Title
BIOACTIVE COATING GLASS AND COMMERCIAL BIOGLASS ENHANCE GENE EXPRESSION, PROTEIN EXPRESSION, AND MATRIX FORMATION OF HUMAN PERIODONTAL LIGAMENT FIBROBLASTS DURING OSTEOGENESIS

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by

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THESIS

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BIOACTIVE COATING GLASS AND COMMERCIAL BIOGLASS ENHANCE GENE EXPRESSION, PROTEIN EXPRESSION, AND MATRIX FORMATION OF HUMAN PERIODONTAL LIGAMENT FIBROBLASTS DURING OSTEOGENESIS

J.B. OWYOUNG

We explore the potential of the ionic products of bioactive glass to induce the up- or down-regulation of genes and transcription factors involved in differentiation and the expression of enzymes and proteins during differentiation. The present study tested the hypothesis that bioactive glass ions enhance the osteogenic potential of human Periodontal Ligament Fibroblasts (hPDLFs) and that a direct connection exists between enhanced gene expression and enhanced matrix protein synthesis.

Experimental bioactive glass (6P53-b) and commercial Bioglass (45S5) conditioned medium was measured for their ion concentrations using inductively coupled plasma mass spectrometry (ICP-MS) prior to addition to hPDLF primary cultures. Gene expression was measured using quantitative reverse transcription-polymer chain reaction (qRT-PCR). Protein expression (ALP, osteocalcin) was measured using enzyme-linked immunosorbent assay (ELISA). A homogeneous, colorimetric method for determining the number of viable cells in proliferation was also conducted (Promega, Madison WI). Statistical comparisons ($p < 0.05$ for statistical significance) were made between GCM-treated cells and control-treated cells using two-way ANOVA.

It was observed that increased silicon, calcium, and sodium concentrations were contained in glass conditioned medium (GCM) as compared to control media (DMEM) after 72 h. Cell number continued to increase over 16 days for both treatment groups and control, both in the presence of ascorbic acid and in its absence. Osteocalcin reached its highest expression level at day eight, with enhanced expression in glass conditioned media compared to control. Average alkaline phosphate over-expression was observed at day two and eight compared to control. Gene expression was

supported by ELISA results, which showed OC protein expression was at its highest concentration at day sixteen, with enhanced expression in glass conditioned media compared to control. AP was over-expressed on days 8 and 16 compared to control.

These results are significant because to this point, no connection has been made to the enhanced gene expression by hPDLFs exposed to bioactive glass ions and their enhanced downstream matrix protein expression. The introduction of a novel bioactive glass powder to hPDLFs may have the potential to produce mineralized matrices and hence the ability to regenerate bone and cementum within the periodontium.

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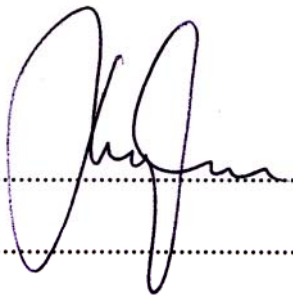

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Committee Chairperson

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SYNOPSIS

Introduction and Specific Aim (Background):

There remains great benefit in developing tissue engineering scaffolds that can predictably stimulate tissue growth, repair, and regeneration. A new family of bioactive glasses (50-59 wt% SiO2) may enhance the osseointegration potential. We explore the potential of the ionic products to induce the up- or down-regulation of genes and transcription factors (intracellular markers) involved in differentiation and the expression of enzymes and proteins (extracellular markers) during differentiation. The present study tested the hypothesis that bioactive glass ions enhance the osteogenic potential of human Periodontal Ligament Fibroblasts (hPDLFs) and that a direct connection exists between enhanced gene expression and enhanced matrix protein synthesis. Therefore, we propose that the introduction of a novel bioactive glass powder to hPDLFs may have the potential to produce mineralized matrices and hence the ability to regenerate bone and cementum within the periodontium.

Materials and Methods

Experimental bioactive glass (6P53-b) and commercial Bioglass (45S5) are melt-derived glasses that were used in the present study (Table 1).

Table 1. Composition (wt. %) of Bioglass (45S5) and experimental bioactive glass (6P53-b)

	SiO_2	Na_2O	K_2O	MgO	CaO	P_2O_5
45S5	45.0	24.5			24.5	6.0
6P53-b	52.7	10.3	2.8	10.2	18.0	6.0

The 6P53-b and 45S5 glass conditioned medium was measured for their ion concentrations using inductively coupled plasma mass spectrometry (ICP-MS) prior to addition to human periodontal ligament fibroblast primary cultures (at confluence in multi-well plates). The cells were induced to differentiate by adding ascorbic acid (50 μg mL^{-1}) to glass conditioned medium (GCM) and control media. Cells were lysed for both mRNA and protein for sample analysis. Gene expression (Col1α1, Col1α2, alk phos, osteocalcin) was measured using quantitative reverse transcription-polymer chain reaction (qRT-PCR) and amplification curve results were analyzed using a sigmoidal curve fitting method (Qiu et al. 2007). Protein expression (ALP, osteocalcin) was measured using enzyme-linked immunosorbent assay (ELISA). A homogeneous, colorimetric method for determining the number of viable cells in proliferation was also conducted (Promega, Madison WI). Statistical comparisons ($p < 0.05$ for statistical significance) were made between GCM-treated cells and control-treated cells using two-way ANOVA (independent variables: time, treatment; dependent variables: gene, protein expression).

Results

The dissolution kinetics profile of commercial bioglass (45S5) in cell culture medium (DMEM), displayed a diffusion limited process at early time points. After 8 hours, the diffusion showed a log increase (linear) and suggests that the diffusion may be reaction limited in the later phase. The pH increase occurs as a result of the ion exchange between Ca+ and Na+ ions in the glasses and H3O+ protons in the media. H+ ions have an affinity for bioglass (Sepulveda et al 2002). The dissolution kinetics of experimental bioglass (6p53-b) (Figure 6) display a similar trend to that of commercial bioglass (45S5).

Inductively coupled plasma mass spectroscopy (ICP-MS) analysis of glass ion concentrations in the glass conditioned media (GCM) showed the behavior of melt-derived 45S5 and 6p53-b glass powders in dissolution. It was observed that increased silicon, calcium, and sodium concentrations were contained in glass conditioned medium (GCM) as compared to control media (DMEM) after 72 h. Increased levels of magnesium were seen in the 6P53-b media.

Cell number continued to increase over 16 days for both treatment groups and control in the absence of ascorbic acid. Glass conditioned medium did not appear to inhibit cell proliferation compared to control (DMEM). Ascorbic acid appeared to slow cell proliferation compared to control (DMEM) as cell number increase was slightly less than in its absence.

q-PCR Gene Expression

Osteocalcin reached its highest expression level at day eight, with enhanced expression in glass conditioned media compared to control (45S5 >>6p53-b >control). Expression of osteocalcin was higher in cells treated with 45S5 glass conditioned media at day two, eight, and sixteen (ANOVA, $p < 0.05$ for statistical significance) as compared to control.

The average alkaline phosphate expression reached its highest level at day two for 45S5 and day eight for 6p53-b. Over-expression was observed at day two and eight, for both 45S5 and 6p53-b, compared to control (ANOVA, $p < 0.05$ for statistical significance).

Collagen 1 Type 1α1 expression reached its highest level at day sixteen for 45S5 and day two for 6p53-b. Over-expression was observed at day two and sixteen, for both 45S5 and 6p53-b, compared to control (ANOVA, $p < 0.05$ for statistical significance).

Collagen 1 Type 1α2 reached its highest expression level at day sixteen for 45S5 and day two for 6p53-b. Over-expression was observed at day two and sixteen, for both 45S5 and 6p53-b, compared to control (ANOVA, $p < 0.05$ for statistical significance).

Protein Expression

Intact human osteocalcin ELISA results showed osteocalcin protein expression was at its highest concentration at day sixteen, with enhanced expression in glass conditioned media compared to control (45S5 >>6p53-b >control). Expression of osteocalcin was higher in cells treated with glass conditioned media at day eight and sixteen (ANOVA, $p < 0.05$ for statistical significance). AP was over-expressed (significant difference: *, $p< 0.05$) on days 8 and 16 compared to control.

Conclusions

This study tested the hypothesis that bioactive glass ions enhance osteogenesis and that there is a direct connection between enhanced gene expression and proteins associated with matrix production. These results are significant because to this point, no connection has been made to the enhanced gene expression by hPDLFs exposed to bioactive glass ions and their enhanced downstream matrix protein expression. Future research will explore the underlying extracellular and intracellular mechanisms that cause enhanced differentiation and impact mineralized-tissue formation. The introduction of a novel bioactive glass powder to hPDLFs may have the potential to produce mineralized matrices and hence the ability to regenerate bone and cementum within the periodontium.

INTRODUCTION

In periodontal treatment therapy, the complete and predictable restoration of the periodontium following trauma or infection remains a critical objective. Bone replacement grafts (BRG) remain among the most widely used therapeutic strategies for the correction of periodontal osseous defects. Bone replacement grafts generally increase bone level, reduce crestal bone loss, and reduce probing depth compared to open flap debridement (OFD) procedures in the treatment of intrabony defects (Reynolds MA et al. 2003). The constant need for periodontal reconstruction has led to the development of high technology materials, with improved biocompatibility, safety, and osteoconductivity.

Bioactive glasses were developed during the past few decades. Commercially available, 45S5 Bioglass® (Novabone-C/M®, Porex Surg., Newnan, GA) has been a promising alternative for bone replacement and augmentation because of its bone-binding and osteoconductive properties (Moreira-Gonzalez et al. 2005).

Despite some clinical success, there remains great benefit in developing tissue engineering scaffolds that can predictably stimulate tissue growth, repair and regeneration. A new family of bioactive glasses (50-59 wt. % SiO_2) may enhance the osseointegration potential (Saiz et al. 2002, Foppiano et al. 2006). Direct bone bonding may be possible as bioactive glasses have been shown to form a hydroxyapatite surface layer and release calcium and silicon ions in the presence of osteoblasts (Foppiano et al. 2004, V. G. Varanasi et al. 2006). The use of bioactive materials in tissue engineering strategies for the repair and regeneration of bone continues to be a promising area of interest.

Current reports have shown the capacity of periodontal ligament cells to function as osteoblasts or cementoblasts under regenerative conditions. Human Periodontal Ligament Fibroblasts (hPDLFs) are the cells essential for periodontal regeneration. They comprise a heterogeneous cell population and consist of several cell subsets that differ in their function. It is known that Human Periodontal Ligament Fibroblasts (hPDLFs) produce osteoblast-related extracellular matrix proteins and show higher ALP activity compared with gingival fibroblasts, indicating that hPDLFs have osteogenic characteristics (Murakami 2003).

At present, the detailed interaction of bioactive glass dissolution products (ions) and hPDLFs has not yet been explored. It is known that PDLFs can differentiate into hard tissue-forming cells, thus playing a key role in the regeneration of periodontal tissues. PDL fibroblasts express transcripts common to bone and cementum, making them potentially capable of forming these tissues while utilizing different molecular pathways of differentiation (Fowler MM and Lallier TE 2002). However, the exact mechanisms involved in osteoblastic differentiation of PDL cells are still poorly understood (Murakami 2003, Nojima N 1990, Arceo N 1991, Cho MI 1992).

In this aim, we intend to characterize the interaction of bioactive glass dissolution products with hPDLF cells. The in vitro dissolution behavior of glass powders may be maximized, in order to optimize the effects of these ions during hPDLF differentiation. We will explore the potential of the ionic products to induce the up- or down-regulation of genes and transcription factors (intracellular markers) involved in differentiation and the expression of enzymes and proteins (extracellular markers) during differentiation. The improved differentiation may be observed in vitro, by monitoring collagen type 1 (Col1α1) and alpha 2 (Col1α 2), and its downstream enhancement of alkaline phosphatase (ALP) and osteocalcin.

Thus, the present study tested the hypothesis that bioactive glass ions enhance the osteogenic potential of hPDLFs and that a direct connection exists between enhanced gene expression and enhanced matrix protein synthesis. The key osteogenic markers to be investigated here are collagen type 1 alpha 1 (Col1α1) and alpha 2 (Col1α2), alkaline phosphatase (ALP), and osteocalcin.

Therefore, we propose that the introduction of a novel bioactive glass powder to hPDLFs may have the potential to produce mineralized matrices and hence the ability to regenerate bone and cementum within the periodontium.

REVIEW OF THE LITERATURE

Clinical Periodontal Regeneration

Periodontal disease is marked by subsequent loss or damage due to infection affecting tooth-supporting tissue including the bone, cementum, and periodontium. These structures maintain the attachment of teeth to the alveolar bone and have shown the ability to regenerate adjacent cellular matrices. Periodontal disease may be implicated in the loss of regenerative potential of bone-associated proteins in human periodontal ligament fibroblasts (hPDLFs) (Sawa et al 2000).

Although fresh autografts remain the optimal material for all forms of surgery seeking to restore structural integrity to the skeleton, it is evident that the supply of such tissue is limited. There are few sources of donor bone in the body, and harvesting bone from other sites can cause significant morbidity. Furthermore, the amount of bone that can be obtained is limited, and the shape and size of the graft may render its implantation technically difficult, if not impossible (Kaufmann et al 2000).

A wide range of graft materials have been applied and evaluated clinically, including autografts, allografts, xenografts, and synthetic/semi-synthetic materials. Overall, the use of specific biomaterials/biologicals was more effective than open flap debridement (during surgical periodontal treatment therapy) in improving attachment levels in intraosseous defects (Reynolds MA et al. 2003).

Any successful regenerative material for periodontal application must satisfy the clinician in several ways. The initial requirement is that it should be easy to handle and can be placed in the defect where it should remain until, and after, the procedure is completed. Once in place it must allow restoration of the periodontiurn, including bone, and at the same time prevent epithelial downgrowth. A successful material must interact with repairing bone, repairing connective tissue and gingival epithelium: all of these

interactions taking place in an environment which is already infected and reactive due to periodontal disease. Problems of storage and certification of allogenic freeze-dried bone make it increasingly unpopular for what have become known as social-legal reasons. For these reasons, particulate bioactive ceramics have been proposed and applied to periodontal defects since the mid-1970s (Levin MP 1974).

Bioglass is an alloplast, a synthetic graft or inert foreign body implanted into tissue. Presently, six basic types of alloplastic materials are commercially available: nonporous hydroxyapatite (HA), hydroxyapatite cement, porous hydroxyapatite (replamineform), beta tricalcium phosphate, PMMA and HEMA polymer (a calcium layered polymer of polymethylmethacrylate and hydroxyethylmethacrylate), and bioactive glass. It has been reported that porous and non-porous HA materials and PMMA and HEMA polymer are nonresorbable while tricalcium phosphate and bioactive glass are bioabsorbable (AAP 2001).

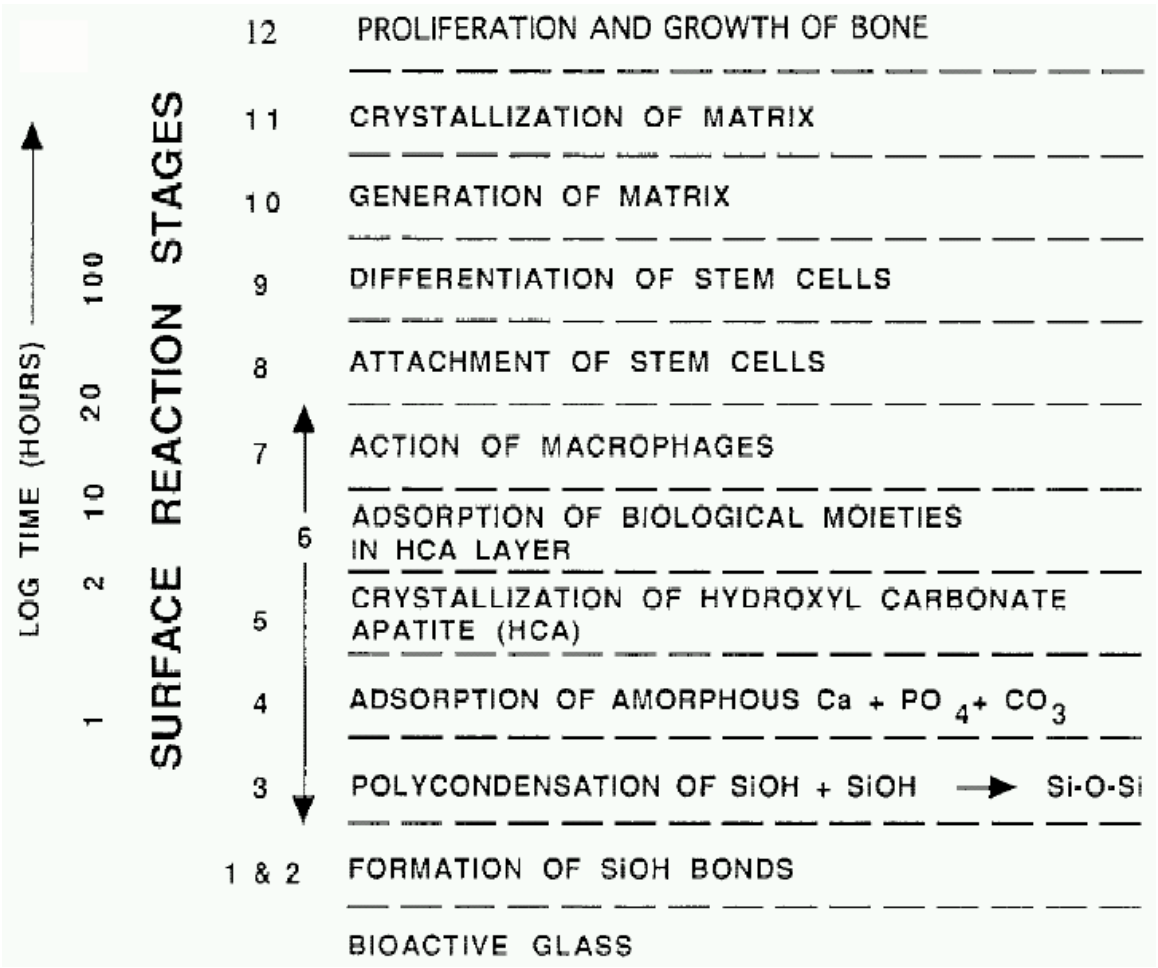
Clinical success of bioglass in regenerative periodontal treatment has been well demonstrated. Wilson and Low showed that two bioglass materials were found to be easily manipulated, were haemostatic and osteoproductive allowing restoration of both alveolar bone and periodontal ligament in a monkey model (Wilson and Low 1992). In humans, bioactive glass treatment of intraosseous defects by means of bone grafting has shown statistically significant improvement of the bony lesion in surgical periodontal treatment (Ong et al. 1998, Park et al. 2001, Zamet et al. 1997). Despite some clinical success, it is necessary to improve tissue engineering scaffolds that can predictably stimulate tissue growth, repair and regeneration.

