THE ANALYSIS OF EMDOGAIN BINDING AFFINITY FOR DIFFERENT PARTICULATE BONE GRAFT MATERIALS.

A Thesis
Submitted to
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MASTER OF SCIENCE

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by
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ABSTRACT

Objectives: Traditional guided tissue regeneration procedures use particulate bone graft materials and occlusive membranes with the primary aim of reconstitution of the supporting periodontal tissues. Currently, the Food and Drug Administration has cleared only four treatment modalities for true periodontal regeneration. These materials are autogenous bone, demineralized freeze dried bone allograft, LANAP (Millennium Dental Technologies INC, Cerritos, CA) and Emdogain (Institut Straumann AG, Basel, Switzerland). The biologically inactive nature of many commercially available bone graft materials provides an opportunity for the addition of certain biologic materials to enhance the healing response. The development of an adequate carrier for biologic agents is a crucial step in the creation of a bioactive graft material. This experiment uses Emdogain (Institut Straumann AG, Basel, Switzerland) to study the specific characteristics of protein binding and release on three different commonly used bone graft substrates.

Methods: Emdogain gel (Institut Straumann AG, Basel, Switzerland) was dissolved in dilute acetic acid to extract amelogenin protein from the polyglycolic alginate carrier and buffered with phosphate buffered saline to create 1% Emdogain working solution. Emdogain solution was added over 100mg of allograft, xenograft, or synthetic particulate graft material each in a 1.5 ml microcentrifuge tube and incubated for 24hr. The supernatant was removed and stored at 4^{o}C, 1ml PBS wash added to each tube, removed and replaced at various time points. The PBS washes were stored at 4^{o}C. Total protein concentrations were quantified using bicinchonic acid (BCA) protein assay reagent kit. ELISA was performed with amelogenin antibody to specifically determine the quantity of

amelogenin remaining in the supernatant after initial coating of particulate bone graft materials and to study the kinetics of release of amelogenin at various time points.

Results: BCA protein quantification revealed a high mean total concentration of protein from the allograft bone particulate. In contrast, the mean total protein concentration of the xenograft bone particulate was below the detectable limit. The mean total protein concentration of the alloplast was low, but still within the detectable range. The kinetics of amelogenin release were detected using an amelogenin-specific ELISA, performed with a monoclonal mouse amelogenin antibody. A very high concentration of amelogenin was in the initial supernatant from the xenograft bone particulate, with a steep decline in concentration after the first and second washes to undetectable levels in the third and fourth washes. The initial supernatant from the allograft bone particulate had the lowest concentration of amelogenin in solution, however, the concentration of amelogenin increased in the first and second washes, then declined to levels below the initial concentration in the third and fourth washes. The concentration of amelogenin in the initial supernatant from the alloplast bone particulate had low levels and declined in the first two washes, then began to increase in the third wash, peaking at high level in the fourth wash.

Conclusions: An efficient ELISA assay for amelogenin in solution was developed using a single monoclonal anti-amelogenin antibody. Using this ELISA, the kinetics of amelogenin release from different bone graft particulates could be observed. The kinetics were unique to each type of particulate.

It is unclear which type of release is most favorable for optimal bioavailability of Emdogain to promote healing a periodontal defect. More studies are needed to determine the response of periodontal fibroblasts to different concentrations of Emdogain over time.

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CHAPTER 1

INTRODUCTION

Regeneration of periodontal supporting tissues in humans requires a complicated healing cascade. Inflammatory mediators that are present during the periodontal disease process must be down regulated while cellular signaling molecules that contribute to new bone and tissue formation must be up regulated. The migration of particular cells into the periodontal defect are crucial to regenerative healing, a characteristic that further complicates therapeutic attempts at achieving true regeneration. True regeneration requires histologic evidence of reconstitution of the periodontal apparatus, including alveolar bone, cementum, and the periodontal ligament (Bowers, 1989).

As described by Gottlow, Nyman, Karring & Lindhe (1984), certain principles are required for successful guided tissue regeneration, a procedure that attempts to regenerate periodontal tissues lost by disease process. These basic tenants include primary closure of the wound; angiogenesis to supply undifferentiated mesenchymal cells, creation and maintenance of adequate space for bone fill, and wound stability for the duration of the healing course. Traditional guided tissue regeneration utilizes particulate bone graft covered with an occlusive membrane and primary closure of the patient's gingival tissues to achieve regrowth of the periodontium. A variety of particulate materials are commercially available, however the majority of these materials do not result in true periodontal regeneration, but rather lead to repair.

Autogenous bone harvested from intraoral sites or sometimes from the iliac crest or femur and demineralized freeze-dried bone allograft from cadavers both have osteoinductive potential. This is a desirable trait for a graft material, but the level of osteoinductivity that these materials contribute may not be sufficient to alter the healing response (Schwartz et al, 1996). Xenografts, harvested from bovine, porcine, or equine sources are another commonly used type of bone particulate. These, along with synthetic bone grafts, such as beta tricalcium phosphate, serve as osteoconductive scaffolds but contain no level of osteoinductive potential.

