocket between the muscle groups.

The test article was then inserted into the muscle pouch just below the femur. Both muscle pockets were sutured by placing a single suture in the muscle and the skin incision was closed with a suture. A similar procedure was then performed on the opposite leg and the same test material was placed.

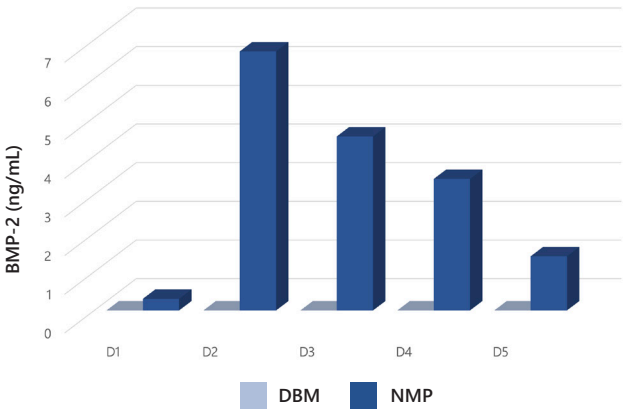
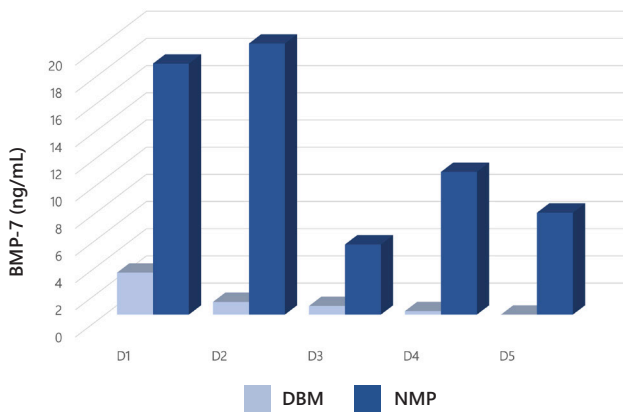
After 28 days the resultant tissue samples were harvested from the animals and the implant along with its surrounding tissue were fixed in 10% NBF. Once fixed the samples were analyzed by  $\mu$ CT for total explant volume, new bone volume and volumetric

mineral density (a measure of the quality of bone). The data was then compared for statistical significance using an analysis of variance (ANOVA). After  $\mu$ CT analysis the implants were decalcified, embedded in wax and sectioned and stained with H&E.

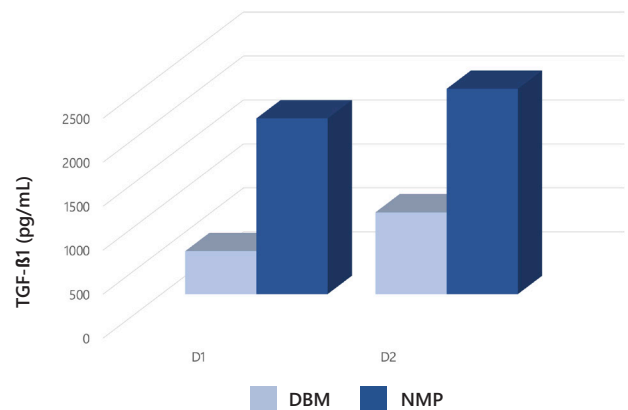
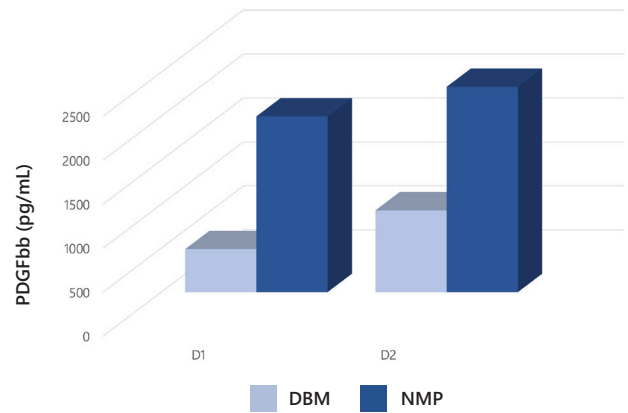
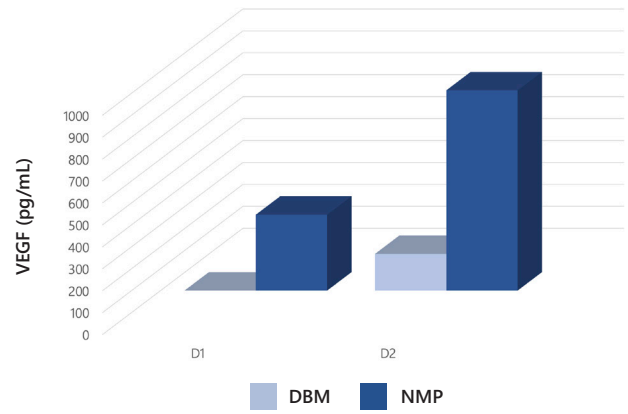
Surgeries were performed by Ibex Research, Logan Utah according to ASTM 2529-13. MicroCT images (Quantum FX, Perkin Elmer, MA) and data collected and analyzed by separate blinded observer. The mineral content of the  $\mu$ CT scans was calculated by comparison to a phantom and the explanted tissue was segmented into bone and non-bone using 200mg HA/cc as the threshold<sup>8</sup>.

