Osteoinductive Characterization of Demineralized Bone Fibers Using In Vitro ELISA-Based BMP-2 and 7 Assays

Chad Ronholdt, MBA - NVB Ventures; Nate Remlinger, PhD - Pinnacle Transplant Technologies; Candice Chen, MS - Pinnacle Transplant Technologies

Background:

Bone formation (osteogenesis) is a complex dance between various growth factors and bone-forming cells that continuously create, remodel, and maintain the natural bone structure. This process is driven by a subset of the Transforming Growth Factor-Beta (TGF-β) superfamily of cytokines and associated proteins, commonly referred to as Bone Morphogenic Proteins (BMPs).1 These BMPs are typically locked on the surface or within the bone and must be extracted using a series of demineralization and digestion steps. A rapid in vitro assay is a desirable early stage complement to the development process that can be used to guide formulation and methodology decisions based on the levels of extractable (bioavailable) BMPs from a demineralized bone matrix. OBJECTIVE: Develop an in vitro immunoassay (ELISA) to predict the bioavailability of two growth factors (BMP-2 and BMP-7) in human demineralized bone fibers (DBFs).

Methods:

All donors used for this research investigation were consented for research and processed at Pinnacle Transplant Technologies (PTT) using proprietary processing methods. The initial phase of testing included test articles (TA) obtained from eight (8) donors in addition to a negative control (NC) sample whereby mineralized cancellous was heat-inactivated by autoclaving at 127°C for 2 hours. A competitive demineralized fiber product with marketed osteoinductivity claims using an in vivo athymic rodent model2 was used as a tissue reference control (TC).

The initial digestion protocol was adapted from methods described by Blum, et al.2 and optimized for DBF formulations. All test articles and controls were normalized via weight and digested in collagenase and the extracts were centrifuged and analyzed via ELISA.

Results:

Research donors for this study were 78% male with an average age of 68.5 and a range of 31 to 85 years old. Twenty (20) individual collagenase extractions were plated in duplicate for a total of forty (40) datapoints per BMP assay. All values are represented as picograms of BMP per gram of dry weight tissue. Statistical analysis of the normalized data was performed within and between the donors using one-way ANOVA and demonstrated that there was no statistical difference of the BMP-2 or BMP-7 values within each donor ($p < 0.05$, data not shown). BMP values averaged 3.4% variability within each of the test article replicates. BMP-2 test article values ranged from 13,889 to 94,169 pg/g, and BMP-7 values ranged from 36,091 to 253,684 pg/g. When compared to a demineralized fiber product with confirmed osteoinductivity in the in vivo ASTM method2, PTT demineralized fibers outperformed the competitive tissue control by at least 3x within the BMP-2 assay and 1.4x within the BMP-7 assay (Table 1).

Conclusion:

The results from this investigation demonstrate that in vitro BMP-2 and BMP-7 ELISA assays can be used as a predictive tool to assess the bioavailability of growth factors present in demineralized fibers, as well as the reproducibility within and between different manufacturing processes. The data presented also strongly suggests that the Pinnacle Transplant Technologies' DBF fibers possess higher expression levels of BMP-2 and BMP-7 growth factors as compared to a confirmed competitive osteoinductive fiber product.

Osteoinductive Characterization of Human Demineralized Bone Fibers using *in vitro* ELISA-based BMP-2 and BMP-7 Assays

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Candice Chen*, MS; Chad J. Ronholdt†, MBA; Nathaniel T. Remlinger, PhD*

*- Pinnacle Transplant Technologies, Phoenix, AZ

† - New Vision BioLOGIC, Dallas, TX

INTRODUCTION

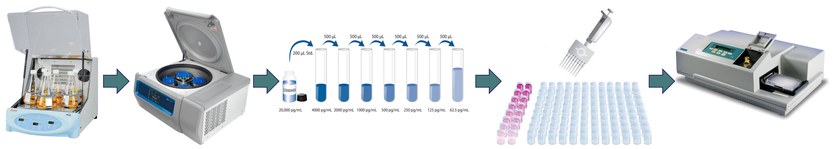
Bone formation (osteogenesis) is a complex dance between various growth factors and bone-forming cells that continuously create, remodel, and maintain the natural bone structure. This process is driven by a subset of the Transforming Growth Factor-Beta (TGF-β) superfamily of cytokines and associated proteins, commonly referred to as Bone Morphogenic Proteins (BMPs).[1] These BMPs are typically locked on the surface or within the bone and must be extracted using a series of demineralization and digestion steps. A rapid *in vitro* assay is a desirable early stage complement to the development process that can be used to guide formulation and methodology decisions based on the levels of extractable (bioavailable) BMPs from a demineralized bone matrix.

OBJECTIVE: Develop an *in vitro* immunoassay (ELISA) to predict the bioavailability of two growth factors (BMP-2 and BMP-7) in human demineralized bone fibers (DBFs).