Currently, there are only four treatment modalities cleared by the Food and Drug Administration for true periodontal regeneration (Garrett, 1996). These are autogenous bone, demineralized freeze-dried bone allograft, LANAP (Millennium Dental Technologies INC, Cerritos, CA) and Emdogain (Institut Straumann AG, Basel, Switzerland). The biologically inactive nature of many commercially available bone graft materials provides an opportunity for the addition of certain biologic materials to enhance the healing response.

One of these biologic materials is enamel matrix derivative, marketed as Emdogain (Institut Straumann AG, Basel, Switzerland). Enamel matrix derivative is harvested from the developing tooth buds of six-month old piglets. Amelogenin comprises up to 90% of the composition of enamel matrix derivative. The remaining 10% of the proteins are proprietary (Esposito et al, 2004). The biological activity of amelogenin and the other proteins in enamel matrix derivative are of interest in the current periodontal literature. It is unknown whether amelogenin is the primary protein contributing to the enhanced healing response when Emdogain is used (Miron et al, 2013).

A Cochrane systematic review (Esposito, 2004) explored the clinical efficacy reported in the literature of enamel matrix derivative in comparison with open flap debridement, guided tissue regeneration, and bone grafting. Guided tissue regeneration showed statistically significant reduction in probing depth and increase of recession when compared to Emdogain. When compared to flap surgery, Emdogain showed significant improvements in attachment levels and probing depths. No studies were available to compare the use of Emdogain versus bone grafting. This review did not include studies that combined Emdogain with any bone graft materials. The conclusions of this review indicate that Emdogain results in similar clinical outcomes as guided tissue regeneration, with the additional benefits that it is simpler to use, may not require antibiotic coverage, and has no reported postoperative infections or adverse outcomes.

The combination of enamel matrix derivative and bone graft materials has shown favorable clinical results in the periodontal regeneration of intrabony defects. In a systematic review by Matarasso et el (2015), intrabony defects treated with a combination of Emdogain and bone graft showed greater clinical attachment gain, greater decrease in probing depths, and less recession than intrabony defects treated with Emdogain alone. These authors concluded that Emdogain combined with bone grafts may result in additional clinical improvements, however the potential influence of the type of bone graft material on clinical outcomes was unknown.

A randomized controlled trial by Gurinsky, Mills, and Mellonig (2004) evaluated the treatment outcomes of intrabony defects with Emdogain versus Emdogain combined with demineralized freeze-dried bone allograft.

Both treatment modalities resulted in significant improvements in soft tissue parameters, including probing depth reduction and clinical attachment gain. The combination of Emdogain and demineralized freeze-dried bone allograft showed statistically significant improvements in bone fill, crestal resorption, and percentage of sites gaining greater than 50% and 90% bone fill when compared to Emdogain alone. The results of this study indicate that the addition of Emdogain to demineralized freeze-dried bone allograft may enhance the hard tissue parameters of healing in infrabony defects.

The combination of enamel matrix derivative and particulate anorganic cancellous bovine derived bone xenograft failed to demonstrate additional clinical benefit. In a randomized controlled clinical study by Scheyer et al (2002), Emdogain combined with bovine derived xenograft to treat infrabony defects in humans had similar clinical results when compared with sites treated with bovine derived xenograft alone. No statistically significant differences were found in probing depth reduction, gain in clinical attachment, bone fill and percent bone fill between the two treatment groups. The authors concluded that both treatment modalities were effective in the treatment of human intrabony defects.

Emdogain as an adjunct to beta tricalcium phosphate/hydroxyapatite, an alloplastic material, was researched by Nery et al (2017) in a split mouth design. Systemically healthy patients who required maxillary sinus floor augmentation were treated on one side with a combination of alloplast + Emdogain and with only alloplast on the contralateral side. Biopsies were obtained from the grafted sites and analyzed histologically and histomorphometrically.

No significant differences were found between the two treatment groups in bone or soft tissue parameters and both treatment groups resulted in adequate amounts of new bone formation to accommodate dental implant placement.

The development of an adequate carrier for biologic agents is a crucial step in the creation of a bioactive graft material. This experiment uses Emdogain (Institut Straumann AG, Basel, Switzerland) to study the specific characteristics of protein binding and release on three different commonly used bone graft substrates. Freeze-dried bone allograft, deproteinized bovine bone matrix xenograft and beta tricalcium phosphate allograft were selected for comparison. These commercially available materials are frequently used in clinical practice in the United States. They are osteoconductive materials, each with unique particle sizing and morphology.

CHAPTER 2

MATERIALS AND METHODS

PREPARATION OF 1% EMDOGAIN SOLUTION

To extract the proteins from the polyglycolic alginate gel, 0.3ml (30mg/ml) of Emdogain was dissolved in 3ml of 0.1% acetic acid and centrifuged at 4°C at 4000 rpm for 10 minutes. Dulbecco's phosphate buffered saline was added to total 300mL of solution.