Bioactive Glass History and Overview

Bioactive glass is often described as a bone substitute. It is a transparent bioactive material with proven ability to bond to bone and connective tissue. Surface-reactive ceramics, glasses, and glass-ceramics attach directly by chemical bonding with the bone (bioactive fixation). This material is made from calcium salts, phosphate, sodium salts, and silicon. The addition of silicon allows for the formation of a silica gel layer over the bioactive glass particles. This layer promotes formation of a hydroxycarbonate-apatite layer onto which osteoblasts are said to proliferate and form bone (Hench LL 1990, Froum SJ et al 1998).

45S5 bioactive glass was formulated by Hench in 1969 and is currently used as a bone regenerative material because of the formation of an apatite phase on the surface of the glass after immersion in physiological fluid. A widely accepted explanation concerning the stages of Bioglasss reactions is the one proposed by Clark and Hench. Briefly, these involve cation release from the glass with consequential increase of pH in the solution, formation of a silica-rich layer and precipitation of a Ca–P rich layer that further crystallizes as hydroxycarbonate apatite (HCA) itself. Bioglass forms a bone-like surface hydroxyapatite (HA) layer that facilitates direct bone cell attachment. It contains a highly porous surface and underlying layers for bone matrix ingress. Bioglass releases calcium and phosphate ions to cells as raw materials for bone matrix formation (Hench 1990).

The diagram below illustrates the 12 reaction stages of bioactive glass to bone formation. The sequence of interfacial reactions involved in forming a bond between bone and a bioactive glass is described (Hench LL 1998):



Bonding of bone to bioactive glasses involves 12 reaction stages. The first five stages occur very rapidly on the surface of most bioactive glasses because of fast ion exchange of alkali ions with hydrogen ions from body fluids (stage 1), network dissolution (stage 2), silica-gel polymerization (stage 3), and chemisorption and crystallization of the HCA layer (stages 4 and 5). The surface reactions lead to the biochemical adsorption of growth factors (stage 6) and the synchronized sequence of cellular events (stages 7–12) that result in rapid formation of new bone (Hench LL 1998).

The bone-binding ability (of bioglass) occurs through the development of a biological apatite layer when the materials are exposed to body fluids or simulated body fluids. For silica-containing bioactive glasses, the main reactions stages are as follows: an ion exchange with hydrogen ions from the surrounding medium; silicate glass network

dissolution; and the condensation of hydrated silanols on the surface to form a rich silica layer that catalyzes the precipitation of a Ca-P film; this layer crystallizes to form hydroxycarbonate-apatite (HCA) with further incorporation of carbonate ions. Studies in vivo have shown that biological species are incorporated into the silica-rich and apatite layers, promoting a continuous compliant bond of the material with the bone tissue and soft tissue (Sepulveda et al 2002).

Many different factors influence the mechanism and rate of glass dissolution, such as glass composition, pH and ionic strength of the leaching solution, surface area of glass in contact with the solution, liquid phase volume, glass surface roughness, glass surface features (e.g. presence of silanol groups, or unsaturated cations) (Cerruti et al. 2005).

The inductive properties of bioactive glasses have been demonstrated in various ways. Previous results from our laboratory (Foppiano 2004, Varanasi 2006) indicate that bioactive glasses may play an active role in pre-osteoblast differentiation. There is evidence to show that the ionic products of bioactive glass dissolution appeared to induce the up-regulation of Col1 and Runx2 that are noted markers for pre-osteoblast differentiation to a mineralizing phenotype (Foppiano 2004). Osteoblasts were seen to enhance the expression of key osteogenic markers when exposed to the ionic products of bioactive glass dissolution. Expression of other markers (e.g., collagen type 1, core binding factor a, runx2, and alkaline phosphatase) was also over-expressed when exposed to glass ions during the first 10 days of differentiation (Foppiano et al 2007).

Several reports have shown that the enhancement of mineralization is due to the presence of specific ions released by the biomaterials. Refitt et al. studied the effect of dietary levels of orthosilicic acid had on osteogenesis in vitro (Refitt et al 2003). They noted the increased expression of collagen type 1 synthesis and alkaline phosphatase enzymatic activity when exposed to silicon. There are possible stimulatory effects of the ionic products of Bioglass 45S5 dissolution on human osteoblast proliferation and

therefore simple inorganic chemical solutions may deliver mitogenic stimuli (Xynos et al. 2000). It is suggested that not only will the bioglass provide space-maintenance when placed in a periodontal osseous defect, but that the specific dissolution products (ions) of bioactive glass may play a role in periodontal regeneration when used as a bone replacement graft.

Human Periodontal Ligament Fibroblasts

Due to their unique localization, the periodontal ligament cells have the responsibility of producing, maintaining and remodeling the periodontal ligament, cementum and bone. This heterogeneity has been suggested to be of fundamental importance for the normal functioning as well as wound healing of connective tissue. This is particularly the case in the periodontium, which is a complex tissue made up of cementum, alveolar bone, periodontal ligament and gingiva. This diverse composition makes periodontal wound healing a more complex process than general soft tissue healing because of the interaction between hard and soft connective tissue as well as epithelium (Melcher AH 1976).

Evidence exists suggesting that a proportion of periodontal ligament fibroblasts express osteoblast-like properties that gingival fibroblasts lack. Periodontal ligament fibroblasts form mineralized nodules in culture and produce alkaline phosphatase to a greater extent than gingival fibroblasts. These findings suggest that periodontal ligament fibroblasts possess the ability to form and possibly regenerate both hard and soft connective tissue extracellular matrices (Arceo N et al. 1991).

It has been suggested that periodontal ligament fibroblasts (PDLFs) are the cells essential for periodontal regeneration. PDLFs comprise a heterogeneous cell population and consist of several cell subsets that differ in their function. It is known that PDLFs produce osteoblast-related extracellular matrix proteins and show higher alkaline

phosphate (ALP) activity compared with gingival fibroblasts, indicating that PDLFs have osteogenic characteristics. Several reports indicate that PDL cells have the potential to differentiate into cementoblasts and osteoblasts, thus playing a key role in the regeneration of periodontal tissues. However, the exact mechanisms involved in osteoblastic differentiation of PDL cells are still poorly understood (Murakami 2003).

Gene Expression

Ionic products of bioactive glass dissolution may induce the expression of a particular gene that has a downstream effect on other genes or matrix protein expression. The elevated in vitro mRNA expression for alkaline phosphatase, collagen type 1 alpha 1, and osteocalcin may be used to identify cells with the potential to facilitate hard tissue formation and hence periodontal regeneration. Ivanovski et al (2001) showed that the elevated in vitro mRNA expression for osteocalcin, osteopontin and bone sialoprotein may be used to identify cells with the potential to facilitate hard tissue formation and hence periodontal regeneration.

To our knowledge, the detailed interaction of bioactive glass dissolution products (ions) and hPDLFs has not yet been explored. Our set of studies aims to relate gene to extracellular matrix protein production. The intended genes include collagen type 1α1 and collagen type 1α2 which may impact downstream marker expression of proteins, alkaline phosphatase and osteocalcin.

Collagen Type 1 α 1 encodes the pro-alpha1 chains of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Type I is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis and tendon. Mutations in this gene are associated with osteogenesis imperfecta types I-IV, Ehlers-Danlos syndrome type VIIA, Ehlers-Danlos syndrome Classical type, Caffey Disease and idiopathic osteoporosis. Reciprocal translocations between chromosomes

17 and 22, where this gene and the gene for platelet-derived growth factor beta are located, are associated with a particular type of skin tumor called dermatofibrosarcoma protuberans, resulting from unregulated expression of the growth factor. Two transcripts, resulting from the use of alternate polyadenylation signals, have been identified for this gene (Garnero et al 2009).

Collagen Type 1 α 2 encodes the pro-alpha2 chain of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Mutations in this gene are associated with osteogenesis imperfecta types I-IV, Ehlers-Danlos syndrome type VIIB, recessive Ehlers-Danlos syndrome Classical type, idiopathic osteoporosis, and atypical Marfan syndrome. Symptoms associated with mutations in this gene, however, tend to be less severe than mutations in the gene for the alpha1 chain of type I collagen (COL1A1) reflecting the different role of alpha2 chains in matrix integrity. Three transcripts, resulting from the use of alternate polyadenylation signals, have been identified for this gene (Garnero et al 2009).

Alkaline phosphatase is an enzyme belonging to a group of membrane-bound glycoproteins. Although its physiological function still remains unclear, ALP may play a key role in the formation and calcification of hard tissues, and its expression and enzyme activity are frequently used as markers of osteoblastic cells. ALP exists in both a soluble form and bound to plasma membranes, but ALP activity is principally due to the membrane-bound form. The activity of alkaline phosphatase is required to generate the inorganic phosphate needed for hydroxyapatite crystallization. Extracellular inorganic pyrophosphate is a potent inhibitor of hydroxyapatite formation. Alkaline phosphatase hydrolyzes extracellular inorganic pyrophosphate. The product of this gene is a membrane bound glycosylated enzyme that is not expressed in any particular tissue and is, therefore, referred to as the tissue-nonspecific form of the enzyme. A proposed function of this form of the enzyme is matrix mineralization; however, mice that lack a

functional form of this enzyme show normal skeletal development (Togari A et al. 1993, Beertsen W et al. 1990).

Osteocalcin, the major non-collagenous protein of bone, is a specific product of the osteoblast and its synthesis is stimulated by 1, 25-Dihydroxyvitamin D3. A fraction of that synthesized does not accumulate in bone but is secreted directly into the circulation. Animal studies indicate serum osteocalcin is derived from new cellular synthesis rather than from the release of bone matrix protein during resorption. Furthermore, serum osteocalcin concentrations are related to histomorphometric measurements of bone formation but not resorption. Deviations from normal concentrations of circulating osteocalcin are a consequence of changes in the synthesis or degradation of the protein. Such changes may result from physiological alterations in skeletal homeostasis that accompany normal development or may be associated with specific disease states. While the precise biological function of osteocalcin is not known, it is the most specific marker of osteoblastic function described to date (Hauschka et al. 1989). Osteocalcin is known to localize in bone, cementum and dentin, and in the formative cells of these hard tissues. It may play a role in delaying nucleation and in preventing excessive crystal growth (Ducy P et al. 1996).

Endogenous reference genes often referred to as housekeeping genes, are routinely used to normalize gene expression levels (Murthi et al. 2008). Expression of the target gene is usually normalized relative to that of a reference gene, which is either known or assumed to express stable levels of transcripts in most tissue or at least in the tissues being compared. A number of reference gene transcripts, such as those encoding the small or large subunits of ribosomal RNA (rRNA), β-actin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), have been used as reference genes in RT-qPCR analyses (Bustin 2000). Transcripts of rRNA genes are present at levels several orders of magnitude greater than transcripts of protein-coding genes, and their measurement

requires dilution of the sample if they are to be used as a reference for measurement of mRNA. Diluting samples can be inconvenient and introduces a potential source of error. A major assumption of real-time PCR analysis is that the reference gene is expressed at a constant level in all tissues and at all stages of development and should not be affected by the experimental treatment (Bustin, 2000).

Although rRNA is considered a stable reference because of its abundance, this may not be the case with transcripts of other reference genes that are commonly used. The selection of an appropriate reference gene is very important for RTqPCR analysis in order to obtain consistent and reliable results. The use of reference genes that are expressed at equivalent levels relative to rRNA in diverse samples also makes it possible to assess differences in the absolute amount of transcripts of target genes in such samples. Reverse transcriptase quantitative polymerase chain reaction (qRT-PCR) is a powerful tool to obtain data about gene expression. Normalization enables one to determine the change in mRNA levels of a gene of interest from multiple samples versus one or more reference gene(s), often a housekeeping gene. Reference genes are selected on the basis of constitutive expression across samples to allow the reliable quantification of changes in gene expression. At present, a universally optimal reference gene has not been identified for any organism that can be used across different tissue types or disorders (Benn 2008).

Results are reported relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. In addition to this long established metabolic function, GAPDH has recently been implicated in several non-metabolic processes, including transcription activation, initiation of apoptosis, and ER to Golgi vesicle shuttling (Tarze et al 2007).

PURPOSE

The goal of this investigation is to evaluate the idea that bioactive glass ions enhance the differentiation/proliferation potential of human periodontal ligament fibroblast cells suggesting possible osteogenic potential and that a direct correlation exists between enhanced gene expression and enhanced matrix protein synthesis. The key osteogenic markers to be investigated in the present study are collagen type 1α1, collagen type 1α2, alkaline phosphatase (ALP), and osteocalcin.

HYPOTHESIS

It is hypothesized that the in vitro dissolution behavior of glass powders can be maximized, in order to optimize the effects of these ions during PDLF differentiation.

The bioactive glass dissolution products (ions) will enhance differentiation and/or proliferation of human Periodontal Ligament Fibroblast (hPDLFs) thus increasing osteogenic potential.

The null hypothesis is that bioactive glass dissolution products (ions) will have no effect on differentiation and/or proliferation of Periodontal Ligament Fibroblast (PDLFs).

SIGNIFICANCE OF THIS RESEARCH

To our knowledge, the detailed interaction of bioactive glass dissolution products (ions) and human periodontal ligament fibroblasts (hPDLFs) has not yet been explored. Therefore, the introduction of a novel bioactive glass powder to hPDLFs may have the potential to produce mineralized matrices and hence the ability to regenerate bone and cementum within the periodontium.

MATERIALS AND METHODS

Specimen Fabrication

Experimental bioactive glass (6p53-b) and commercial Bioglass (45S5) are melt-derived glasses that were used in this study (Table 1).

The bioactive glass powder specimens, purchased commercially, were prepared and fabricated by Mo-Sci Specialty Products L.L.C. (North Rolla, MO) to a specific particle size range of 100-300 microns. Verification of the specific particle size range was completed by Mo-Sci Specialty Products L.L.C. with a sieve technique prior to use in the present study.

The particle size of the bioactive glass powders was chosen with aims to optimize *in vitro* dissolution behavior, so that the effects of these ions during human periodontal ligament fibroblast (hPDLF) differentiation would be maximized.

Shapoff and coworkers (1980) described that particle size should be considered a variable in periodontal application. Their studies found that a smaller particle size (100-300 microns) had more osteogenic potential than a larger one (1000-3000 microns).

Sepulveda et al. (2002) determined that the rates of dissolution for two types of bioactive glass powders increased as the particle size decreased. Thus, particle size range can be used as a means to control the release rate of active ions that stimulate the gene expression and cellular response for tissue proliferation and repair.

In the present work, we analyzed and modeled the dissolution kinetics of both 45S5 (commercial bioglass) and 6p53-b (experimental bioglass). The early stages of reactivity of the two bioactive glass samples (45S5 and 6p53-b) were evaluated over 72 hours. Ion release, measured with ion-coupled plasma mass spectroscopy, and pH variations are reported to describe these materials.

Cell-free testing of both bioactive glass powders (45S5 and 6p53-b) was completed prior to cell-culture work as an initial screen to evaluate the bio- or cyto-compatibility of materials with human Periodontal Ligament Fibroblasts (hPDLF). This allows for tighter and more stringent controls over testing of these materials with the primary cell culture. Stage 1 included cell-free *in vitro* testing to study dissolution kinetics of the bioactive glass powders. This allows for understanding of dissolution kinetics and determines the point at which Si/Ca leaching is at its zenith. pH was determined to optimize hPDLF cell viability during subsequent experiments as pH variations of glass conditioned medium (GCM) are reported in Stage 1 of the present study. The dissolution kinetic profile has been well-described for 45S5 but has not been well-described for 6p53-b.

Dissolution Kinetics Experimental Protocol

The selected amount of bioactive glass powders (0.8g) was dissolved in a 50ml centrifuge tube with 50ml of un-supplemented medium (Dulbecco's Modified Eagle's Medium, DMEM, UCSF Cell-Culture Facility San Francisco, CA), under continuous stirring (stirring rate = 75rpm), at a temperature of approximately 37°C in a water bath. The centrifuge tube was sealed immediately after introduction of the bioactive glass to prevent evaporation and stored in an incubator set at 37°C for 24 hours prior to time-point 0 (Cerruti et al. 2005).

