A Validated In Vitro Alkaline Phosphatase Assay and Its Correlation with In Vivo New Bone Formation for Osteoinductive Potential Assessment of Demineralized Bone Matrix

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Introduction: Human demineralized bone matrix (DBM) has been used widely in clinical application to repair skeletal defects and to treat periodontal diseases. The osteoinductive potential of DBM may vary from preparation to preparation [1, 5, 8]. Currently, an in vivo bioassay utilizing a rodent model is considered the gold standard to assess the potential of DBM to form new bone. However, this bioassay is semi-quantitative, time-consuming and expensive. Therefore, it is advantageous to develop a rapid and reliable assay to assess the osteoinductive potential of DBM for both research and product quality control [2]. In vitro alkaline phosphatase (AP) assays have been used to assess the osteoinductive potential over the last ten years [3, 7]. However, to date there is no standard testing protocol for an in vitro AP assay and very few studies have correlated the in vitro AP assay and the “gold standard” in vivo bioassay [4]. The purpose of the present study was to develop and validate an in vitro AP assay that demonstrated acceptable correlation with new bone formation in vivo in a nude mouse model.

Materials and Methods: In vitro AP assay: C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. DBM samples were digested in collagenase (Sigma) at 37°C. The DBM digestes were centrifuged, the supernatants were aliquoted to the volume equivalent of 10 mg of DBM, and were introduced into each well of the C2C12 cell seeded 24-well plate on day two. Recombinant human BMP-2 (rhBMP-2) was used as a positive control for AP induction [6]. AP standards (Sigma) were used as a positive assay control. C2C12 cell culture (without the addition of DBM digest or rhBMP-2) was used as a negative control. Cells were incubated in a 37°C incubator containing 5% CO2 for 3 days. Cells were then detached from culture plates and cell lysates were generated for AP assay and BCA protein assay (Pierce). The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of substrate para-nitrophenyl phosphate (pNPP) and cell lysate at 37°C. The AP activity of each sample was calculated using pNP standard and was normalized by weight of protein (nmol pNP/min/mg protein). In vitro AP assay validation: Linearity of the AP standard, pNP standard, and BSA protein standard were tested. Detection limit (LOD) and quantitation limit (LOQ) of the AP assay and BCA assay were evaluated. Precision of the in vitro AP assay was analyzed by testing the mean, standard deviation, and coefficient of variation (CV) from a minimum of 3 separate experiments for the same lot of DBM samples. The accuracy of the in vitro AP assay was analyzed through relative error (RE) of pNP standard, spiking recovery, and correlation with the gold standard – in vivo bioassay [4]. The purpose of the present study was to develop and validate an in vitro AP assay that demonstrated acceptable correlation with new bone formation in vivo in a nude mouse model.

Results: The results of the in vitro AP assay for nine DBM lots are shown in Figure 1. The acceptance criteria for linearity of AP standard, pNP standard, or BSA protein standard was an R-squared value greater than 0.98. All standards used in this study met the acceptance criteria. All AP assay results and BCA assay results were higher than the detection limit with the exception of the negative control. Coefficients of variation for DBM samples and negative controls were 35.5% and 5.95% respectively. Average relative error for pNP standard was -4.76%. Average AP recovery in cell lysate was 94.4%. The normality tests demonstrated normal distribution of AP activity (p > 0.05). The results of the in vivo bioassay are shown in Figure 1. The Pearson correlation (R) between AP activity and histomorphometry was 0.95. The R-squared value for AP activity versus histomorphometry yielded a correlation of 0.90, suggesting the in vitro AP assay predicts new bone formation in vivo 90% of the time. The linear relation between AP activity and histomorphometry was highly significant (p < 0.0005).

Discussion: This in vitro AP assay proved to be repeatable and accurate. Furthermore, the in vitro AP assay demonstrated very strong correlation with new bone formation in vivo in a nude mouse model suggesting its suitability as an alternative method to predict the osteoinductive potential of DBM samples. The in vitro AP assay developed and described herein is a timely, accurate, cost effective method that can reliably be used to assess the osteoinductive potential of DBM.