DOT BLOT TEST OF AMELOGENIN ANTIBODY

To verify the utility of two commercially available anti-amelogenin antibodies for detection of amelogenin in Emdogain solution, a dot blot test was completed using a monoclonal Amelogenin Antibody F-11: sc-365284 (Santa Cruz Biotechnology Inc, Dallas, TX) and a polyclonal Amelogenin Y-linked Antibody C-Term (Aviva Systems Biology, San Diego, CA). Serial dilutions 1:10 were made, seven in total beginning with undiluted 1% Emdogain solution. Dilutions were dot blotted onto a PVDF membrane and air-dried. The PVDF membrane was hydrated with methanol, rinsed 3 times with phosphate buffered saline wash on a rocking platform for 3 minutes each time. 3ml of 3% bovine serum albumin was applied to the membrane and rocked on rocking platform for 30 minutes. 3µg of test antibodies were added to the BSA solution over the membrane and incubated for 24 hours in 4°C. The membrane was washed 3 times with 1% TBS tween on rocking platform for 5 minutes each wash. The appropriate secondary antibody was prepared; IRdye 680LT goat anti-mouse IgG with 0.02% SDS and 0.1% tween 20 with 1:20000 dilution and IRdye 680RD goat anti rabbit IgG with 1:15000 dilution. These were applied to the membrane and placed on rocker for several hours.

The membranes were rinsed with TBS tween 6 times on rocking platform for 5 minutes each rinse. The membranes were imaged on a Licor imager at appropriate light wavelengths.

TEST ELISA OF EMDOGAIN SOLUTION

To test the efficacy of the purchased anti-amelogenin antibodies in an ELISA, wells in a 96 well plate were coated with 50µl anti-amelogenin antibody in coating buffer (0.02M PBS+ 0.138M NaCl+ .0027M KCl, pH 7.4) for 24hr at 25°C. The plate was washed 3 times with PBS Tween, then blocked with 200µl 3% BSA and stored overnight at 4°C. The plate was washed 3 times with PBS Tween. Serial dilution 1:2 of 1ml Emdogain solution with phosphate buffered saline (7 dilutions total) were prepared and applied to wells. The plate was incubated at 4°C for 24 hours. The plate was washed 3 times with PBS Tween and blocked with 200 µl 3% BSA for 1 hour at 4°C. The amelogenin antibody solutions were prepared with 13.6µl of the rabbit polyclonal antibody (Aviva Systems Biology, San Diego, CA) in 3.4ml of 1% BSA and 17µl of the mouse monoclonal antibody (Santa Cruz Biotechnology Inc, Dallas, TX) in 1.7ml of 1% BSA respectively.

The plate was washed 3 times with PBS Tween and 100ml of antibody was applied and incubated for 24 hours at 4°C. The plate was washed 3 times with PBS Tween and the appropriate secondary antibody HRP conjugate was applied. To wells coated with the Amelogenin Y-linked rabbit Antibody C-Term (Aviva Systems Biology, San Diego, CA) 100 µl of anti-rabbit HRP at 1:1000 dilution (3.4ml BSA+ 3.4 µl anti-rabbit HRP IgG) was applied.

To wells containing Amelogenin mouse Antibody F-11: sc-365284 (Santa Cruz Biotechnology Inc, Dallas, TX) 100 µl of anti-mouse HRP at 1:1000 dilution (1.7ml 1% BSA + 1.7 µl anti-mouse HRP IgG) was applied. Wells were washed 5 times with PBS Tween.

200 µl of prepared TMB substrate reagent (BD OptEIA) was added to wells and incubated at room temperature. 50 µl of stop solution ($2NH_2SO_4$) was added and the absorption was measured using a Versa Max plate reader at the appropriate wavelength.

COATING OF BONE GRAFT MATERIALS WITH 1% EMDOGAIN SOLUTION

The 3 different particulate bone graft materials that were used in this experiment include Straumann Allograft Cortical & Cancellous freeze dried bone allograft (Institut Straumann AG, Basel, Switzerland) with granule size 250-1000 µm, Straumann Xenograft deproteinized bovine bone matrix (Institut Straumann AG, Basel, Switzerland) with granule size 200-1000 µm and beta-tricalcium phosphate allograft (Osteohealth Company, Shirley, NY) with granule size 500-1000 µm.

1 ml of 1% Emdogain solution was applied to each graft material in a 1.5ml microfuge tube. 1 ml of phosphate buffered saline was applied to control tubes. Samples were incubated with Emdogain solution (n=4) or control PBS (n=1) for 24 hours at 4°C. After incubation, tubes were centrifuged at 170G for 5min at 4°C and supernatant was removed and replaced with 1ml phosphate buffered saline. Samples were then incubated for 24 hours at 4°C, centrifuged, supernatant removed and replaced with 1ml phosphate buffered saline.