The dissolution experiments were conducted in triplicate over 3 days (72 h) for each commercial bioglass (45S5), experimental bioglass (6p53-b), and control (DMEM). At the scheduled time points (1,2,4,8,24,48,72 hours), the sample was vortexed for 3 seconds and 1 ml of the solution was removed with a sterile pipette at a consistent distance from the glass powder interface (at the 25ml marked line of the centrifuge tube), and introduced into a new 15 ml centrifuge tube. The pH of the Glass Conditioned Media

(GCM) solutions was measured with a portable, digital Accumet 1003 pH meter by Fisher, at a temperature of approximately 37°C in a water bath.

The 9 volt Fisher Accumet 1003 portable pH meter (Fisher Scientific. Pittsburgh, PA) measures pH (range -6 to 20); mV (range +/-999); Ion (range: 0.001 to 99900) and temperature (0 to 100C). This meter uses a pH/ATC combination electrode.

Sample Preparation for Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

At each time point listed above, 1 ml of GCM was removed in a similar manner and stored at room temperature in a 15 ml centrifuge tube for future analysis with ICP-MS. Samples were prepared for ion-coupled plasma mass spectroscopy according to the guidelines of the Interdisciplinary Center for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) at the University of California, at Davis. 100ul of each sample to be analyzed was placed in new 15 ml centrifuge tube and diluted with 3% nitric acid under a fume hood (1:100).

Characterizations of the ions in solution (or corrosion products) were evaluated by the Interdisciplinary Center for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) at the University of California, at Davis. Inductively coupled plasma mass spectrometry (ICP-MS) is a fast, precise, and accurate multi-element analytical technique for the determination of trace element abundances. Because of its high sensitivity (detection limit at parts per trillion levels) and capability of analyzing both solution and solid samples, ICP-MS has broad applications in many fields including the earth, environmental, materials, biological, and medical sciences. The UCD/ICPMS has two ICP quadrupole mass spectrometers (Agilent Technologies 7500a and 7500ce). (http://icpms.geology.ucdavis.edu/InstrumentationQuad.html)

Specimen and Glass Conditioned Media Preparation

Bioactive glass specimens were assembled using a melt-derived method and purchased commercially from Mo-Sci Specialty Products L.L.C. (North Rolla, MO). Compositions are described in detail at the beginning of this section and also in Table1.

45S5 (commercial) and 6P53-b (experimental) powders of similar particle size (100-300 μm) were soaked in Dulbecco's modified Eagle medium (DMEM) for 3 days in order to pre-condition the glass and prepare glass conditioned media (GCM). This procedure allows release of ions leading to a stable pH. This ion extract was filter-sterilized (0.2 μm) and collected. Ion extracts were then supplemented with fetal bovine serum (10% by volume), penicillin-streptomycin (1% by volume), and fungizone (0.5% by volume) to provide the final glass conditioned medium (GCM). Control medium was prepared using D-MEM supplemented with FBS and penicillin-streptomycin/antimycotic in the same percentages.

For mineralization studies, glass conditioned mineralization media was prepared in a similar manner by supplementation of the glass conditioned media and control with ascorbic acid (AA, 50 mg/L), in the absence of beta-glycerol-phosphate(b-GP). This was completed for consistency with the cell culture experiments.

Cell Culture and Cell Proliferation Assay

Human Periodontal Ligament Fibroblast (hPDLF) cell preparation and culture was modified slightly from the technique by Somerman et al. (1988). hPDLF cells were isolated from healthy periodontal ligament tissues of premolar teeth of individuals undergoing tooth extraction for orthodontic reasons. Periodontal ligament tissue was removed from the center (middle one-third) of the root surface with a surgical scalpel. The tissue was minced and transferred to a culture plate. The explants were cultured in

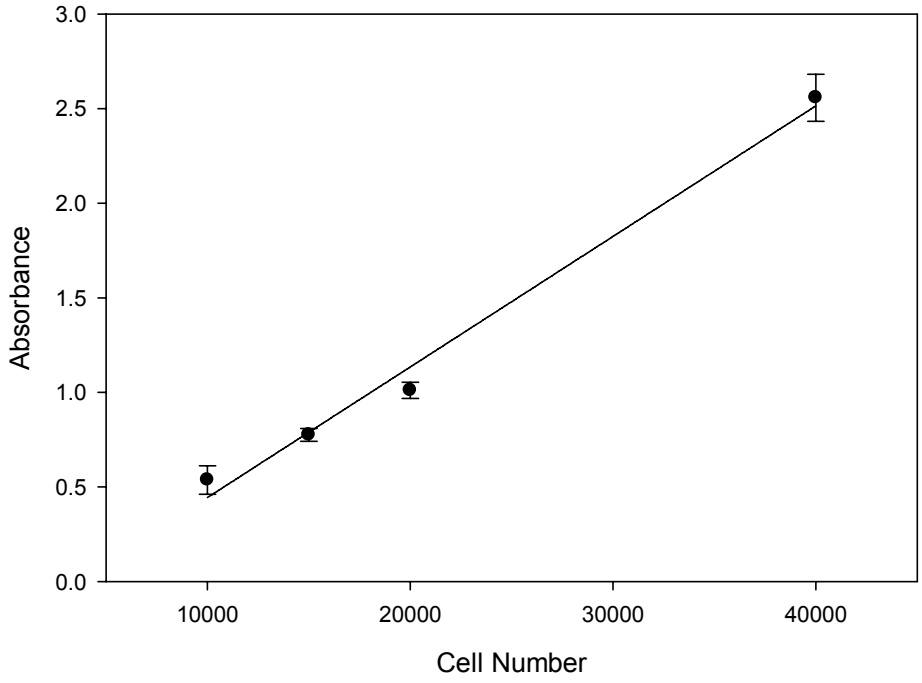
Dulbecco's modified Eagle medium (D-MEM) (UCSF, Cell Culture Facility) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic at 37 degrees C in a humidified atmosphere of 95% air and 5% CO2, until the cells grew out of the explants and became nearly confluent. Every 2 days the medium was changed and growth was monitored. The cells were then trypsinized and cells from passages 5-9 were used for the experiments. Reserve cells were stored in the UCSF Cell-Culture facility, an on-campus cryogenic cell bank, at passage 4 for convenience and future work.

For cell culture experiments, cells were seeded (350,000 – 400,000 cells cm^{-2}) and allowed to attach and proliferate through their doubling time (24 hours) in triplicate in 6-well plates. Cells were counted using a standard hemacytometer and an optical microscope (Nikon TE300, Nikon Incorporated, Tokyo, Japan) and were synchronized with D-MEM, 1% FBS, and 1% pen-strep. Cells were allowed to differentiate in ascorbic acid with control medium (50 mg L^{-1}) for 16 days, with replenishment of media at 2 day intervals. Experiments were carried out in triplicate for 16 days, with samples generated at days 0, 2, 4, 8, and 16. Media extracts were stored at -20°C for future work.

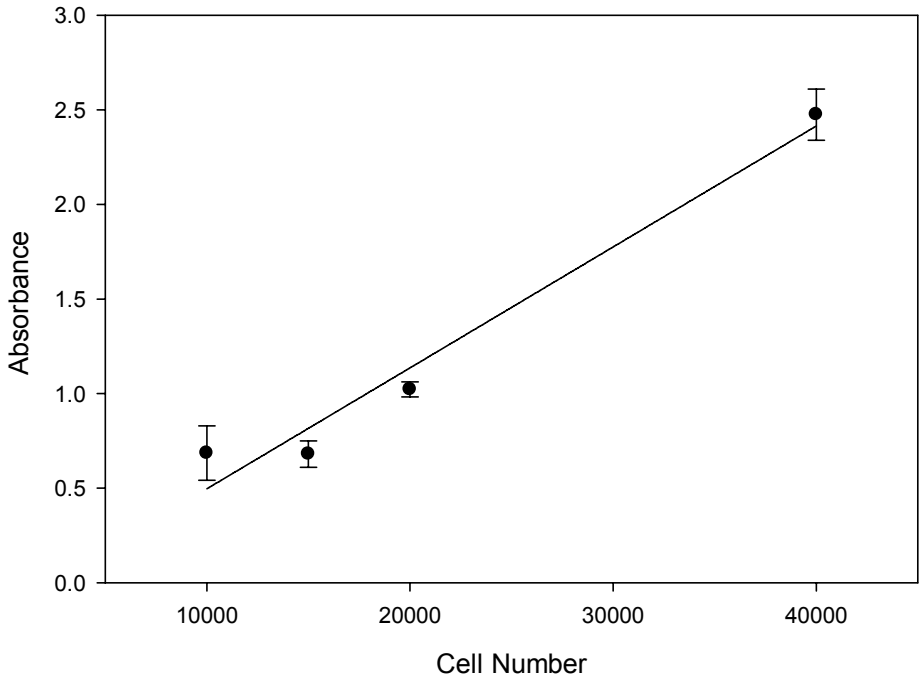
hPDLF cells were then allowed to mineralize by the introduction of mineralization media, which consisted of their respective GCM with ascorbic acid in the absence of glycerol 2-phosphate. Experiments were carried out in triplicate for 16 days, with samples generated at days 0, 2, 4, 8, and 16.

A homogeneous, colorimetric method for determining the number of viable cells in proliferation was conducted (Promega, Madison WI). This assay is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; abbreviated ($MTS^{(a)}$)] and an electron coupling reagent (phenazine methosulfate) PMS. MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The

absorbance of the formazan product at 490nm was measured directly from 96-well assay plates without additional processing. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. A standard curve was developed for the proliferation assay.



Cell Proliferation Standard Curve in the absence of ascorbic acid (r^2=0.98). Each data point represents triplicate results as average ± standard deviation.



Cell Proliferation Standard Curve in the presence of ascorbic acid (r^2=0.95). Each data point represents triplicate results as average ± standard deviation.

Real-Time quantitative PCR

Cells disrupted with RLT Buffer (a buffer containing guanidine thiocyanate and β-mercaptoethanol; CelLytic M, Sigma Aldrich, St. Louis, MO) were collected in microcentrifuge tubes. Total Cellular RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen Pty Ltd), according to the manufacturer's instructions.

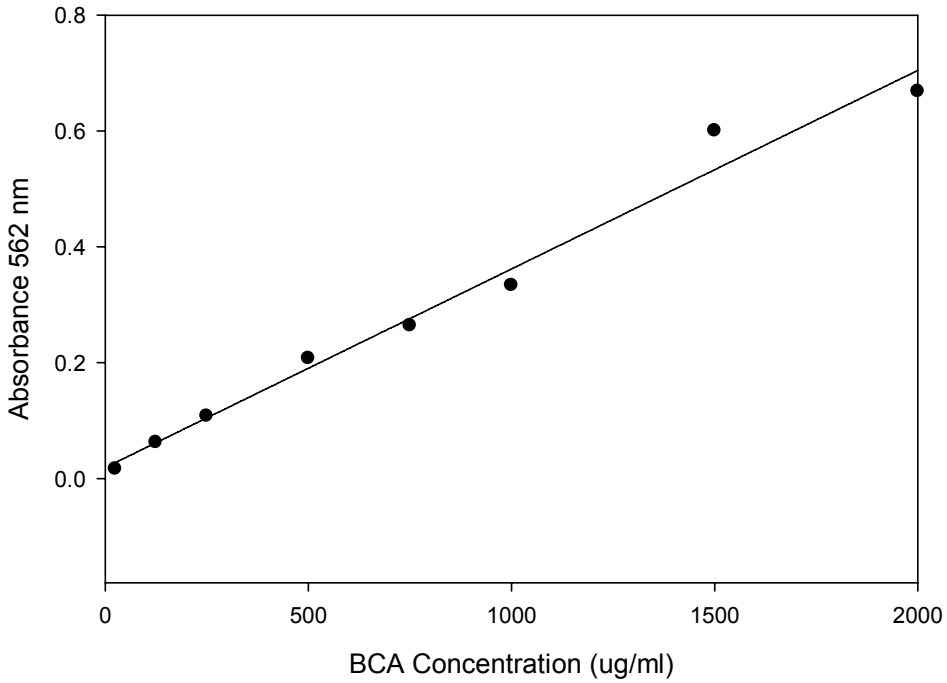
FastStart TaqMan® Probe Master (Roche Applied Science. Mannheim, Germany) was used for running qPCR, a quantitative, real-time DNA detection assay. The product is provided as a 2× concentrated master mix that contains all the reagents (except primers, probe and template) needed for the hydrolysis probe detection format.

A stable, lyophilized enzyme with RNase-Free Buffer and water (RNase-Free DNase Set- Qiagen. Valencia, CA) was used for more complete DNA removal due to the sensitive nature of our RNA application. There was an expectation that we would extract only very small amounts of DNA. This particular set is quality-controlled and optimized for use with the subsequent RNeasy procedures. It also provides efficient on-column digestion of DNA during RNA purification from cells and tissues using RNeasy Kits. The DNase is efficiently removed in subsequent wash steps.

To quantify the expression of alkaline phosphatase and noncollagenous bone proteins (osteocalcin), semiquantative RT-PCR, relative to glyceradldehyde-3-phophate dehydrogenase (GAPDH) was performed. Primers for real-time quantitative PCR were obtained from Applied Biosystems Inc. (Foster City, CA) and selected for minimal amplicon length and number of mRNA gene banks.

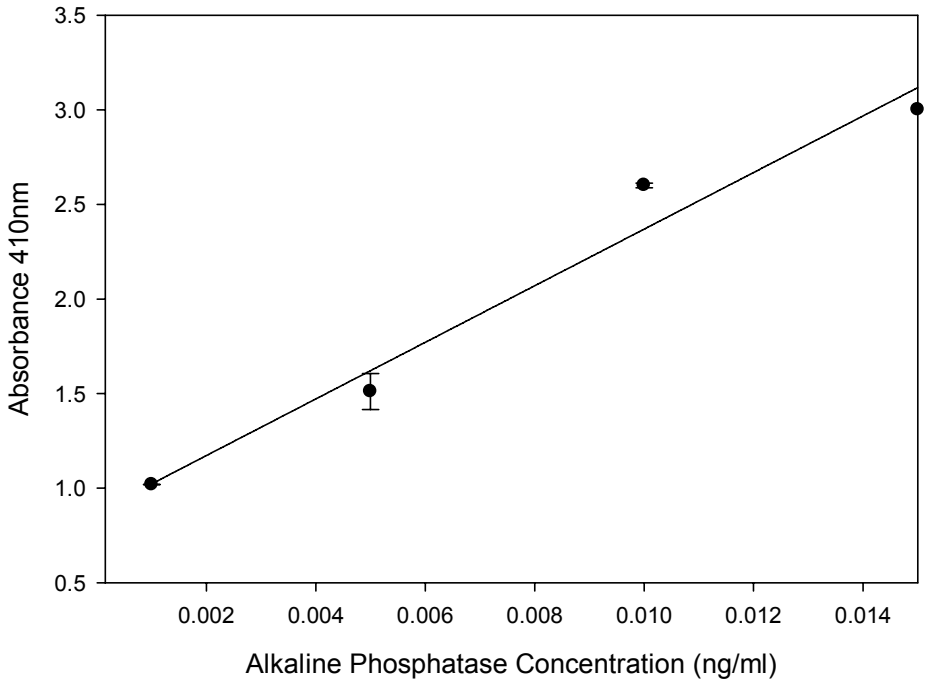
Protein expression assays for hPDLF

At the described time points (0,2,4,8,16 days), cell cultures were scraped and collected in phosphate buffered saline, centrifuged, and collected as a cell pellet. Pellets were treated with the appropriate lysis buffer (Celytic M, Sigma Aldrich, St. Louis, MO). Protease inhibitors (Sigma Aldrich) were used as needed for the prevention of unwanted degradation of proteins during their isolation and characterization. The concentration of total protein was assayed and the expression level of alkaline phosphatase (APF, Sigma Aldrich) and osteocalcin (Biomedical Technology Inc. Stoughton, MA) were evaluated. A standard curve was developed for the total protein, alkaline phosphatase, and osteocalcin assay.



BCA standard curve (total protein) (r^2=0.98). Data points represent absorbance values of known albumin standard stock concentrations.