MATERIALS AND METHODS

All donors used for this research investigation were consented for research and processed at Pinnacle Transplant Technologies (PTT) using proprietary processing methods. The initial phase of testing included test articles (TA) obtained from eight (8) donors in addition to a negative control (NC) sample whereby mineralized cancellous was heat-inactivated by autoclaving at 127°C for 2 hours. A competitive demineralized fiber product with marketed osteoinductivity claims using an *in vivo* athymic rodent model[2] was used as a tissue reference control (TC).

The initial digestion protocol was adapted from methods described by Blum, et al.[3] and optimized for DBF formulations. All test articles and controls were normalized via weight and exposed to 20 units/ml of Type 1 collagenase (Worthington Biochemical Corporation, Lakewood, NJ, Cat # LS004197) for 16 hours while shaking at 275 rpm and at a temperature of 37 ± 3°C. The digested extracts were centrifuged at 1,500 rpm for 20 minutes and the extracts were decanted into fresh tubes. BMP-2 and BMP-7 ELISA assays were performed following manufacturer instructions (Quantikine ELISA, R&D Systems, Minneapolis, MN, Cat # SBP200 and Cat # SBP700). Absorbance was measured at 450nm using a plate reader (SpectraMax M2, Molecular Devices, San Jose, CA). Data was collected and organized using MS Excel to generate linear standard curves and statistical data analysis was performed using Minitab (Ver 21.2, Minitab, LLC, State College, PA). Graphs and plots were generated using GraphPad Prism (Ver 9.5.1.).



Step 1: Collagenase digestion | **Step 2:** Centrifugation | **Step 3:** Prepare standard curves | **Step 4:** Perform ELISA | **Step 5:** Read absorbance

RESULTS

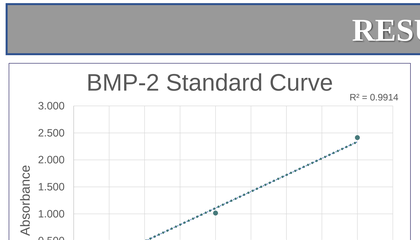


Figure 1. BMP-2 ELISA standard curve. The R^2 value for all BMP-2 assays performed was >0.99.

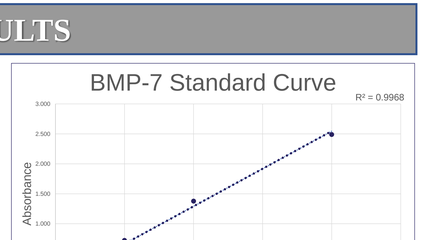


Figure 2. BMP-7 ELISA standard curve. The R^2 value for all BMP-7 assays performed was >0.99.

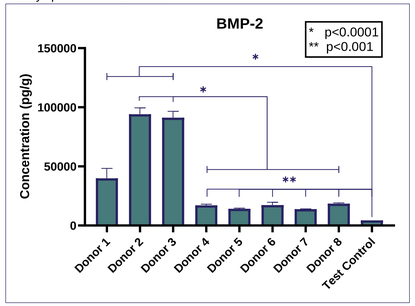


Figure 3. Total BMP-2 concentrations. All DBF tissues possessed BMP-2 levels significantly higher than TC (p<0.001). Donors 1-3 possessed BMP-2 levels significantly higher than TC (p<0.0001) and donors 2-3 had significantly higher BMP-2 levels than donors 4-8 (p<0.0001)

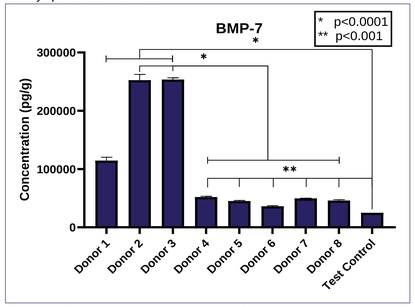


Figure 4. Total BMP-7 concentrations. All DBF tissues possessed BMP-7 levels significantly higher than TC (p<0.001). Donors 1-3 possessed BMP-7 levels significantly higher than TC (p<0.0001) and donors 2-3 had significantly higher BMP-7 levels than donors 4-8 (p<0.0001)

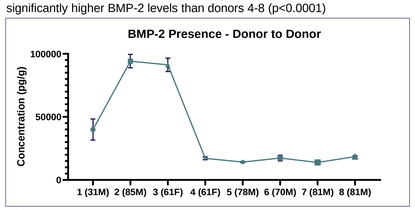


Figure 5. Donor to donor variance in total BMP-2 concentrations.