Samples were then incubated at 4°C for 48 hours, 5 days and 8 days, with removal of supernatant and addition of phosphate buffered saline wash between time points. All supernatant and washes were stored at 4ºC. All experiments were performed individually and test samples were quantified in quadruplicate.

TOTAL PROTEIN QUANTIFICATION

To analyze the total protein concentration of the supernatant collected at various time points, a BCA assay was used. 200 µl of working reagent was added to 20 µl of sample in a 96 well plate and allowed to develop. The plate was read at 595nm wavelength.

To analyze the total protein concentration remaining in the solid bone graft samples following multiple PBS washes and incubations, 1 ml of BCA working reagent was added to graft samples, incubated at 37°C for 30 minutes with gentle shaking on an orbital shaker. 200 µl samples were removed from each tube and added to the wells of a 96 well plate and the plate was read at 595nm wavelength.

AMELOGENIN QUANTIFICATION WITH ELISA

To specifically quantify amelogenin protein released from each particulate bone graft material, an ELISA was conducted using Amelogenin Antibody F-11: sc-365284 (Santa Cruz Biotechnology Inc, Dallas, TX). 100 µl of supernatant and wash samples from each time point were incubated at 4°C overnight in wells of a 96 well plate. The plate was then washed 3 times with PBS Tween, then blocked with 200 µl of 3% BSA. Amelogenin bound to the wells of the plate was detected using the mouse monoclonal anti-amelogenin antibody and the HRP anti-mouse detection antibody as follows: 100 µl of monoclonal mouse anti-amelogenin antibody was dissolved in 10ml 1% BSA.

100 µl of antibody was applied to the plate and incubated overnight at 4°C. The plate was washed 3 times with PBS tween. Anti-mouse HRP (10 µl anti-mouse IgG heavy and light chain HRP + 10 ml 1%BSA) was applied, and the plate was washed 5 times with PBS Tween. 200 µl of TMB substrate was placed in wells and the plate was allowed time to develop. 50 µl of stop solution ($2NH_2SO_4$) was applied and the plate was read with Versa Max plate reader at the appropriate wavelength.

CHAPTER 3

RESULTS

The dot blot test provided evidence that the two antibodies were both effective in detection of amelogenin. The monoclonal antibody, Amelogenin Antibody F-11: sc-365284 (Santa Cruz Biotechnology Inc, Dallas, TX), provided clearer, more luminescent bands when the membrane was imaged.

The ELISA used to test the efficacy of the two antibodies also indicated that both antibodies were effective in detection of amelogenin. In this assay, the monoclonal antibody Amelogenin Antibody F-11: sc-365284 (Santa Cruz Biotechnology Inc., Dallas, TX) was more effective in detecting the amelogenin protein across the concentration range covered by the prepared standards. Therefore, the mouse monoclonal antibody was selected to use in the ELISA to determine the concentration of amelogenin in the samples collected from the Emdogain-coated bone graft particulates. The results of the comparison ELISA of the two anti-amelogenin antibodies are depicted in Figure 1.

For the BCA protein quantification assay, the optical density (OD) values obtained for each of the Emdogain-coated particulates were corrected by subtracting the OD value of the respective PBS-only coated particulates from the Emdogain-coated particulate values. BCA protein quantification revealed a high mean total concentration of protein from the allograft bone particulate. In contrast, the mean total protein concentration of the xenograft bone particulate was below the detectable limit. The mean total protein concentration of the alloplast was low, but was still within the detectable range. Some samples were not on the standard curve.

Therefore, the OD values were used to assess the protein amounts. The corrected mean optical densities of each type of bone graft particulate are depicted in Figure 3.
The kinetics of amelogenin release detected using the amelogenin-specific ELISA, performed with a monoclonal Amelogenin Antibody F-11: sc-365284 (Santa Cruz Biotechnology Inc., Dallas, TX) results are depicted for each bone graft particulate in Figure 5 and Figure 6.

There was a very high concentration of amelogenin in solution in the initial time supernatant from the xenograft bone particulate, and a steep decline in concentration in the first and second washes to undetectable levels in the third and fourth washes. The initial supernatant from the allograft bone particulate had the lowest concentration of amelogenin in solution, however, the concentration of amelogenin increased in the first and second washes, then declined to levels below the initial concentration in the third and fourth washes. The concentration of amelogenin in the initial supernatant from the alloplast bone particulate had low levels and declined in the first two washes, then began to increase in the third wash, peaking at a high level in the fourth wash.

Comparatively, the BCA assay resulted in a distinct pattern of protein release for each type of bone graft particulate, as depicted in Figure 4 and Figure 7. Similar to the kinetics observed in the ELISA, the protein concentration of the xenograft bone particulate measured by BCA had initially high values, which steeply declined after the first wash. The alloplast particulate demonstrated low total protein concentration levels at all time points. The allograft bone particulate had very high initial total protein concentration as measured by BCA, and then declined to low levels after the first PBS wash.