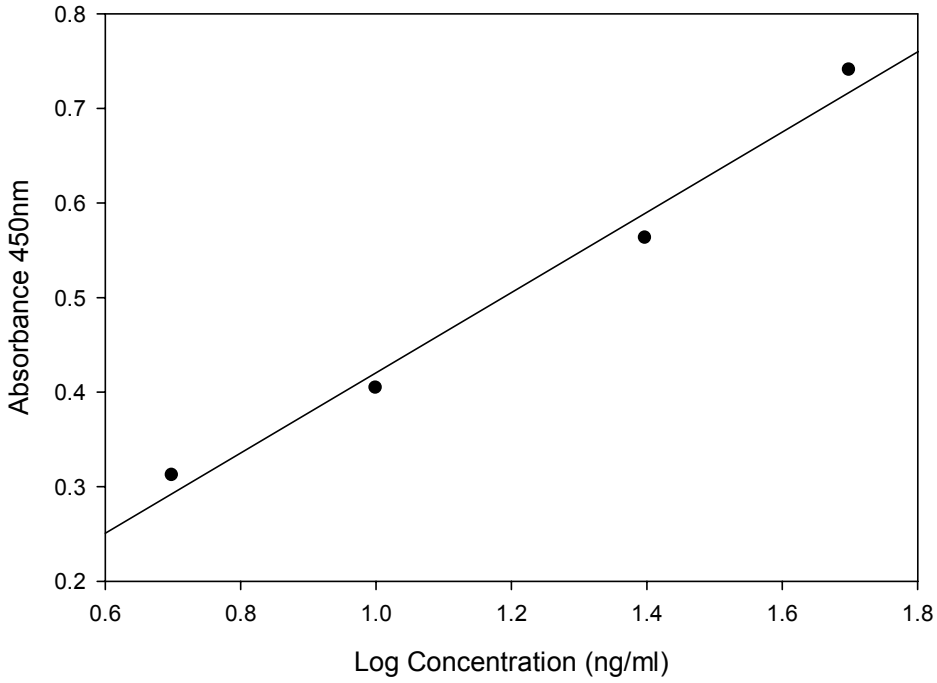
Expression levels of alkaline phosphatase at described data points were evaluated by mixing the extracted proteins with alkaline buffer solution and stock substrate solution, followed by incubation at 37^0C for 15 minutes. Samples were then added to test wells, and deionized water served as control. Plates were incubated at 37^0C for another 15 minutes and 0.05N NaOH was added to each well. The solutions were then transferred to a 96 well plate (200 µL/well) and read in a spectrophotomer at an absorbance of 410nm to record their intensity levels relative to a bovine intestinal alkaline phosphatase standard.



Alkaline Phosphatase ELISA standard curve (r^2=0.97). Each data point represents triplicate results as average ± standard deviation.

The concentration of total osteocalcin at desired data points was evaluated with the BTI Human Osteocalcin RIA Kit (Biomedical Technology Inc. Stoughton, MA). The kit includes 5 individual standards and a simplified 3.5 hour protocol. The assay requires only a 50ul sample and yields a range of 1.0-50 nanograms per ml (sensitivity: 0.5 ng/ml). Serum samples and cell culture supernatants can be measured directly. This stock standard was diluted seven times producing 50ng/mL, 25ng/mL, 12.5ng/mL, 6.25ng/mL, 3.12ng/mL, 1.56ng/mL and 0ng/mL solutions. These solutions were pipetted (n=3 at 25µL) into the provided microtiter plate. Next, 25µL of protein test samples from days 0-16 were pipetted into wells, followed by 100µL of osteocalcin antiserum. The plate was sealed and incubated at 4^0C for 18 hours. The following day, phosphate-saline wash buffer was prepared by diluting the provided 100mL bottle with 400mL deionized

water. Each well was aspirated completely and washed 5 times with 0.3mL/well of phosphate-saline wash buffer. Then, 100µL of streptavidin-horseradish peroxidase was added to each well. The plate was swirled and incubated at room temperature for 30 minutes. During this time, 4mL of TMB solution was mixed with 4mL of hydrogen peroxide solution and set aside. After incubation time, all wells were aspirated and washed 5 times with 0.3mL/well of phosphate-saline wash buffer. Next, 100µL of the TMB-H_20_2 solution was added to each well and left to incubate at room temperature, in the dark, for 15 minutes. Stop solution (100µL/well) was added to all wells, the plate was swirled, and immediately measured with a spectrophotometer at an absorbance of 450nm.



Intact Human Osteocalcin ELISA standard curve (r^2=0.98). Each data point represents triplicate results as average ± standard deviation.

Mineralized Tissue Analysis and Collagen Staining

Cell plates obtained at selected time points (0,2,4,8 days) were analyzed for collagen tissue formation through the Picrosirius Red staining method. The Picro-sirius red method of staining is one of the best understood techniques of collagen histochemistry. The nuclei of hPDLF on glass cover slips were stained with Weigert's haematoxylin for 8 minutes, and then washed for 10 minutes in running tap water. Samples were stained with picro-sirius red (Direct Red 80 Sigma Aldrich, St. Louis, MO) for one hour and washed in two changes of acidified water. Water was physically removed from the slides by vigorous shaking, then the specimens were dehydrated in three changes of 100% ethanol. Finally samples were cleared in xylene (Puchtler et al., 1973; Junqueira et al., 1979).

Cell plates obtained in duplicate at day 16 were analyzed for mineralized tissue formation through the Alizarin Red S staining method. This technique allows the direct demonstration of insoluble calcium cations where Alizarin Red S forms an insoluble orange red lake. The technique is made specific by pH control. Alizarin Red S also forms lakes with other metals such as barium, aluminium, mercury and magnesium. Fortunately, the bright red coloration of calcium can be easily differentiated from the dark red of other metal-lakes (Carson 1990, Fredenburgh et al 1993).

Statistical Analysis

Amplification curve results were analyzed using a sigmoidal curve fitting method (H. Qiu et al. 2007) by SigmaPlot 11.0 (Systat Software, Inc. San Jose, CA) and Excel (Microsoft corp. Seattle, WA). Statistical comparisons ($p < 0.05$ for statistical significance) were made between GCM-treated cells and control-treated cells using two-way ANOVA (independent variables: time, treatment; dependent variables: gene, protein expression).

RESULTS

Specimen Fabrication

Experimental bioactive glass (6P53-b) and commercial Bioglass (45S5) are melt-derived glasses that were used in this study (Table 1). The bioactive glass powder specimens, purchased commercially, were prepared and fabricated by Mo-Sci Specialty Products L.L.C. (North Rolla, MO) to a specific particle size range of 100-300 microns.

Dissolution Profiles of Bioactive Glasses

Figure 1 shows the dissolution kinetics profile of commercial bioglass (45S5) in TRIS Buffered solution described by solution pH as a function of time. pH can be restricted by buffering if needed for cell culture experiments. The pH is limited to 7.2 ± 0.03 after 72 hours of dissolution. 6p53-b can also be controlled in a similar manner (Figure 2).

Figure 3 demonstrates the dissolution kinetics profile of commercial bioglass (45S5) in deionized water (diH2O) described by solution pH as a function of time. pH appears to rapidly increase in the absence of any buffering. Similarly, 6p53-b pH also increases very rapidly, comparable to the 45S5 bioglass (Figure 4).

Figure 5 demonstrates solution pH as a function of time. The dissolution kinetics profile of commercial bioglass (45S5) in cell culture medium (DMEM), displayed a square root of time dependence typical of diffusion limited processes at early time points. After 8 hours, the dissolution slowed and plateaued suggesting that the diffusion may be reaction limited in the later phase. The pH increase occurs as a result of the ion exchange between Ca+ and Na+ ions in the glasses and H3O+ protons in the media. H+ ions have an affinity for bioglass (Sepulveda et al 2002).

The dissolution kinetics of experimental bioglass (6p53-b) (Figure 6) display a similar trend to that of commercial bioglass (45S5).

Inductively coupled plasma mass spectroscopy (ICP-MS) analysis of glass ion concentrations in the glass conditioned media (GCM) is listed after dissolution for 72h (Table 2). These are the approximate final ion concentrations used in the cell culture experiments. It was observed that increased silicon and sodium concentrations were contained in glass conditioned medium (GCM) as compared to control media (DMEM) after 72 h. Increased levels of magnesium were seen in the 6P53-b media.

The behavior of melt-derived 45S5 and 6p53-b glass powders in dissolution is depicted in Figures 7-13, which show the concentration of magnesium, silicon, phosphorous, and calcium species. The dissolution profiles are evaluated in the presence of culture medium (DMEM) for the two materials. Concentrations of ions to baseline concentrations of culture medium (DMEM) are shown in parts per million.

Magnesium concentrations increased over 72h after experimental bioglass dissolution in culture medium (Figure 7). Magnesium was not detected in 45S5 dissolution analyses as expected.

The concentration of silicon released into solution increases rapidly during the first few minutes, and continues over 72 hours (Figures 8 and 9). This initial fast release of silicon ions indicates the first reaction stage of dissolution by breaking up of the outer silica layers of the network. The solid silica dissolves in the form of monosilicic acid Si(OH)4 until the solution is saturated.

Concurrent with the increase in silicon, the concentration of phosphorous decreases rapidly after the first 24 hours of dissolution (Figures 10 and 11). A slight depletion of calcium ions (Figures 12 and 13) is also observed during the same duration of dissolution. The calcium content of the solution (both 45S5 and 6p53-b) increases with reaction time in culture media after similar trends to those of silicon. This behavior typically is attributed to the incorporation of phosphorous ions together with calcium ions from the dissolution medium within the Ca-P rich reaction layer forming on the glass surface, as a result of the solution saturation (Hench 1996).

The dissolution kinetics profiles and corresponding pH values for 6p53-b glass powders in culture media show similar trends to those observed for 45S5 powders. The major changes in elemental concentration of these ions occur during the first few hours of dissolution, are also indicated by the pH increase (Figures 5 and 6).

Cell Proliferation

Cell number (Figure 14) continued to increase over 16 days for both treatment groups and control in the absence of ascorbic acid. Glass conditioned medium did not appear to inhibit cell proliferation compared to control (DMEM). The doubling time is at approximately 2 days. Time appears to be the only independent variable as there was no statistically significant difference ($p < 0.05$) between treatment groups over 16 days. The cell proliferation levels off after 2 days likely due to cell crowding and/or decreased availability of reagent dye.

Cell number (Figure 15) continued to increase in a similar manner over 16 days for both treatment groups and control in the presence of ascorbic acid. Ascorbic acid was added to illicit possible differentiation. Ascorbic acid appeared to slow cell proliferation compared to control (DMEM) as cell number increase was slightly less than in its absence (Tables 3 and 4).

qRT-PCR Gene Expression

Osteocalcin (Figure 16) reached its highest expression level at day eight, with enhanced expression in glass conditioned media compared to control (45S5 >>6p53-b >control). Expression of osteocalcin was higher in cells treated with 45S5 glass conditioned media at day two, eight, and sixteen (ANOVA, $p < 0.05$ for statistical significance) as compared to control.

The average alkaline phosphate expression (Figure 17) reached its highest level at day two for 45S5 and day eight for 6p53-b. Over-expression was observed at day two and eight, for both 45S5 and 6p53-b, compared to control (ANOVA, $p < 0.05$ for statistical significance).

Collagen 1 Type 1α1 expression (Figure 18) reached its highest level at day sixteen for 45S5 and day two for 6p53-b. Over-expression was observed at day two and sixteen, for both 45S5 and 6p53-b, compared to control (ANOVA, $p < 0.05$ for statistical significance).

Collagen 1 Type 1α2 (Figure 19) reached its highest expression level at day sixteen for 45S5 and day two for 6p53-b. Over-expression was observed at day two and sixteen, for both 45S5 and 6p53-b, compared to control (ANOVA, $p < 0.05$ for statistical significance).

Since cell proliferation increased for all treatment groups and control (without statistical significance), results were not reported relative to total protein expression.

Protein Expression

Intact human osteocalcin ELISA results (Figure 20) showed osteocalcin protein expression was at its highest concentration at day sixteen, with enhanced expression in glass conditioned media compared to control (45S5>>6p53-b >control). Expression of osteocalcin was higher in cells treated with glass conditioned media at day eight and sixteen for both GCM (ANOVA, $p < 0.05$ for statistical significance).

Alkaline phosphatase (Figure 21) was represented by optical density values (absorbance 410nm). AP was over-expressed for both GCM (significant difference: *, $p< 0.05$) on days 8 and 16 compared to control.

Mineralized Tissue Analysis and Collagen Staining

Alizarin Red S Calcium staining (with hematoxylin) of hPDLFs grown in the presence of glass powder ions after 16d at 40x magnification (Figures 23-25) shows bright red coloration of calcium formation. The mineralized tissue nodule is much more pronounced in GCM compared to control, which is diffuse.

Optical micrographs show extracellular matrix collagen as a result of hPDLF exposure to control media and ascorbic acid treatments (Figure 27) and GCM with ascorbic acid treatments (Figures 26 and 28) at day two. Cells were fixed in Bouin's fixative for one hour, followed by Fastgreen and Picrosirius stains for thirty minutes each. Imaging shows the presence of type 1 collagen in cell layers. A greater presence of such fibers was observed in GCM (45S5 and 6p53-b) compared to control media over the time course of the experiment. Notice the presence of fibrillar collagen. Please note

that background Fastgreen stained more intensely in more dense tissue layers, hence, the reason for the difference in green background staining. Further, background staining of blue, blue-green, and green are typical for fast green treatments coupled with picrosirius follow-on staining.

DISCUSSION

The data from the present study indicate that the *in vitro* dissolution behavior of bioactive glass powders can be characterized and subsequently optimized. It is known that particle size difference will have a large effect on the dissolution rates of the glass powders. The particle size of the bioactive glass powders was chosen with aims to optimize *in vitro* dissolution behavior, so that the effects of these ions during human periodontal ligament fibroblast (hPDLF) differentiation would be maximized.

Shapoff and coworkers (1980) described that particle size should be considered a variable in periodontal application. Their studies found that a smaller particle size (100-300 microns) had more osteogenic potential than a larger one (1000-3000 microns). Therefore, for consistency with this and other studies, we chose to use this smaller particle size range to maximize the osteogenic potential in our experiments.

Sepulveda et al. (2002) determined that the rates of dissolution for two types of bioactive glass powders increased as the particle size decreased. Thus, particle size range can be used as a means to control the release rate of active ions that stimulate the gene expression and cellular response for tissue proliferation and repair. The particle size remained relatively constant (~100-300μm) throughout our experiments.

In vitro testing of both bioactive glass powders (45S5 and 6p53-b) was completed prior to *in vivo* work as an initial screen to evaluate the bio- or cyto-compatibility of materials with human Periodontal Ligament Fibroblasts (hPDLF). The bioactivity of materials commonly is examined by in vitro procedures involving the dissolution of glasses in aqueous media (Warren et al 1989).

This allows for tighter and more stringent controls over testing of these materials with the primary cell culture. Stage 1 included cell-free *in vitro* testing to study dissolution kinetics of the bioactive glass powders. This allows for understanding of dissolution kinetics and determines the point at which Si/Ca leaching is at its zenith. Our results were similar to the time-dependent dissolution behavior of 45S5 bioactive glass powder that was evaluated in cell culture media commonly used for osteoblasts by Xynos et al. (2000).

Dissolution Profiles of Bioactive Glasses

The measurements for the glass dissolution studies were collected after solution was removed with a sterile pipette at a consistent distance from the glass powder interface (at the 25ml marked line of the centrifuge tube), and introduced into a new 15 ml centrifuge tube. This particular distance may make a difference in pH measurements and ion concentration determination. The distance from the forming hydroxyapatite layer, at the base of the centrifuge tube, may affect the values recorded. This distance is decreased at each time point as more solution is removed from the closed system. One way that the experiment could be conducted would be to replace the volume removed with fresh media. The small volume (2 cc/time point) relative to the 50ml starting volume however, was assumed to be insignificant in the pH and ion concentration measurements in the present study.

The samples were stored in a closed centrifuge tube as compared to the well plates for cell-culture experiments, and the seal inhibits air exchange within the incubator. This lack of incubation air exchange did not allow CO2 to interact with the media, thus maintaining a high pH while glass samples dissolved. Therefore, the exact pH read after

72h may not be the exact pH served to cells because the well plates are not as well-sealed. This is of little concern as the data recorded is relative to control in each assay.

The glass specimens were stored for long periods (weeks) at room temperature in a closed system, one centrifuge tube for each time point. This may affect the ion concentration because although there is no removal of the ions within the closed system, the plastic of the centrifuge tube may leach ions. There is specifically silicon in the plastic of the tubes, although this was consistent for all samples taken.

pH measurement may be affected by how long the sensitive probe tip is left in solution prior to recording. The pH measure was recorded after the digital display remained constant for a few seconds. This was completed in a consistent manner for each sample replicate and treatment group.

Two different DMEM manufacturers were used at the preliminary stages of our Stage 1 cell-free in vitro experiments. For consistency, DMEM from the UCSF cell-culture facility was used for all reported data and cell-culture experiments. However, it was discovered that different DMEM can contain different baseline ion concentrations and thus affect GCM's subsequent behavior.

In our study, ion concentration at each time point (1,2,4,8,24,48,72h) is representative of three separate ICP-MS analyses. Blanks were used but when compared between trials, appeared to show different baseline ion concentrations. This may be attributed to the plastic leaching specific ions into solution and thus being detected. It may also be due to the particular lot and varying compounds added to the medium upon production. Lot number and chemical composition of all compounds added during media preparation were gathered from the manufacturer.

Single-charged ions, such as sodium and potassium, may mask the sensitive detection of each like-ion. There was a large standard deviation for ICP-MS analysis for these ion concentrations although the pH trend, representative of the exchange with H+, displayed a very robust and consistent increase over time (Figure 5 and 6).

We found the dissolution kinetics profile of commercial bioglass (45S5) in DMEM to be similar to those reported in the literature (Xynos et al 2000).

It was observed that increased silicon and sodium concentrations were contained in glass conditioned medium (GCM) as compared to control media (DMEM) after 72 h. High levels of magnesium were seen in the 6P53-b media. Sepulveda et al (2002) demonstrated similar dissolution profiles for the elemental release of silica. They showed silicon concentration released from a fine 45S5 powder (~5-20μm), for instance, is approximately 94 ppm after 4 h and reaches 143 ppm after 22h in culture media.