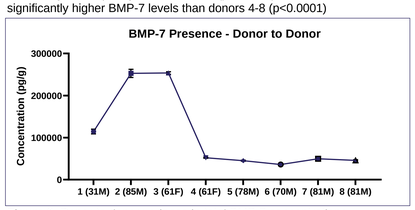


Figure 6. Donor to donor variance in total BMP-7 concentrations.

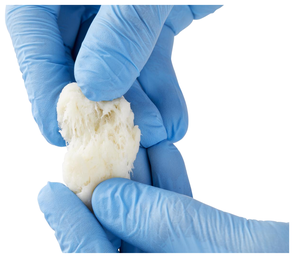


Figure 7. All BMP-2 and BMP-7 *in vitro* ELISA assays used Evoke Demineralized Bone Fiber, shown above, as the test article.

Donor # (Age,Sex)	BMP-2 (pg/g)	BMP-7 (pg/g)
1 (31M)	39,907 ± 8405	114,478 ± 5,740
2 (85M)	94,169 ± 5,333	252,633 ± 9,809
3 (61F)	91,237 ± 5,332	253,684 ± 3,008
4 (61F)	17,125 ± 945	52,065 ± 1,235
5 (78M)	14,214 ± 501	45,104 ± 1,082
6 (70M)	17,326 ± 2,324	36,091 ± 1,187
7 (81M)	13,899 ± 139	49,679 ± 287
8 (81M)	18,473 ± 614	45,911 ± 1,623
Negative Control	1,402 ± 891	-53‡ ± 490
Tissue Reference Control	4,323 ± 0	24,885 ± 618

Table 1. Total BMP-2 and BMP-7 concentrations for DBF donors 1-8, Tissue Control, and Negative Control samples, represented as average ± standard deviation. ‡Value assumed to be zero.

DISCUSSION

Research donors for this study were 78% male with an average age of 68.5 and a range of 31 to 85 years old. Twenty (20) individual collagenase extractions were plated in duplicate for a total of forty (40) datapoints per BMP assay. All values are represented as picograms of BMP per gram of dry weight tissue.

All BMP-2 and 7 test article values were statistically higher ($p > 0.001$) when compared to the negative control, although the NC did exhibit low levels of BMP-2 (1402 pg/g). This suggests that perhaps the heat inactivation cycle did not completely destroy the target proteins and/or there is low-level non-specific binding of extraneous protein remnants within the control samples. To control for this non-specific binding, the average NC BMP values were subtracted from TAs and TCs to normalize the data.

Statistical analysis of the normalized data was performed within and between the donors using one-way ANOVA and demonstrated that there was no statistical difference of the BMP-2 or BMP-7 values within each donor ($p < 0.05$, data not shown). BMP values averaged 3.4% variability within each of the test article replicates. BMP-2 test article values ranged from 13,889 to 94,169 pg/g, and BMP-7 values ranged from 36,091 to 253,684 pg/g.

Statistical differences were observed between the donors for both BMP-2 and BMP-7 (Figures 3,4). All donors possessed BMP levels at statistically higher concentrations than the tissue control ($p < 0.001$ and <0.0001). Donors 2-3 possessed BMP-2 and -7 levels at statistically higher concentrations than donors 4-8 ($p < 0.001$). There was no immediate correlation of age or sex with the different donors, although the small sample sizes precluded deeper investigation. As observed in Figures 5 and 6, two donors of the same age and gender (61F) demonstrated significantly different levels of BMP. Furthermore, the youngest donor (31M) did not demonstrate the highest levels of BMP as one might expect. These results could reflect both the natural donor to donor variation in bioavailability of growth factors within human demineralized bone fibers as well as the inherent processing variability within the manufacturing processes used at PTT.

When compared to a demineralized fiber product with confirmed osteoinductivity in the *in vivo* ASTM method[2], PTT demineralized fibers outperformed the competitive tissue control by at least 3x within the BMP-2 assay and 1.4x within the BMP-7 assay (Table 1).

CONCLUSION

The results from this investigation demonstrate that *in vitro* BMP-2 and BMP-7 ELISA assays can be used as a predictive tool to assess the bioavailability of growth factors present in demineralized fibers, as well as the reproducibility within and between different manufacturing processes. The data presented also strongly suggests that the Pinnacle Transplant Technologies' DBF fibers possess higher expression levels of BMP-2 and BMP-7 growth factors as compared to a confirmed competitive osteoinductive fiber product.

CONTACT and REFERENCES

Nate Remlinger, PhD. Director of Research and Product Development, PTT. O: 623-277-5478 E: nremlinger@pinnacletransplant.com

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2 - ASTM F2529-13 Standard Guide for In-vivo evaluation of Osteoinductive Potential for Materials Containing Demineralized Bone (DBM).

3 - Blum, B., et al. (2004). Measurement of Bone Morphogenetic Proteins and Other Growth Factors in Demineralized Bone Matrix. *Orthopedics, 27*(1 Suppl), 161-165.