CHAPTER 4
CONCLUSIONS

There was a high protein concentration for the allograft bone particulate. The processing of this bone graft material does not remove the native proteins, so the high levels detected in the initial washes probably reflect the native protein still present in the sample. The bovine derived xenograft bone particulate is deproteinized in commercial processing. This material had undetectable concentrations of total protein in the BCA assay, which either indicates that the proteins in Emdogain remained tightly bound to the bone graft particulate, or that they were almost completely washed out of the material after serial washes. The beta-tricalcium phosphate alloplast does not contain any native proteins, therefore, the protein concentration measured by the BCA analysis can be attributed to the proteins in Emdogain.

An efficient ELISA assay for amelogenin in solution was developed using a single monoclonal anti-amelogenin antibody. Using this ELISA, the kinetics of amelogenin release from different bone graft particulates could be observed. The kinetics were unique to each type of particulate. Allograft seems to initially bind Emdogain, then slowly release it over time. Alloplast also appears to bind Emdogain upon coating of particles, but binds them for a longer period of time, after which there is a large quantity of Emdogain released. Xenograft apparently binds Emdogain irreversibly as there was little or no detectable for 8 days.

It is unclear which type of release is most favorable for increased bioavailability of Emdogain in a periodontal defect. More studies are needed to determine the response of periodontal fibroblasts to different concentrations of Emdogain over time.

If substantivity of Emdogain is required, the results of this experiment indicate that Xenograft would be the most favorable material to combine it with. Alternatively, if rapid release of Emdogain is most biologically favorable, then Allograft is the material of choice.

FIGURES

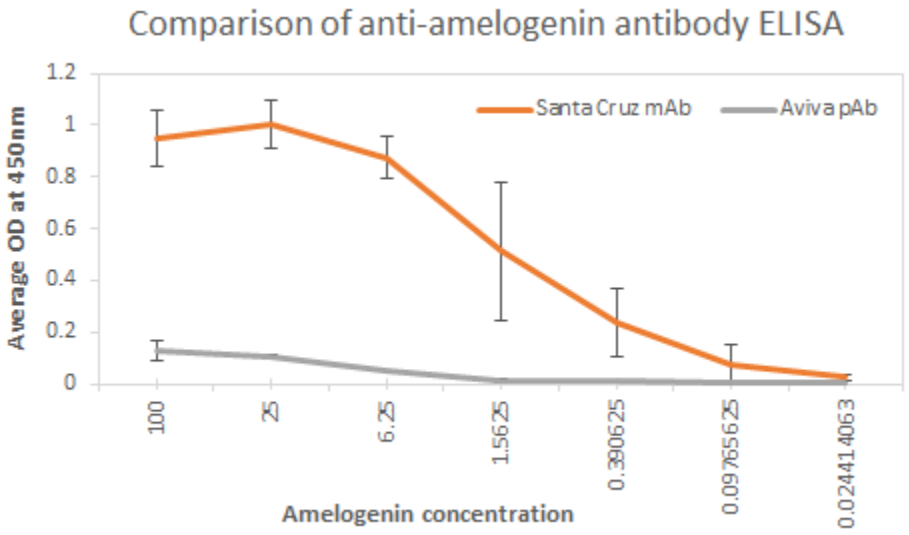

Figure 1: Average optical density vs concentration of amelogenin for HRP & TMB ELISA used to test efficacy of a monoclonal and polyclonal anti-amelogenin antibody