We found that a novel bioactive glass releases Silicon and Magnesium ions to cells and displayed similar dissolution kinetic profiles to those of commercial bioglass. These data support an earlier report by Keeting et al. (1992) showing that solutions containing high Si concentrations are mitogenic for bone cells.

Magnesium is not contained in commercial bioglass but showed continued release in 6p53-b. The increased magnesium ion concentration may provide some benefit in terms of increasing osteogenic potential as seen in osteoblasts. However, throughout literature, the storage solutions recommended for PDL cell survival are often depleted of magnesium compounds. Magnesium may actually reduce the ability of human PDLF cells to proliferate and differentiate. Nonetheless, there were expressed osteogenic markers in the experimental bioglass GCM treated cells in our study.

Silicon may be involved in the mechanism of collagen processing during bone matrix formation, and has been found to enhance the expression level of collagen type I. Magnesium is involved in integral binding of cells to bioceramics, and its high level in 6P53-b media may suggest that cells are more likely to adhere to an implant's surface in this type of environment.

Calcium concentrations contained in glass conditioned medium (GCM) compared to control media (DMEM) after 72h were not increased (Table 1). This relative relationship is different than what is reported in literature and was not expected in our studies. The absolute values however, are representative of previously reported bioglass dissolution (53.33 ± 8.73 for 45S5 and 57 ± 3.62 for 653-b). The dilemma is believed to be a result of inaccurate ICP-MS values of the control DMEM. As mentioned previously, the control blanks showed inconsistent baseline ion concentrations from different manufacturers and relative comparisons are unreliable. Despite this confusion, the trends for calcium were expected and representative of the incorporation of phosphorous ions together with calcium ions from the dissolution medium within the Ca-P rich reaction layer forming on the glass surface.

Cell Proliferation

The periodontal ligament fibroblast cells used in the present study acted as any healthy primary cell culture. The results obtained from our proliferation studies, however, are slightly different compared to those of Messer et al. (2006), who suggested a doubling time of approximately 80h for PDLF. We found that doubling time was earlier (~48h). The difference may be due to the stages of cell cycle. Davenport et al. (2003) found that the hPDLF proliferation rates were not significantly different between the

experimental treated cells and positive control, and therefore could be considered similar to the earlier fluctuations due possibly as a result of the cell cycle (Davenport et al 2003).

Differences may also be attributed to the detection method used and the surface area on which the cells were grown. We found that the cell proliferation levels off after 2 days likely due to cell crowding and/or decreased availability of reagent dye. Dental cell populations used for the reports of Aubin et al. (1995) were highly heterogeneous, demonstrating variability. The heterogeneity may be due to some plasticity in the cell population, samples likely contain cells of varying inherent differentiation capacity, ie. different subpopulations (osteoprogenitor, preosteoblast, and mature osteoblast), or may contain cells of varying origin (mesenchymal and ectodermal). Thus may be the case in our results compared to others reported in literature.

pH was determined to understand hPDLF cell viability during subsequent experiments as pH variations of glass conditioned medium (GCM) are reported in Stage 1 of the present study. There may be concern as one introduces a basic (pH >7) glass conditioned media during cell culture experiments. The color of GCM was an intense pink compared with supplemented media. This visual difference, however, became insignificant after cell incubation as the color in each treatment group and control was the same orange color. The incubation environment may act to regulate the pH, in essence acting as an enhanced buffer. Cell number continued to increase over 16 days for both treatment groups and control in the presence of ascorbic acid. Ascorbic acid is known to change the acidity of solution. Ascorbic acid appeared to slow cell proliferation compared to control but was not detrimental to their survival.

During the assay, control media was placed in the well-plate for each group, including treatment groups, prior to all spectrophotometer readings replacing the GCM. This was in accordance with the protocol for the MTS assay in order to achieve an accurate measurement and confirmed with the manufacturer's support technician. There were also negligible differences among readings from a calibration that was completed prior to the assay. Lastly, the replacement allowed us to conserve GCM for future work as it can be difficult to prepare.

q-PCR Gene and Protein Expression

On the basis of our findings, the effects of these ions during hPDLF differentiation, have been shown to enhance differentiation and proliferation of human periodontal ligament fibroblasts (hPDLFs) thus increasing osteogenic potential. Quantitative polymerase chain reaction results were confirmed with comparable protein expression increases.

Our results are consistent with, Lindroos et al. (2008), who demonstrated that dental cell sources, including periodontal ligament stem cells, exhibit comparable surface marker and bone-associated marker profiles as those of other mesenchymal stem cell sources, yet distinct from the buccal mucosa fibroblasts. They may provide the potential for clinical applicability as dental stem cells in hard tissue regeneration.

We showed that osteocalcin was over-expressed when human periodontal ligament fibroblasts were exposed to bioactive glass ions. Osteocalcin reached its highest expression level at day eight, with enhanced expression in glass conditioned media compared to control (45S5>>6p53-b >control). This is consistent with the findings of Varanasi et al. (2006) who observed that bioactive glass dissolution of silicon enhances osteocalcin expression from differentiation to mineralization of osteoblasts. This

similarity may suggest that the human periodontal ligament fibroblasts may in fact contain osteoblast-like cells within their heterogeneous cell population.

During PCR work, only one technician completed all of the pipette work per well plate. This decreased the error while introducing the nominal reaction volumes by minimizing inter-well differences. The PCR work was completed twice. The first time with duplicates of each replicate per gene and this was compared to a second assay with the same samples. The error was minimized due to improved operator experience and data was reported from this assay.

All data for PCR analysis was included and had less than or equal to 10% standard error. We can be confident while reporting this data as the sigmoidal curve fitting method gave r-squared values >0.95 and $p < 0.05$ for all replicates and treatment groups.

Cell protein lysates were scraped with plastic cell scrapers manually and stored in -80°C Cell Lytic buffer solution until used for the protein assays. Media at each time point was also extracted and saved for future analysis. The unfreezing of samples was minimized as the protein could be altered at each cycle.

The cells used in the present study were passage 5-9, as reserve cells were stored at passage 4. PDLF may lose the osteocalcin production potential with passage (Nohutcu et al 1997). The production ability of bone-associated proteins in PDLF may change in response to cell growth conditions involving differential interaction with cementoblasts and mitogenic stimulation, and culture aging with passage. The production ability may be reduced with increased passage in PDLF with the impairment of the potential to proliferate and differentiate into other homologous cell types.

Sawa et al (2000) discovered that senescent PDLF (passage 8) did not include cells which have a detectable reactivity with anti-osteocalcin immunohistochemically and the reactive intensity was significantly weaker in the senescent culture than in the culture in passage 2 by ELISA. It is suggested that the production potential of osteocalcin is impaired in PDLF with aging in culture. Shimonishi et al (2008) were unable to detect osteocalcin mRNA in human periodontal ligament fibroblasts cultured alone through RT-PCR. This may be the reason that we found enhanced gene expression in some but not all of the days of treatment.

Shimonishi et al (2007) showed the expression of mRNA for alkaline phosphatase was significantly higher in periodontal ligament fibroblasts than in gingival epithelial cells suggesting that these cells may play a potential role in the mineralization process. The same group found weak osteocalcin mRNA expression and were unable to detect any significant differences in the relative intensities of osteocalcin mRNA among three different cell populations (including PDLF). Over-expression for GCM was observed at day two and eight compared to control (ANOVA, $p < 0.05$ for statistical significance). Our findings are consistent with the notion that periodontal ligament fibroblasts have great regenerative potential.

There was evidence of periodic collagen expression observed in the present study. Aubin et al. (1995) showed that over a time course of osteoblast differentiation in vitro, the expression of type 1 collagen was found to be high and then to decrease; the level of alkaline phosphatase increased but decreased when mineralization was well progressed; and osteocalcin appeared with mineralization.

Collagen staining showed consistent trends with the q-PCR results. Alizarin red S staining for day 16 cells displayed interesting visual evidence for possible mineralization. The GCM conditions showed increased contrast and a more intense red color under optical light microscopy (40x) when compared to control. The combination of our gene and protein expression results and histological evaluation may provide evidence for the ability of human periodontal ligament fibroblasts to produce mineralized matrices similar to the activity demonstrated within osteoblast-like cell lines. To our knowledge, this is the first study to show a positive effect of bioglass, or any inorganic material, to induce differentiation of hPDLF to a partially mineralizing phenotype.

Future work will focus on demonstrating mineralization when hPDLF are treated with glass conditioned media. This heterogeneous cell population will be further characterized. Gene expression activity of genes such as PDGF, runX2, and other osteogenic markers would be interesting to study. The exact time of optimal mineralization can be pursued by observing cellular activity between days 8 and 16 and perhaps beyond day 16. Despite the limitations of our study, observations at additional time points may provide stronger evidence. Future work may include histological evaluation through H and E staining and Alizarin Red S staining. There are many interesting aspects of the interaction of human periodontal ligament fibroblasts with bioglass that future research can continue to investigate.

CONCLUSIONS

This study tested the hypothesis that bioactive glass ions enhance osteogenesis and that there is a direct connection between enhanced gene expression and protein matrix production. These results are significant because to this point, no connection has been made to the enhanced gene expression by hPDLFs exposed to bioactive glass ions and their enhanced downstream matrix protein expression. Future research will explore the underlying extracellular and intracellular mechanisms that cause enhanced differentiation and impact mineralized-tissue formation. The introduction of a novel bioactive glass powder to hPDLFs may have the potential to produce mineralized matrices and hence the ability to regenerate bone and cementum within the periodontium.

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TABLES

Composition (wt. %) of Bioglass (45S5) and experimental bioactive glass (6P53-b)						
	SiO_2	Na_2O	K_2O	MgO	CaO	P_2O_5
45S5	45.0	24.5			24.5	6.0
6P53-b	52.7	10.3	2.8	10.2	18.0	6.0

Table 1. Experimental bioactive glass (6p53-b) and commercial Bioglass (45S5) are melt-derived glasses that were used in this study.

Table 1. Ion Extract Concentration (ppm) after 72 h						
	Si^{4+}	Na^{+}	K^{+}	Ca^{2+}	Mg^{2+}	$P0_4^{3-}$
D-MEM	2.536	3498	255.1	103.5	22.19	32.53
45S5+ D-MEM	56.84 ±2.73	4476.0 ±218.4	224.43 ±11.8	53.33 ±8.73	18.67±1.28	15.4 ±3.12
6P53b+ D-MEM	65.01± 1.18	4264.67 ±43.11	230.3 ±3.47	57±3.62	40.25±0.22	13.11±1.39

Table 2. Inductively coupled plasma mass spectroscopy (ICP-MS) analysis of glass ion concentrations in the glass conditioned media (GCM) is listed after dissolution for 72h. These are the approximate final ion concentrations used in the cell culture experiments.

Table 3.Number of cells after 16 d in the absence of ascorbic acid	
D-MEM	44168.12 ± 905.69
45S5+ D-MEM	36023.19 ± 8384.97
6P53b+ D-MEM	33863.77 ± 7770.95

Table 3. Number of cells after 16d in the absence of ascorbic acid

Table 4.Number of cells after 16 d in the presence of ascorbic acid	
D-MEM	33547.73 ± 486.2
45S5+ D-MEM	29285.86 ± 5858.96
6P53b+ D-MEM	24606.68 ± 499.13

Table 4. Number of cells after 16d in the presence of ascorbic acid

FIGURES

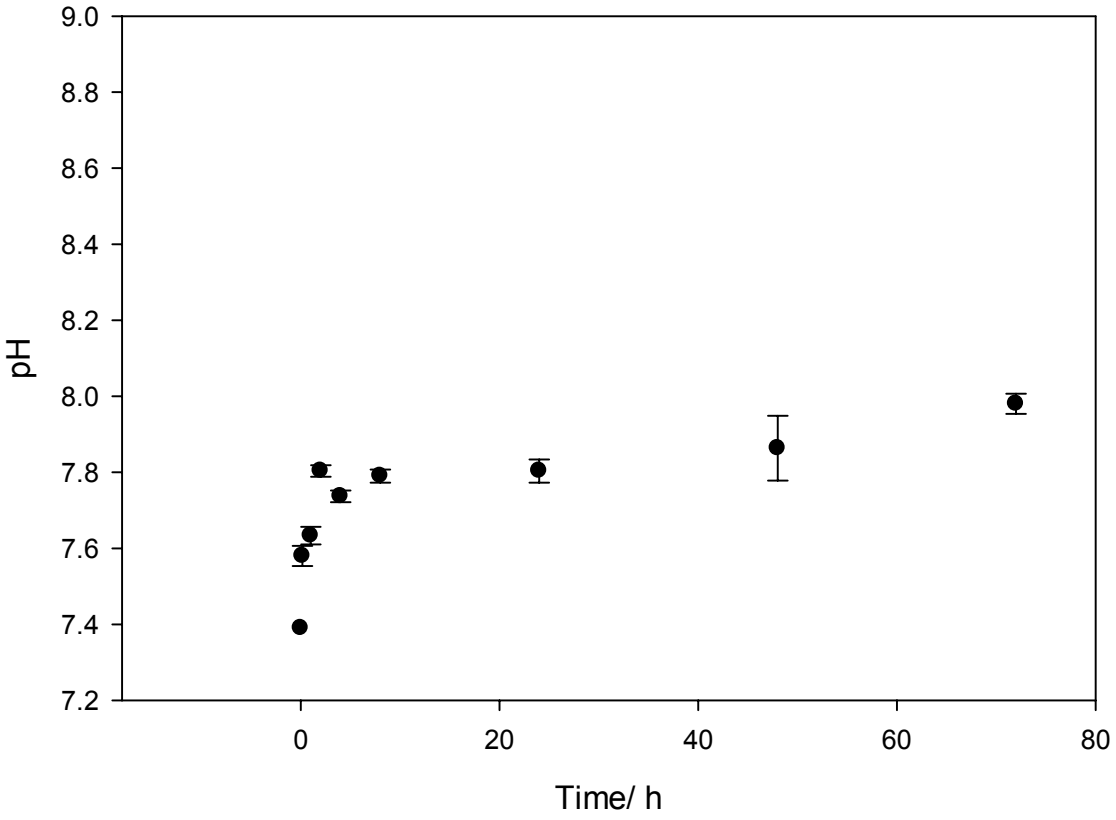

Figure 1. The dissolution kinetics profile of commercial bioglass (45S5) in TRIS Buffered solution described by solution pH as a function of time. pH can be restricted by buffering if needed for cell culture experiments.

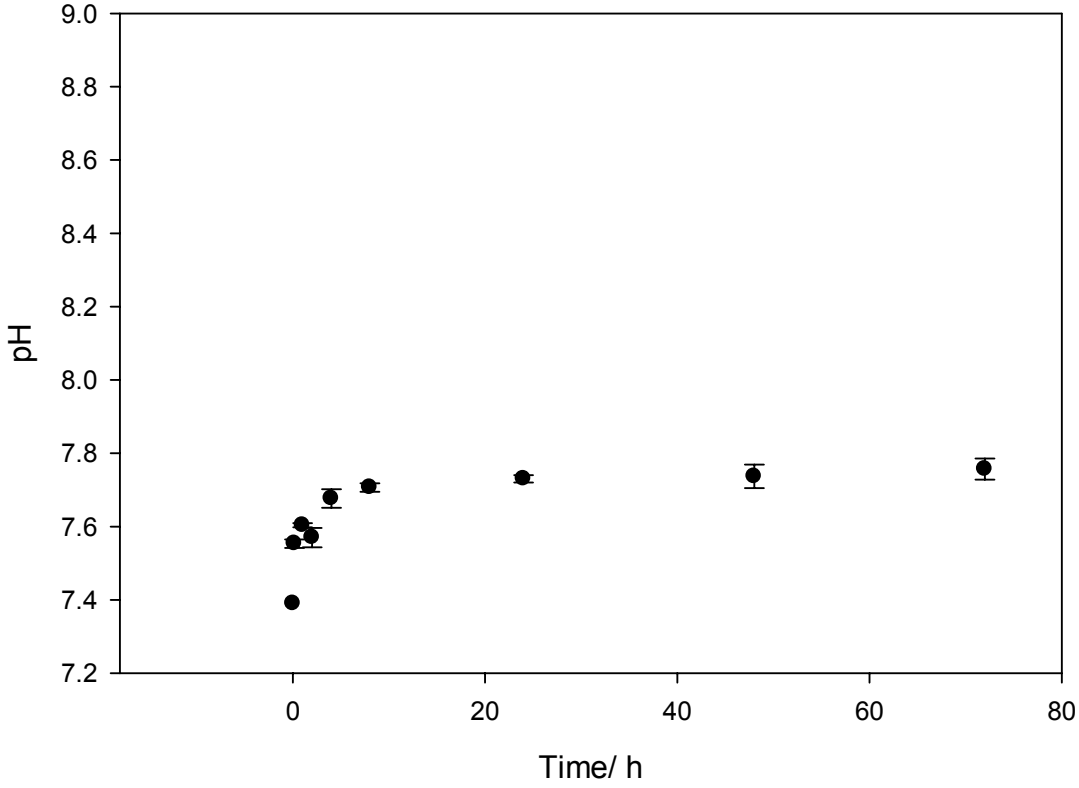

Figure 2. The dissolution kinetics profile of experimental bioglass (6p53-b) in TRIS Buffered solution described by solution pH as a function of time. pH can be restricted by buffering if needed for cell culture experiments.