## Results

### Bioavailability of Growth Factors



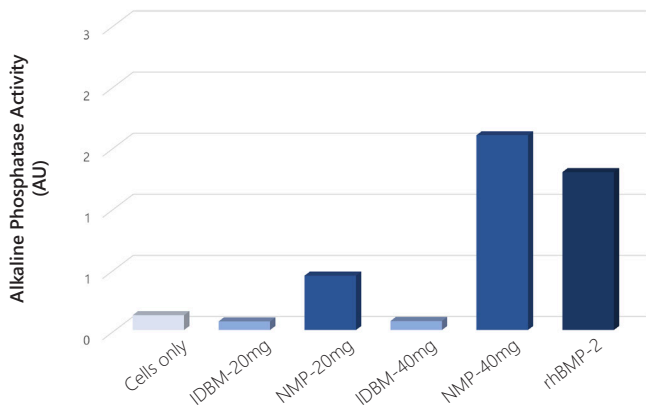
Ground cortical bone particulate was obtained from 5 separate donors. BMP-7 and BMP-2 bioavailability in NMP samples was higher than for matched DBM samples (mean $\pm$ SD).



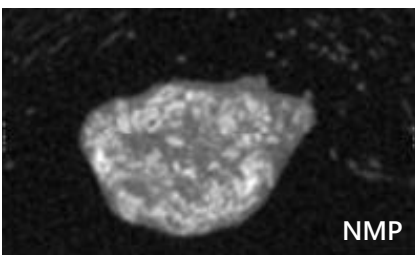
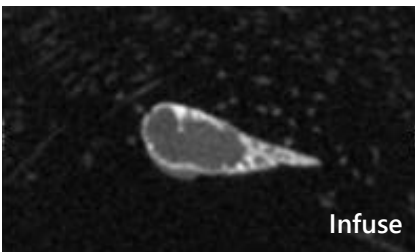
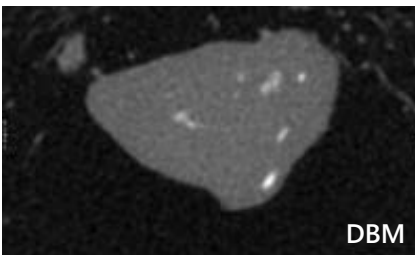
Cortical bone from 2 different donors was processed into DBM or NMP. Bioavailability of PDGFbb, VEGF and TGF- $\beta$ 1 was higher in the NMP samples than the DBM samples for both donors.

### In Vitro Osteoinductive Activity

The 40mg of NMP had significantly higher ALP activity than cells only, 40mg inactive DBM or 20mg of NMP or DBM (n=4; mean ±SD. 1-way ANOVA P<0.001 for each).



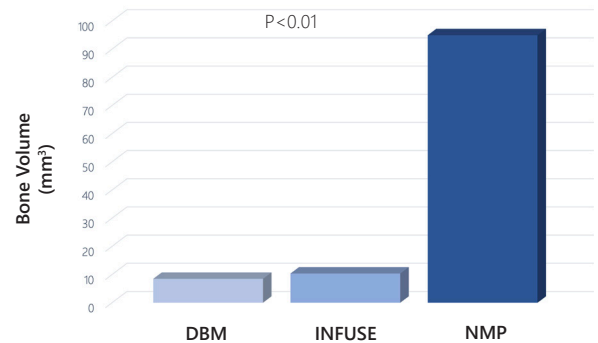
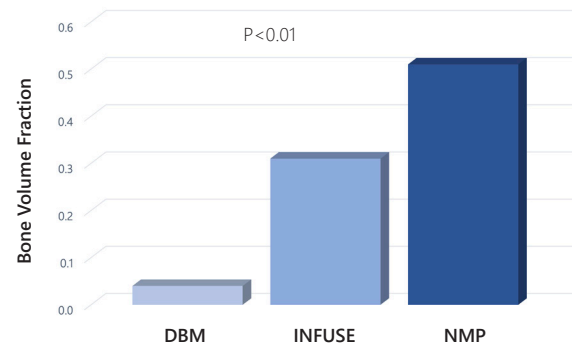
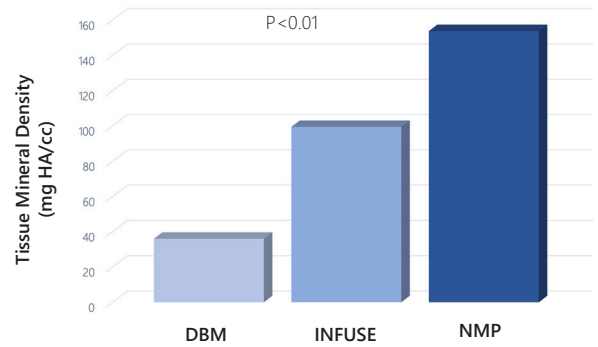
### In Vivo Osteoinductive Activity



MicroCT slice views of DBM, Infuse and NMP explants. Central gray mass shows the explanted tissue. White areas indicate areas of high mineral density.