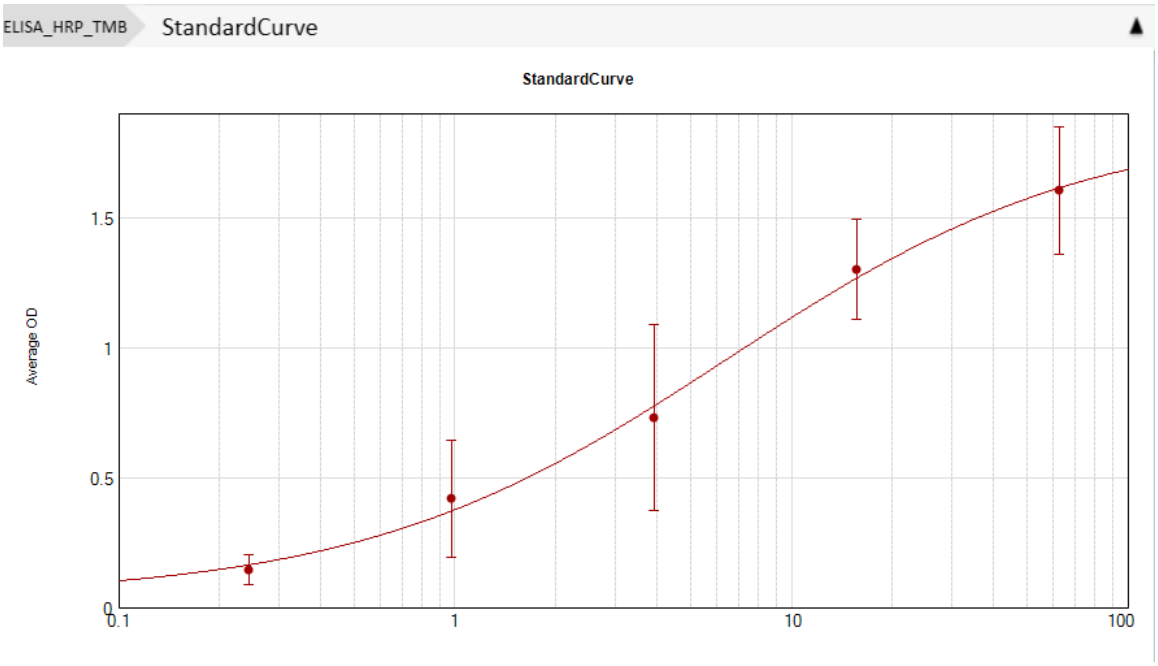

Figure 2: Standard curve of average optical density vs concentration of Emdogain solution used to quantify Amelogenin in samples beginning with 1% Emdogain solution and subsequent PBS washes at time points 24 hours, 48 hours, 5 days, and 8 days.

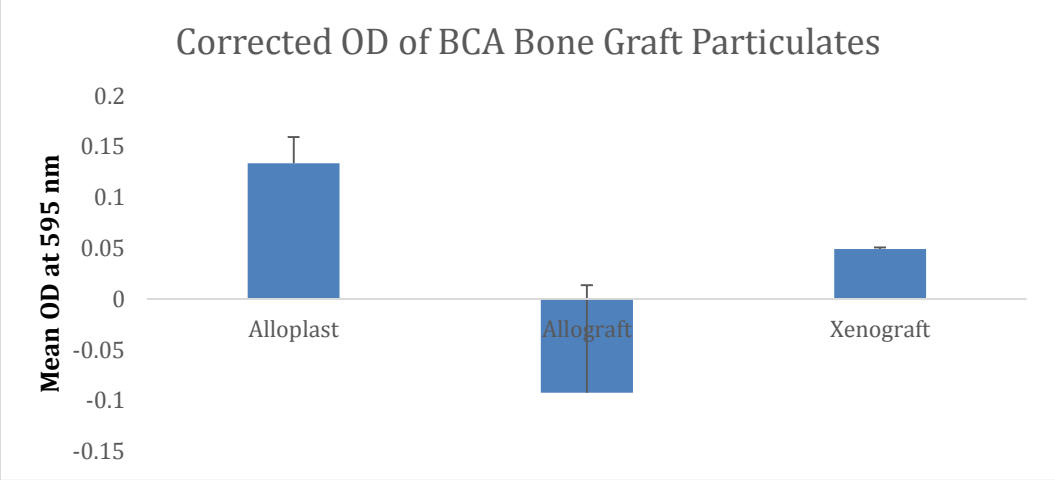

Figure 3: Corrected values of mean optical density representing total protein concentration derived from BCA assay of each type of bone graft particulate.

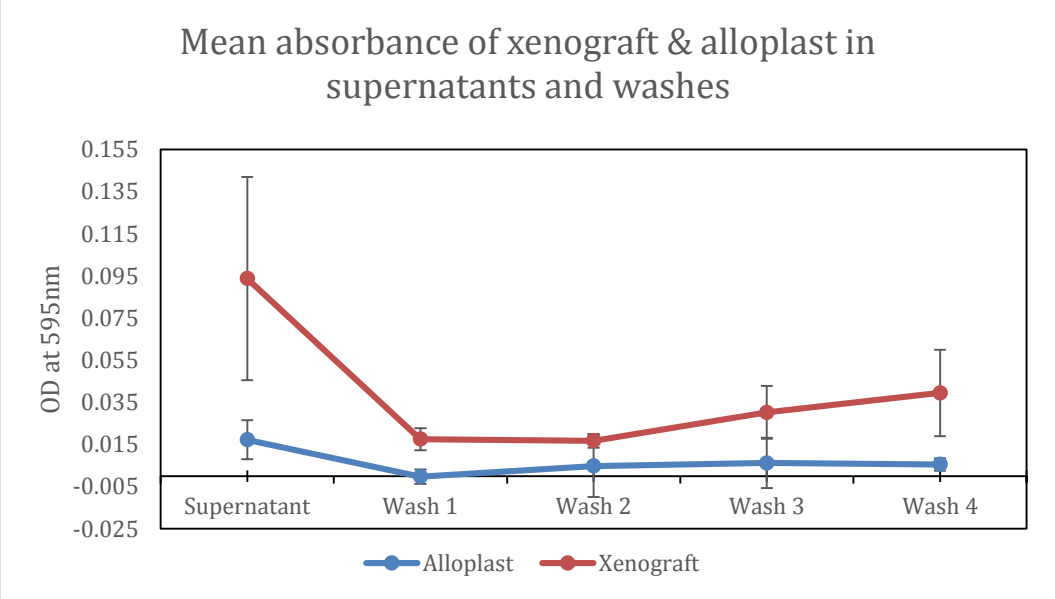

Figure 4: Mean absorbance of supernatant collected after 24hr incubation with Emdogain solution and subsequent PBS washes at 24hrs, 48 hrs, 5 days and 7 days of xenograft and allograft bone particulates measured with BCA assay and read at 595 nm