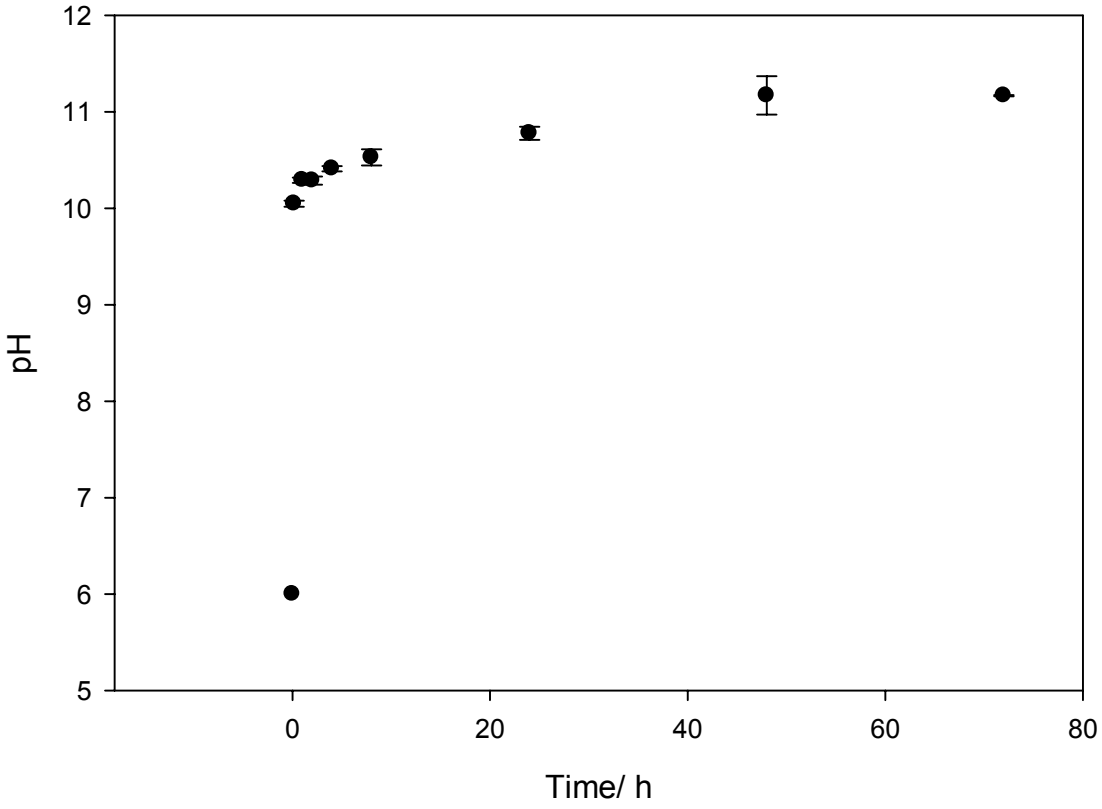

Figure 3. The dissolution kinetics profile of commercial bioglass (45S5) in deionized water (diH2O) described by solution pH as a function of time. pH appears to rapidly increase in the absence of any buffering.

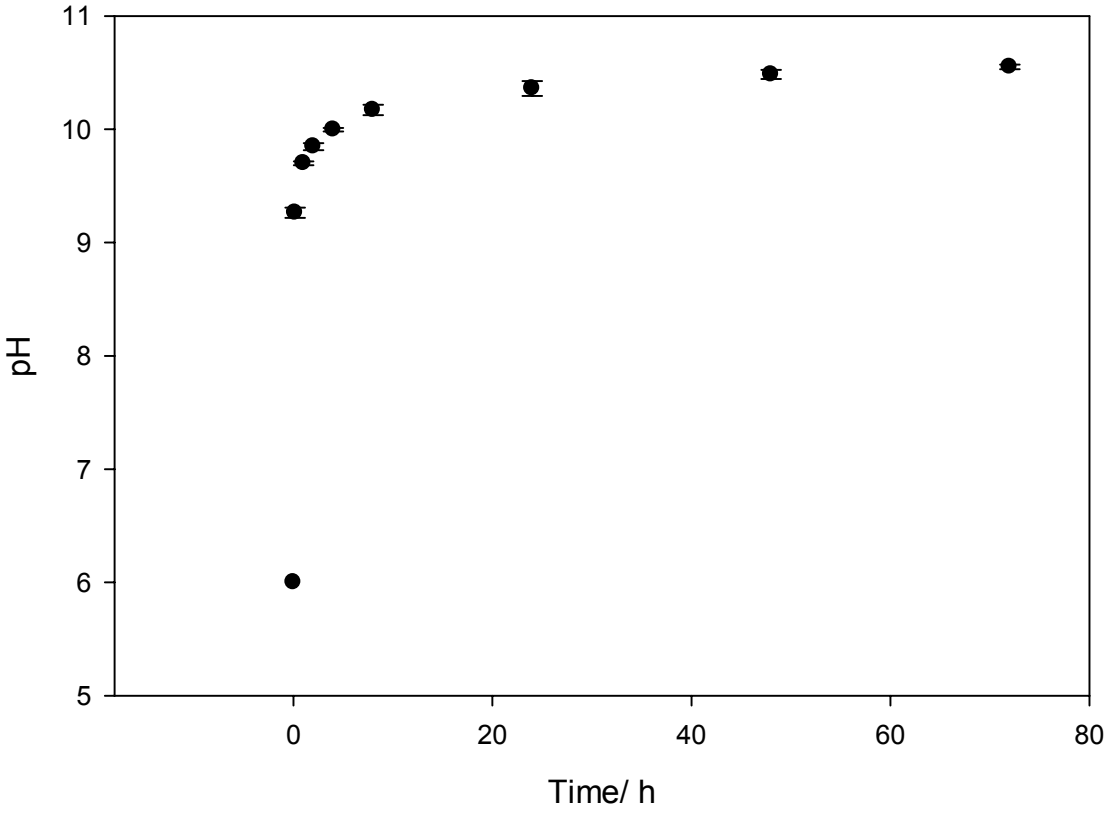

Figure 4. The dissolution kinetics profile of experimental bioglass (6p53-b) in deionized water (diH2O) described by solution pH as a function of time. pH also increases very rapidly, similar to the 45S5 bioglass.

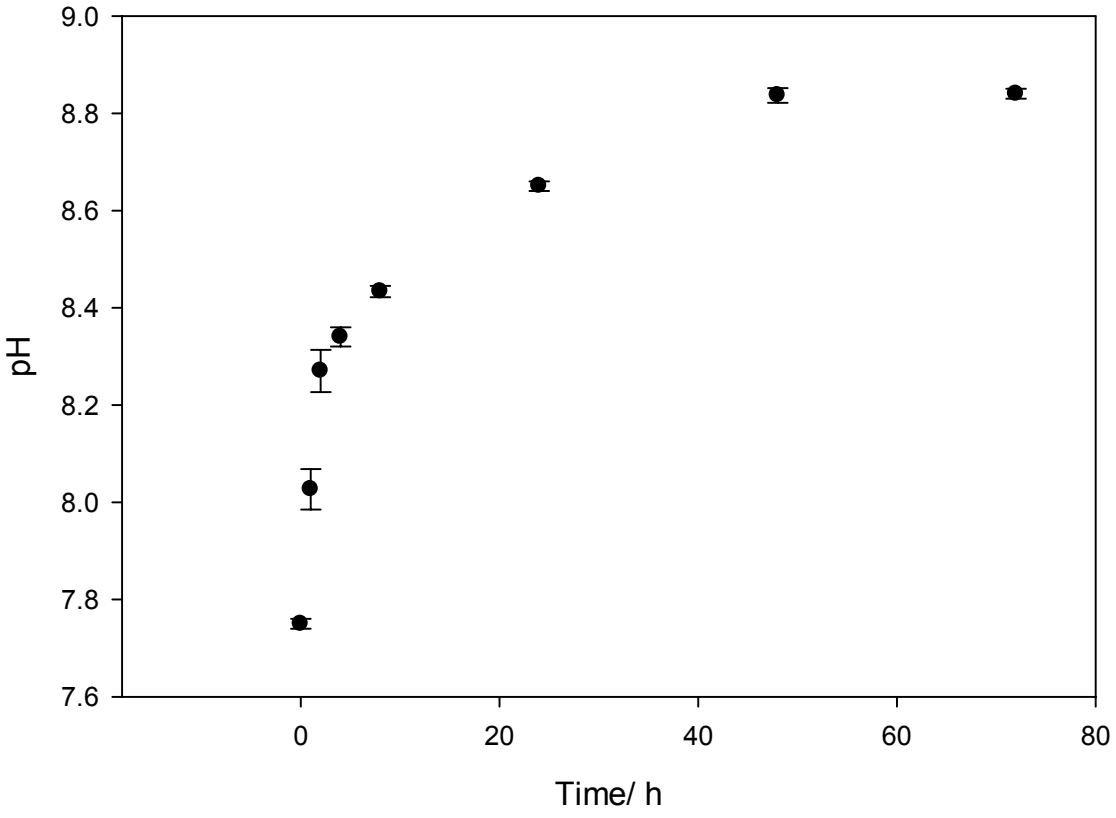

Figure 5. The dissolution kinetics profile of commercial bioglass (45S5) in DMEM described by solution pH as a function of time.

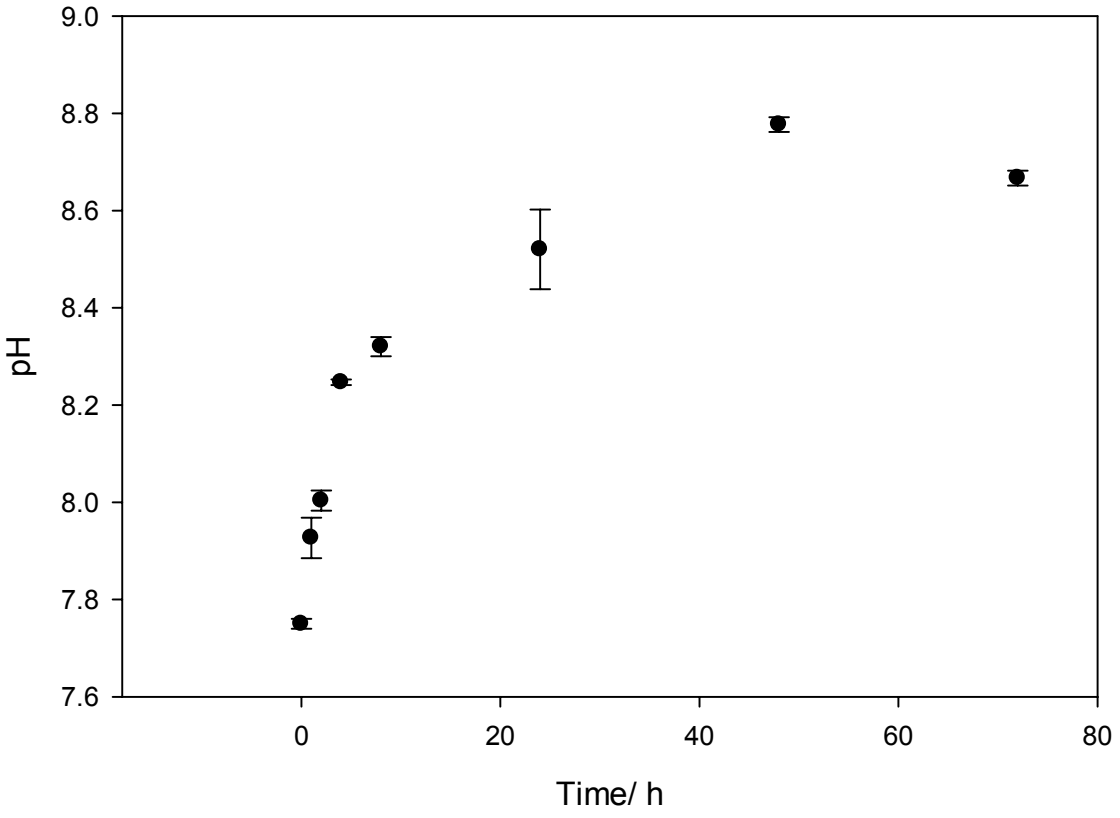

Figure 6. The dissolution kinetics of experimental bioglass (6p53-b) in DMEM displayed a similar trend to that of commercial bioglass (45S5).

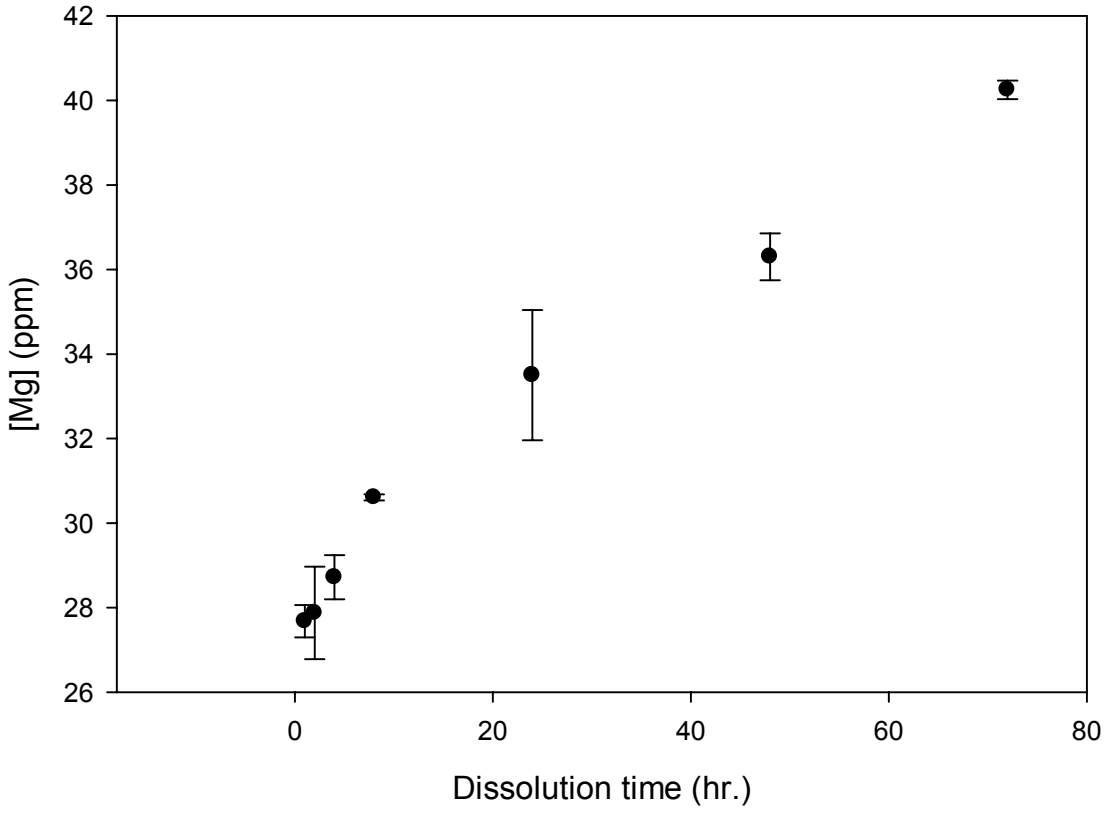

Figure 7. Elemental concentration [Magnesium] measured by ICP-MS analysis of solution medium after dissolution of experimental bioglass 6p53-b in culture medium (DMEM). Relative concentrations of ions to baseline concentrations (~ 28 ppm) of culture medium (DMEM) are reported in parts per million (ppm).

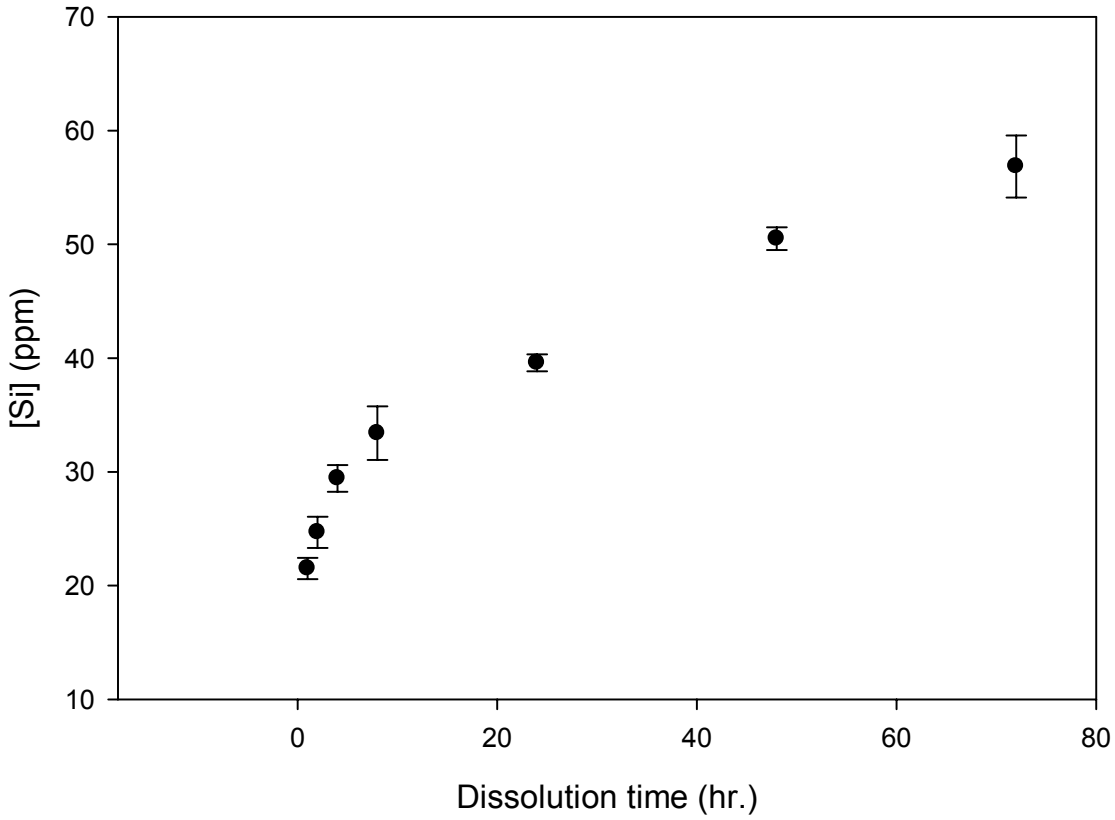

Figure 8. Elemental concentration [Silicon] measured by ICP-MS analysis of solution medium after dissolution of melt-derived 45S5 in culture medium (DMEM). The concentration of silicon released into solution increases rapidly during the first few minutes, and continues over 72 hours.