The NMP explant had mineralized tissue throughout the explant while the Infuse implant was smaller and had a thin shell of mineralized tissue surrounding a non-mineralized core.

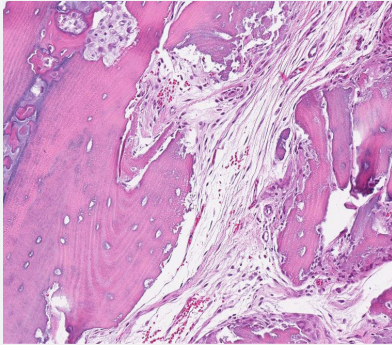
### MicroCT Analysis



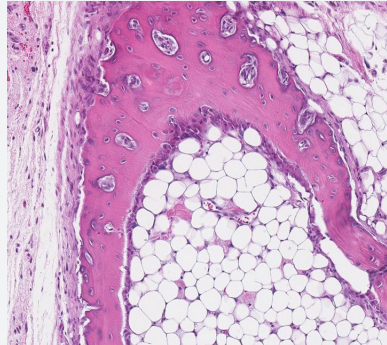
NMP explants had significantly greater Tissue Mineral Density, Bone Volume and Bone Volume fraction than Infuse or DBM explants (1-Way ANOVA, P<0.01 for all comparisons).

## Histology

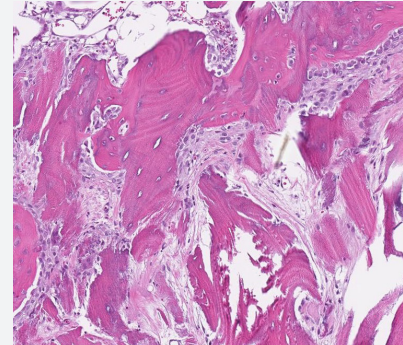
H&E sections of DBM, Infuse and NMP explanted tissue. (Original Magnification 10x)



**DBM** explants had non vital bone matrix surrounded by a mixed cellular infiltrate. Focal areas of new bone and cartilage were seen.



**Infuse** explants had a thin ring of new bone surrounding a central core. The central core is largely acellular, with some areas of new bone.



**NMP** explants had non vital bone particles surrounded by large amounts of new bone with some areas of marrow.

## Conclusions

**NMP** bioimplants had significantly elevated levels of bioavailable BMP-2, BMP-7, VEGF, PDGFbb and TGF- $\beta$ 1 compared to DBM bioimplants processed from the same human allogeneic donor.

**NMP** bioimplants dose dependently stimulated alkaline phosphatase activity in C2C12 cells. These results suggested that in this model NMP bioimplants were more osteoinductive than those treated with rhBMP-2 (Infuse).

**NMP** bioimplants produced significantly more mineralized tissue (bone) than did the Infuse or the DBM bioimplants in vivo. The histological samples confirmed that this tissue was new bone. The bone formed by the NMP bioimplants was seen through-out the implant, while by comparison, the Infuse bioimplants formed a thin shell of bone (eggshell) around an unmineralized core.

## References

- <sup>1</sup> Pietrzak et al. (2011) Cell Tissue Bank 12:81–88
- <sup>2</sup> Devescovi et al. (2008) Chir Organi Mov 92:161–168
- <sup>3</sup> Pietrzak et al. (2012) Cell Tissue Bank 13:653–661
- <sup>4</sup> Pietrzak et al. (2017) J. Craniofac Surg 28: 2183–2188
- <sup>5</sup> Bouaicha et al. (2013) BMC Surgery 13:58.
- <sup>6</sup> Peel et al. (2003) J Craniofac Surg 14: 284–291
- <sup>7</sup> ASTM F2529-13 “Standard Guide for in vivo Evaluation of Osteoinductive Potential for Materials Containing Demineralized Bone (DBM)”
- <sup>8</sup> Rantalainen et al. (2014) Calcif Tissue Int 95:132–140

## Disclosures

NMP grafts are intended for use as a bone void filler for filling voids and gaps in the skeletal system that are not intrinsic to the stability of the bony structure.

NMP grafts are marketed by Induce Biologics USA <https://www.inducebiologics.com>

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