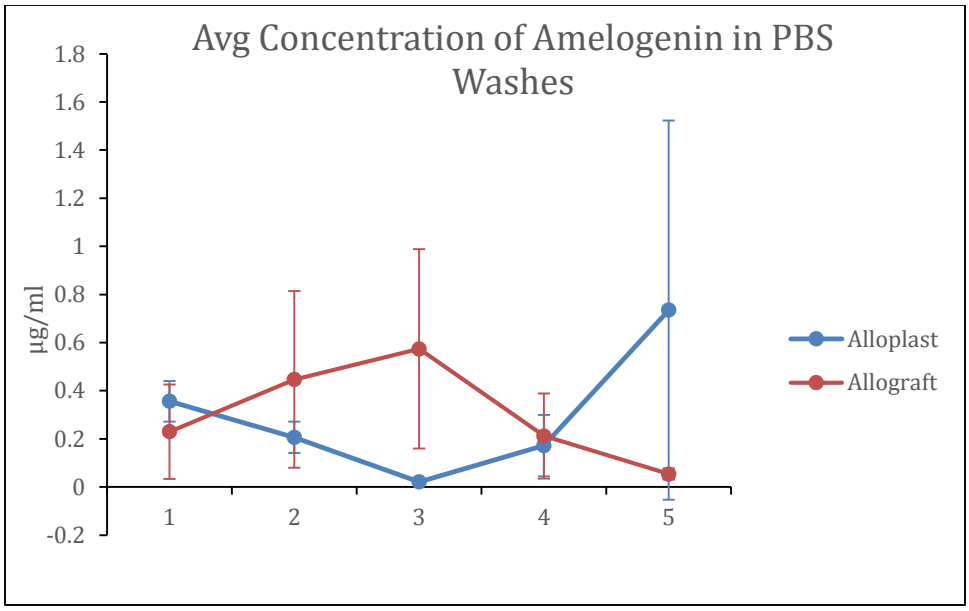

Figure 5 Average concentration in µg/ml of amelogenin after initial 24hr incubation (1) with Emdogain solution and subsequent PBS washes after 24 hours (2), 48 hours (3), 5 days (4) and 8 days (5) measured by ELISA

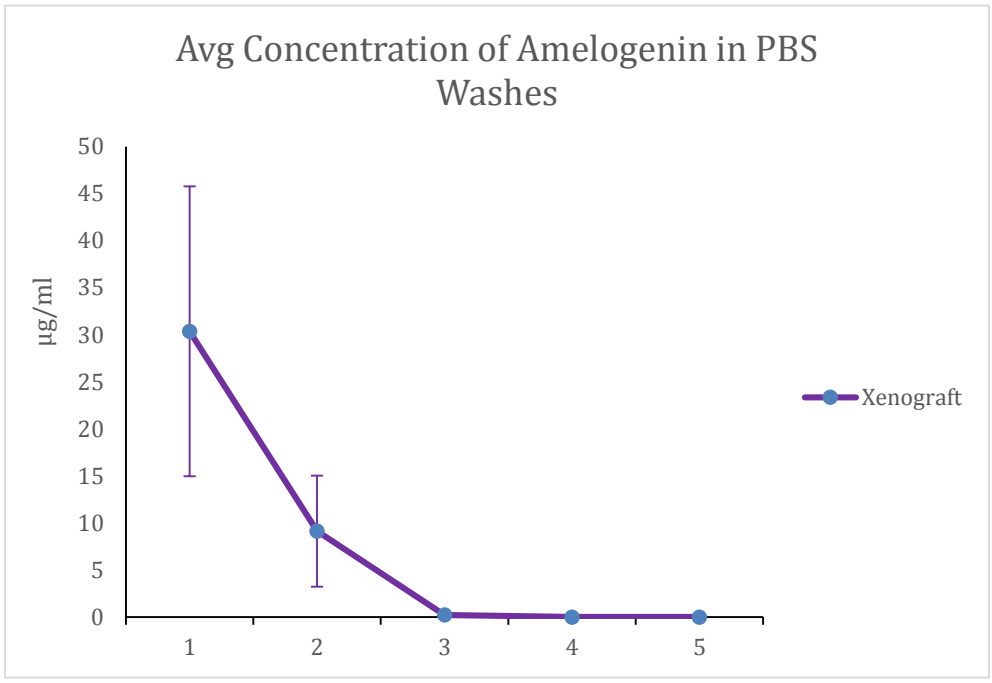

Figure 6: Average concentration in µg/ml of amelogenin after initial 24hr incubation in Emdogain solution (1) and subsequent PBS washes after 24 hours (2), 48 hours (3), 5 days (4) and 8 days (5) measured by ELISA