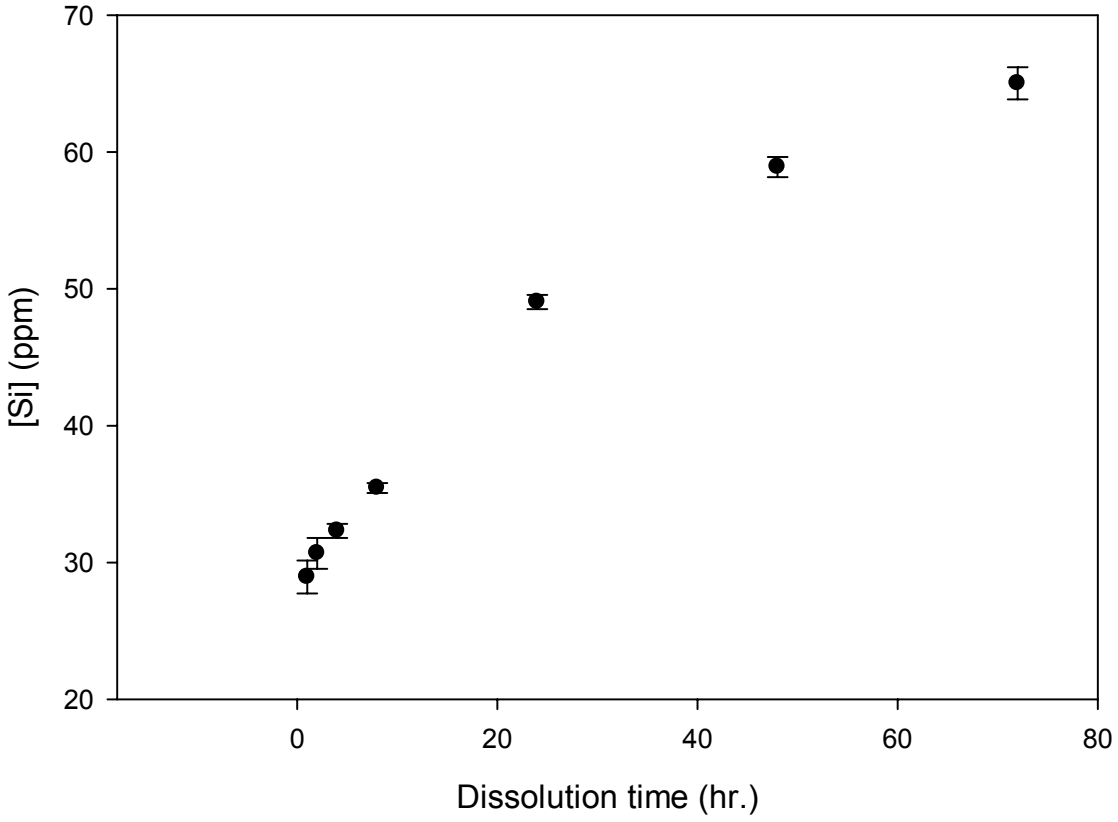

Figure 9. Elemental concentration [Silicon] measured by ICP-MS analysis of solution medium after dissolution of experimental bioglass 6p53-b in culture medium (DMEM). Silicon leaching in experimental bioglass shows a similar trend to that of commercial bioglass.

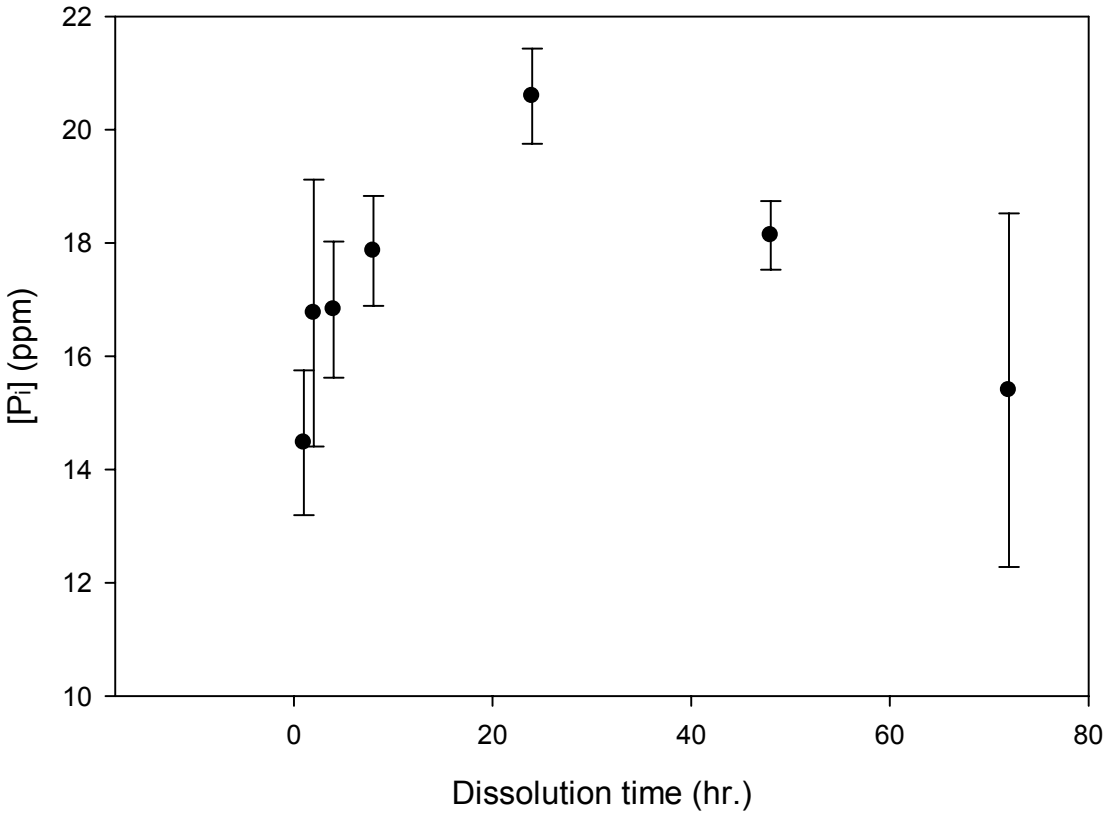

Figure 10. Phosphate leaching from commercial bioglass (45S5) appears to peak around 24 hours. Concurrent with the increase in silicon, the concentration of phosphorous decreases rapidly after the first 24 hours of dissolution.

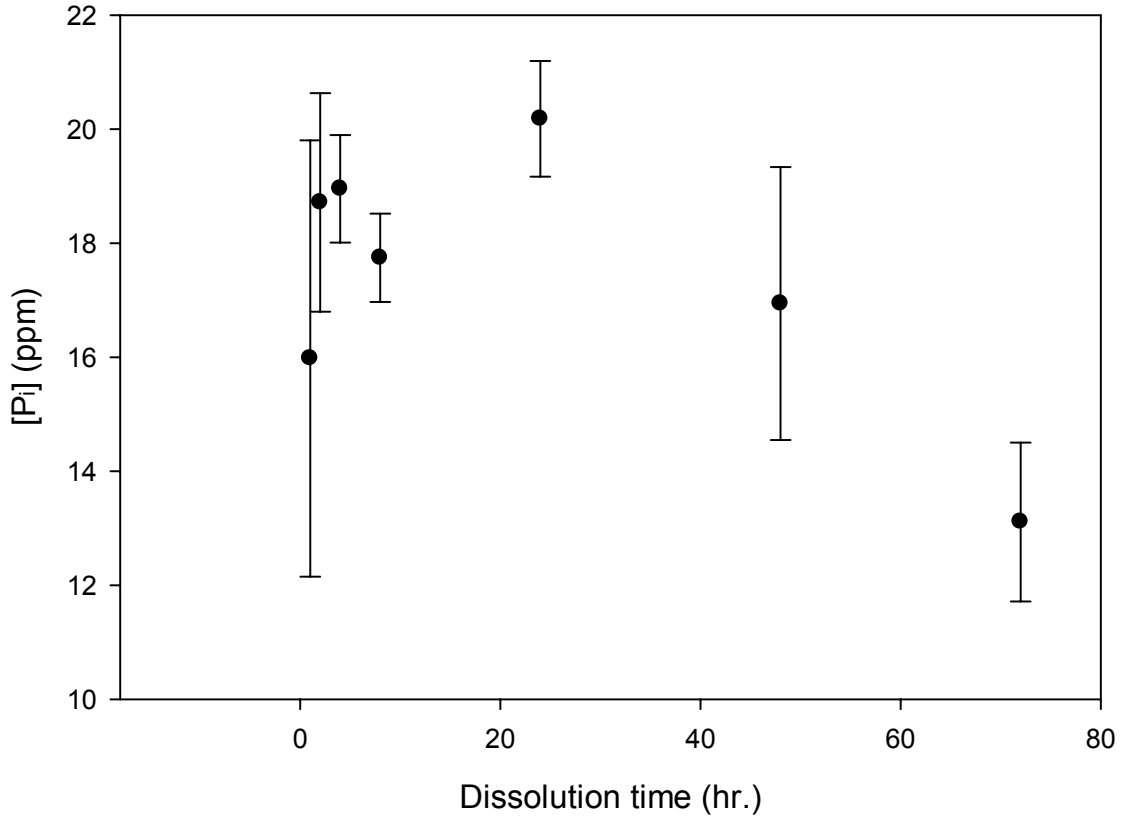

Figure 11. Phosphate leaching in experimental bioglass (6p53-b) appears to peak prior to 24 hours in a similar manner.

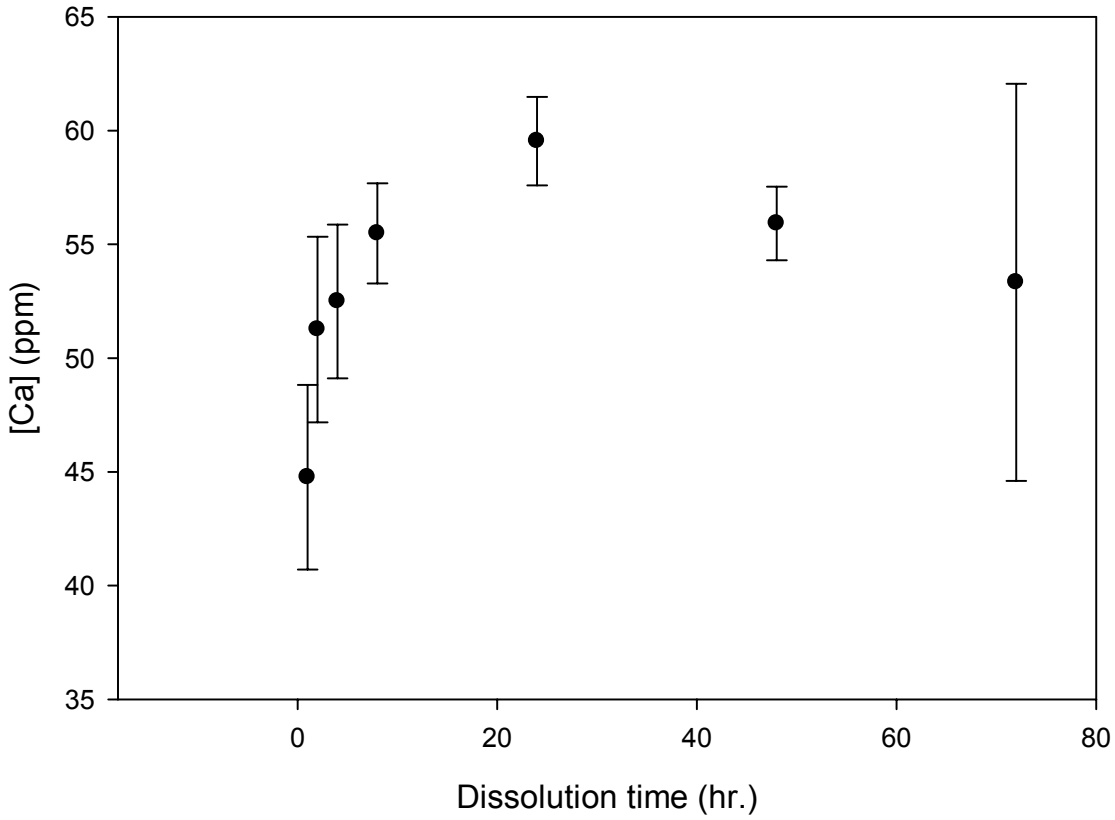

Figure 12. ICP-MS 45S5 Calcium shows a similar trend to that of phosphate, peaking approaching 24 h. A slight depletion of calcium ions is observed during the same duration of Pi dissolution.

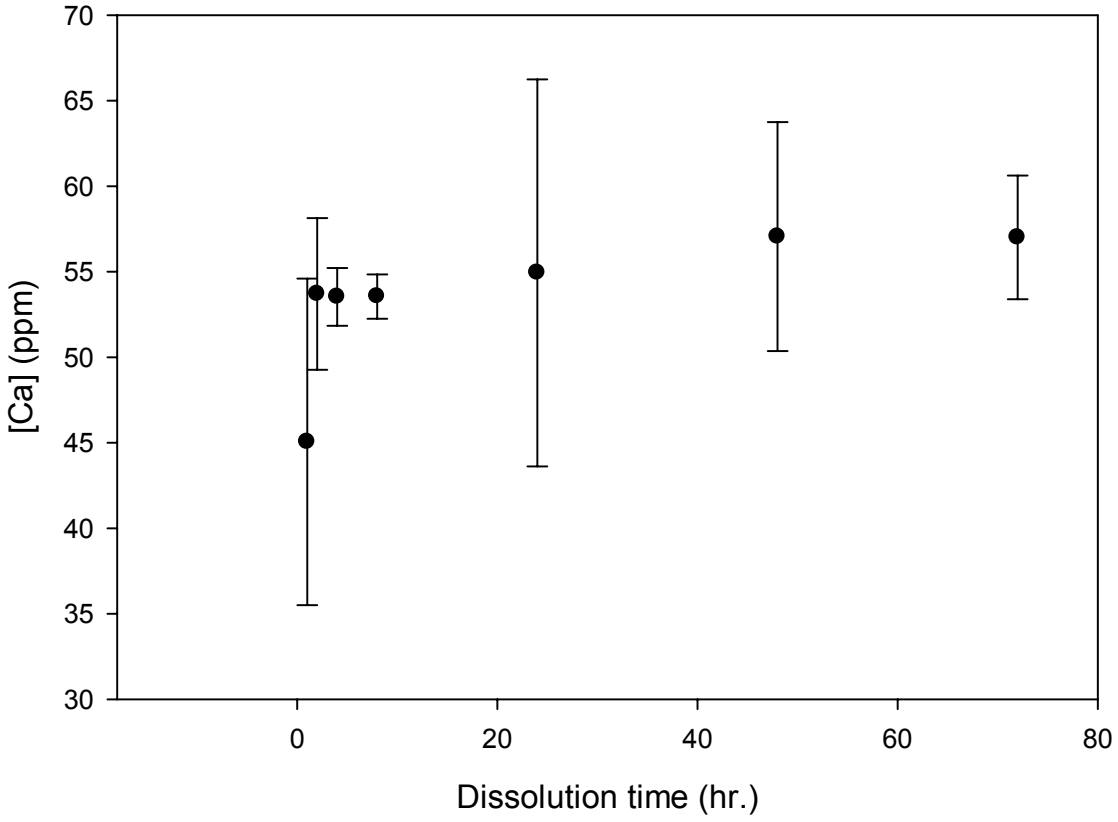

Figure 13. Elemental concentration [Calcium] measured by ICP-MS analysis of solution medium after dissolution of experimental bioglass 6p53-b in culture medium (DMEM) indicates that calcium concentration for experimental bioglass (6p53-b) shows a similar trend to that of commercial bioglass, though not as pronounced.

