METHOD OF MEASURING BMP-2 IN DBM WITH AN IN VITRO ASSAY FOR USE AS AN INDICATOR OF OSTEOINDUCTIVITY IN DBM/CaSO₄ BONE GRAFT SUBSTITUTE

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Urist first demonstrated that demineralized bone matrix (DBM) is “osteoinductive” (i.e., it is capable of inducing new bone growth in heterotopic sites [Urist, et al, 1965]). The traditional method of assessing osteoinductivity of DBM, therefore, has been by implantation of the material in heterotopic sites in rodents, such as intramuscular or subcutaneous sites. Extent of osteoinductivity is typically evaluated by measurement of new bone growth, or by an increase of bone markers in cells (e.g., alkaline phosphatase), in the implant sites at 28 days. Testing of human DBM is typically conducted in athymic rats or athymic mice to eliminate an immunogenic response. These animal assays are costly and typically require at least 7-8 weeks for implantation and histology. While Urist first attributed the phenomenon of osteoinductivity to “bone inductive protein”, it has since been established that osteoinductivity is induced by multiple proteins which are now called “bone morphogenetic proteins” (BMPs). Of the BMPs, BMP-2 is one of the most widely studied. Based on this premise, an ELISA-based assay for BMP-2 was developed. This method is less costly and much faster than the traditional animal-based assays. Our approach to developing the assay consisted of four steps: 1) identification of a feasible assay; 2) confirmation of assay repeatability; 3) confirmation of the assay as an indicator of osteoinductivity; and 4) identification of an acceptance/rejection level.

ASSAY DEVELOPMENT

Measurement of proteins in DBM by ELISA is a two-step process: 1) extract proteins from the DBM into solution; and 2) measure levels of selected proteins in the fluid extracts, such as with commercially available ELISA kits (R&D Systems, St Paul, MN).

For over 35 years, BMPs and other growth factors in bone have been isolated from DBM for further examination using a number of extraction methods. Protein extractions conducted by early researchers primarily utilized guanidine-hydrochloride or urea-based solutions, but these typically yielded very low concentrations of proteins (Sampath and Reddi, 1981; Urist, et al, 1979). Jortikka and associates adopted a method utilizing a collagenase solution in an effort to increase the yield of proteins released from the bone (Jortikka, et al, 1993). We adapted the collagenase extraction method used by Jortikka and associates, with a minor modification. We opted to omit the dialysis step, based on early data that indicated higher BMP-2 yields without dialysis (MCo~10 kDa) of extraction fluid samples (mean of 7.2 times higher). Proteins are extracted from 1 gram samples of freeze-dried DBM by soaking DBM overnight (17-18 hours) in Tris-buffer containing collagenase at 37°C in a shaking water bath. Following extraction, samples are centrifuged to settle the residual DBM. Fluid extract samples are poured into clean micro-centrifuge tubes and stored at -20°C until testing.

CONFIRMATION OF DISTINCTION BETWEEN ‘ACTIVE’ AND ‘DEACTIVATED’ DBM

To confirm the ability of the BMP-2 assay to distinguish between ‘active’ and ‘deactivated’ lots of DBM, samples of DBM from ten lots were subjected to protein extraction by soaking in guanidine-hydrochloride, per a DBM ‘deactivation’ method previously described (Mizuno and Glowacki, 1996). These guanidine-extracted samples, as well as non-‘deactivated’ DBM samples from the same donor lots, were then tested in the rat and BMP-2 ELISA assays.

The mean values of BMP-2 levels in the active and guanidine-extracted samples were 1,928±1,179 pg/g DBM (range 200-4,155) and 388±220 pg/g DBM (range 0-703), respectively. Levels of BMP-2 were significantly lower (p=0.002) in the ‘guanidine-extracted’ samples of DBM, confirming the ability of the ELISA test to distinguish between ‘active’ and [theoretically] ‘deactivated’ DBM. The lower amount of protein
corresponded to decreased new bone formation in the rat model with ‘guanidine-extracted’ DBM samples, although the distinction between active and guanidine-extracted samples was clearer from the ELISA results due to less variability in the data within each lot.

CONFIRMATION OF ASSAY REPEATABILITY
To confirm the repeatability of both the extraction and ELISA processes, several studies were undertaken including intra-assay variability (i.e., the effect of location within an assay plate); inter-extraction variability (i.e., the effect of extraction process) and inter-assay variability (i.e., the effect of individual assays); the effect of freezing and thawing on extract samples (multiple times); and the stability of samples over time. The inter-extraction and inter-assay studies were combined into one study to represent the ‘worst case’ (i.e., potential of variability from both steps of the two-step assay process).

To assess variability of samples within one plate, samples from four lots, 16 replicate samples per lot, were assayed. Variability of replicates for each lot was low (3-6%; mean 4.75%).

To simultaneously assess the variability in levels of BMP-2 from different extractions or assays of the same samples, samples of DBM from five lots were extracted three separate times (Extracts A, B, C). Each fluid extract sample was then assayed seven times (Assays 1-7). Thus, samples from each lot were assayed a total of 21 times (3 extracts x 7 assays/extract = 21). Results from one-way ANOVA or Kruskal-Wallace one-way ANOVA on ranks analyses indicated no differences in BMP-2 levels from different extractions or assays.

To assess the effect of freezing and thawing on samples, two different studies were conducted. Fluid extract samples were frozen and thawed up to nine times. Each freeze/thaw ‘cycle’ consisted of freezing samples for a minimum of 12 hours (-20ºC), and subsequently thawing the samples by placing them at room temperature for 3-5 hours. There were minimal, insignificant differences (one-way ANOVA) between BMP-2 levels in all samples regardless of how many times the samples had been frozen and thawed.

CONFIRMATION OF BMP-2 ASSAY AS AN INDICATOR OF OSTEOINDUCTIVITY
To confirm a relationship between levels of BMP-2 and osteoinductivity as determined by the “gold standard” model (i.e., new bone growth achieved in an athymic nude rat, ATNR, muscle pouch model), we implanted samples of 70 lots of DBM / CaSO₄ Putty in athymic nude rats. One gram DBM samples from the same lots were extracted and assayed for BMP-2. Samples were implanted for 28 days in the sub-latissimus dorsi or gluteus superficialis muscles. Explants were decalcified, embedded in paraffin, and cut. Slides were stained with hematoxylin and eosin and evaluated histomorphometrically to determine the area of new bone growth as a percentage of the area of implant and new bone.

There was a strong association between BMP-2 in DBM and bone growth with DBM / CaSO₄ implants (p<0.001 for slope; Power = 1.000). A strong relationship was also observed between level of BMP-2 in DBM and percentage area of new bone growth with DBM implants (p<0.001 for slope; Power = 1.000). Subsequently, there was also a
strong association between bone growth with DBM implants and bone growth with corresponding DBM / CASO4 implants.

DETERMINATION OF ACCEPTANCE THRESHOLD
The minimum acceptable amount of BMP-2 required for lot release is based on a measurement above the amount of BMP-2 which correlates with zero bone growth in the athymic nude rat muscle pouch model. While other criteria were considered (e.g., intercept of the regression line with the x-axis), the selected acceptance level provides a more conservative, rigorous threshold.

CONCLUSION
BMP-2 levels in lots of DBM are strongly associated with osteoinductivity in the in vivo athymic nude rat method of screening DBM / CASO4. Putty. It is the intent of this assay to serve as a quality measurement of each lot of DBM and DBM / CASO4. Putty to ensure that it is acceptable for clinical use.

REFERENCES