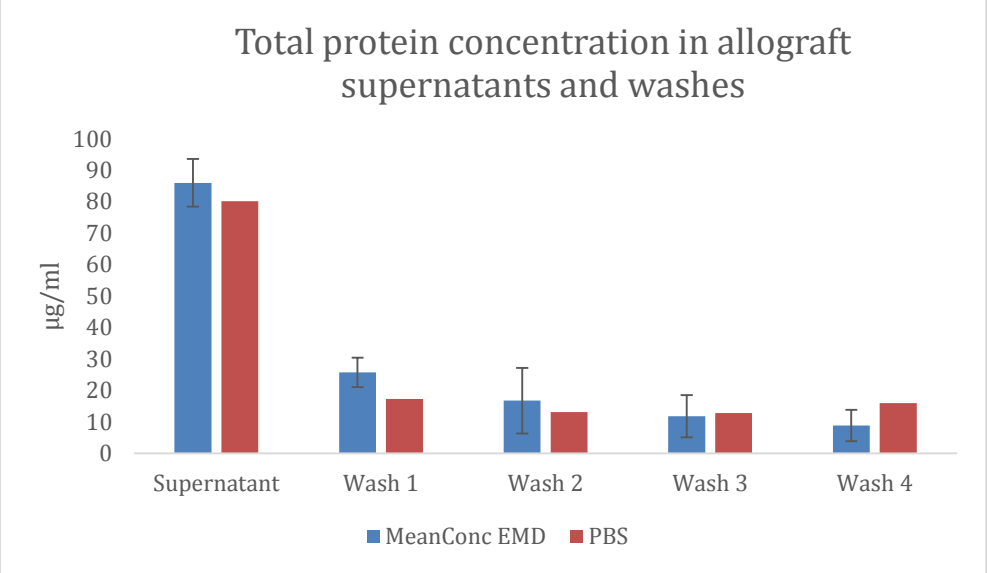

Figure 7: Total protein concentration measured with BCA read at 595 nm from initial 24hr incubation with Emdogain solution and subsequent 24hr, 48 hr, 5 day and 7 day PBS washes of allograft bone particulate

TABLES

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.051	1.435	0.781	0.782	0.79	0.86	0.828	0.861	0.828	0.793	0.833	0.821
B	0.969	0.945	0.896	0.871	0.897	0.94	0.882	0.912	0.812	0.831	0.836	0.805
C	0.823	0.839	0.797	0.823	0.812	0.79	0.861	0.84	0.826	0.857	0.83	0.796
D	0.879	0.839	0.814	0.803	0.872	0.82	0.877	0.809	0.832	0.828	0.8	0.836
E	0.884	0.818	0.889	0.831	0.852	0.88	0.802	0.562	0.862	0.877	0.839	0.843
F	0.831	0.833	0.872	0.872	0.825	0.82	0.748	0.824	0.881	0.935	0.909	0.882
G	0.809	0.814	0.858	0.874	0.721	0.87	0.564	0.747	0.831	0.869	0.874	0.848
H	0.842	0.877	0.943	0.798	0.752	0.91	0.748	0.924	0.901	0.892	0.876	0.83

Table 1: Raw data of total protein concentration in solution from BCA protein assay read at 595nm after initial 24 hour incubation, PBS wash, addition of 1ml of PBS at intervals of 24 hours, 48 hours, 5 days, and 8 days.

Sample	OD_Values	Av OD	Concentration	MeanConc	SD
Alloplast					
PBS	0.031	0.02	-53.048	-61.559	12.037
EMD1	0.142	0.1395	34.261	32.339	2.718
EMD2	0.189	0.1895	71.13	71.522	0.555
EMD3	0.155	0.155	44.537	44.498	0.055
EMD4	0.129	0.131	23.749	25.358	2.274
Allograft					
PBS	1.829	1.8445	1357.382	1369.462	17.084
EMD1	1.717	1.7265	1269.289	1277.015	10.927
EMD2	1.844	1.874	1368.913	1392.564	33.448
EMD3	1.651	1.622	1218.064	1194.963	32.671
EMD4	1.765	1.787	1306.942	1324.317	24.573
Xenograft					
PBS	-0.023	-0.027	-95.172	-98.585	4.826
EMD1	0.021	0.021	-61.127	-60.931	0.277
EMD2	0.023	0.0235	-59.088	-58.813	0.388
EMD3	0.024	0.0235	-58.46	-58.696	0.333
EMD4	0.016	0.0215	-65.128	-60.735	6.212

Table 2 : Data calculations including optical densities and concentrations from BCA of bone graft particulates after multiple PBS washes

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