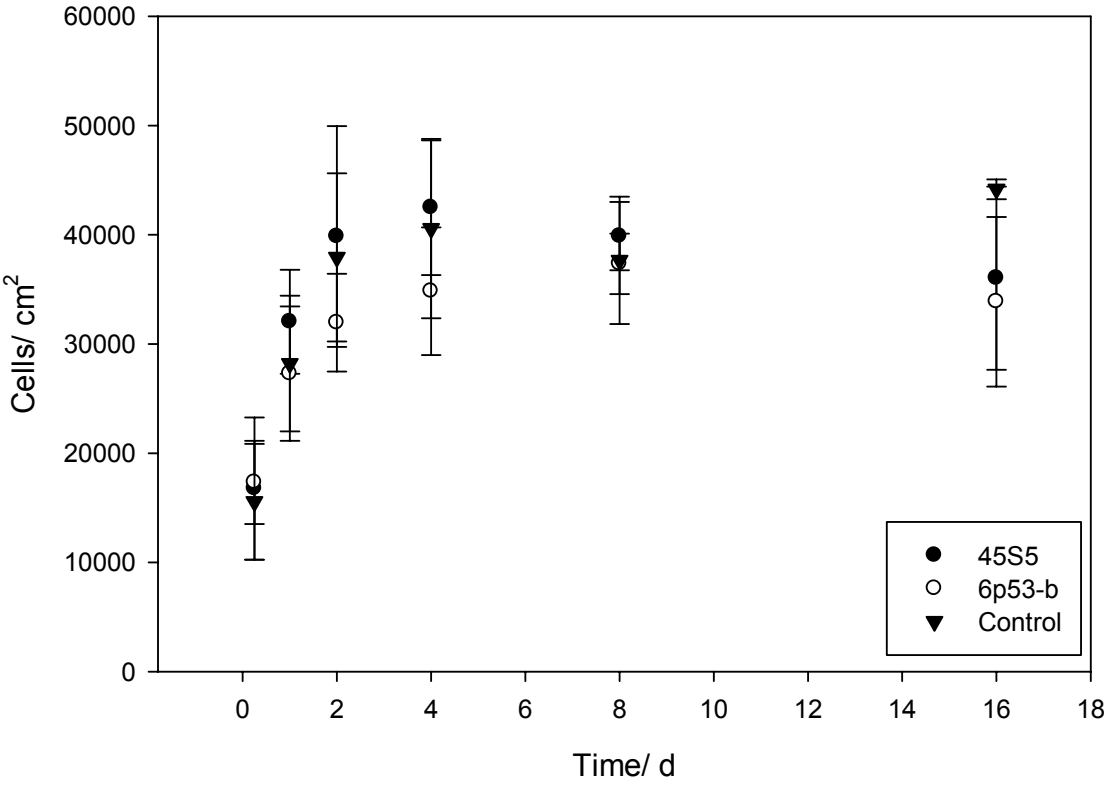

Figure 14. Cell number increased and leveled off after 2 days for both treatment groups and control in the absence of ascorbic acid. Glass conditioned medium did not appear to inhibit cell proliferation compared to control (DMEM).

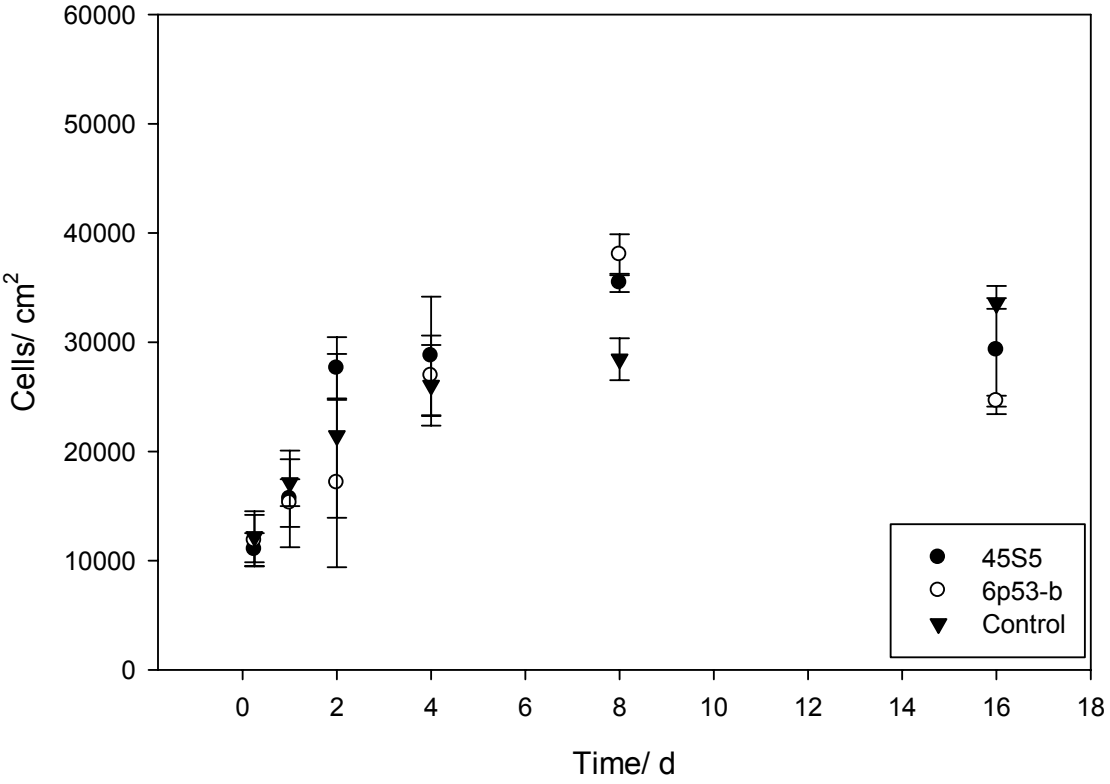

Figure 15. Cell number continued to increase in a similar manner over 16 days for both treatment groups and control in the presence of ascorbic acid. Ascorbic acid appeared to slow cell proliferation compared to control (DMEM) as the increase was less in its absence.

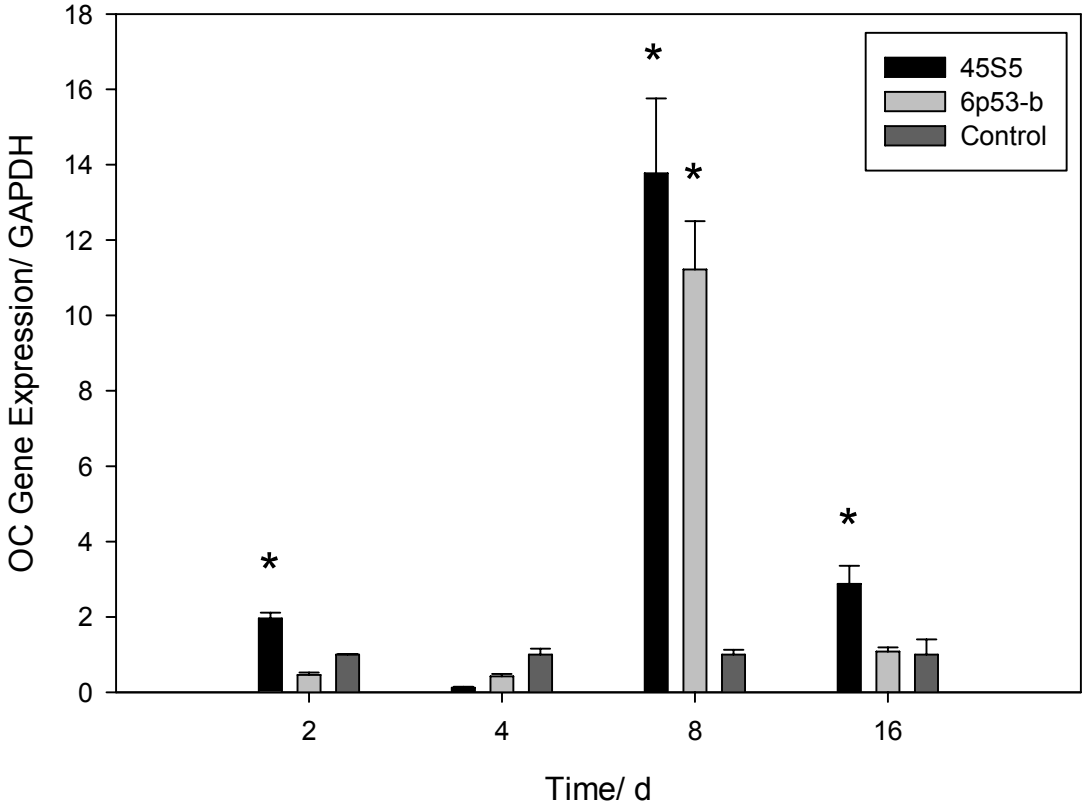

Figure 16. Graphical representation of relative q-PCR standardized by glyceraldehyde-3-phosphate dehydrogenase (mean ± standard deviation, n=3, significant difference: *, $p < 0.05$). Osteocalcin gene expression normalized to GAPDH.

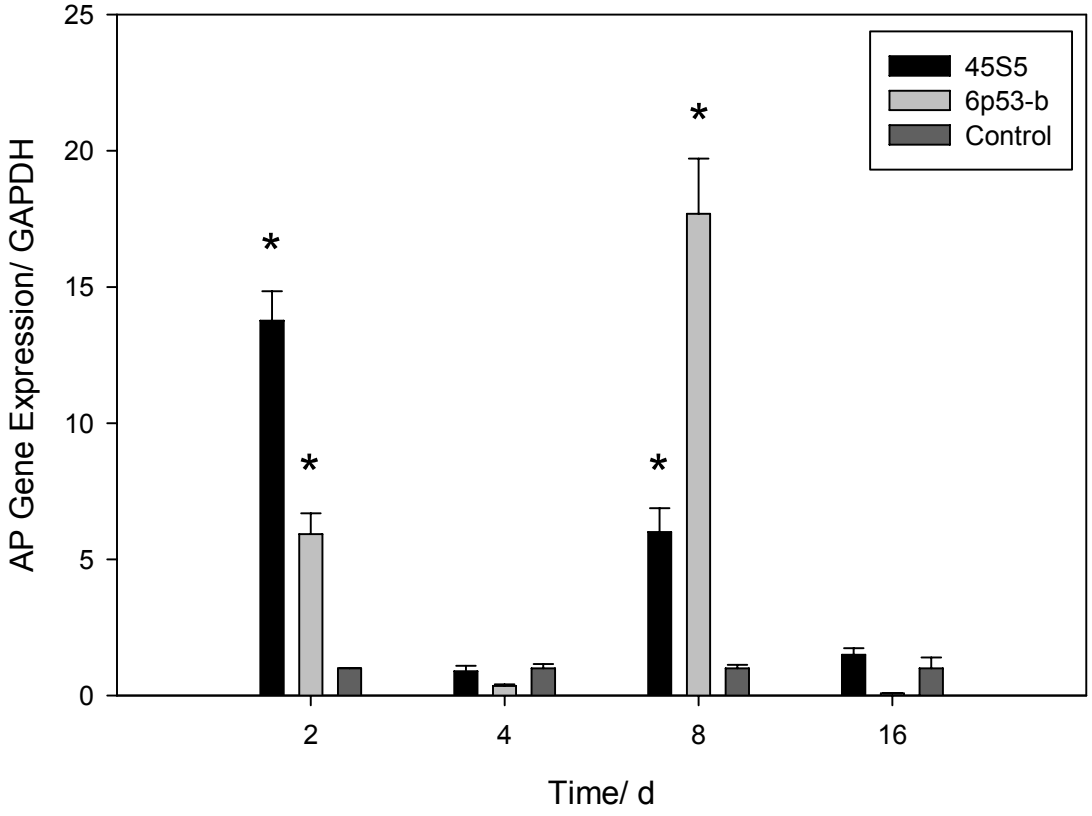

Figure 17. Graphical representation of relative q-PCR standardized by glyceraldehyde-3-phosphate dehydrogenase (mean ± standard deviation, n=3, significant difference: *, $p < 0.05$). Alkaline phosphate gene expression normalized to GAPDH.

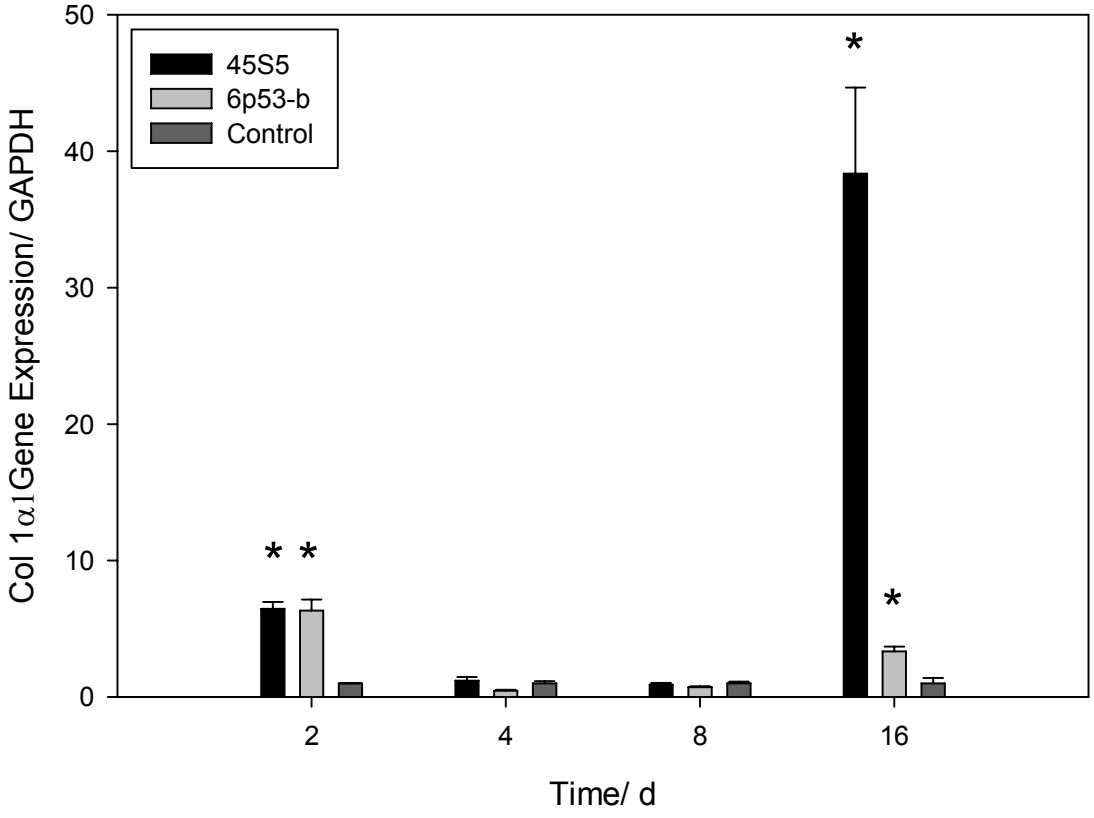

Figure 18. Graphical representation of relative q-PCR standardized by glyceraldehyde-3-phosphate dehydrogenase (mean ± standard deviation, n=3, significant difference: *, $p < 0.05$). Collagen Type 1 α1 gene expression normalized to GAPDH.

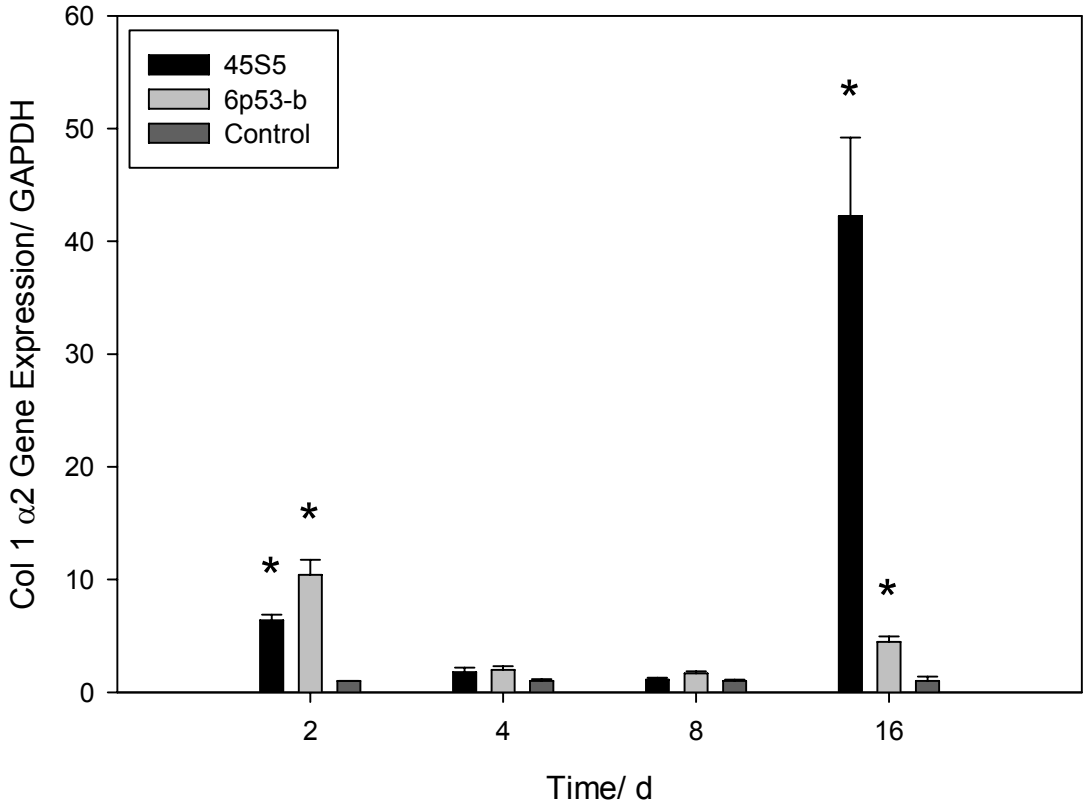

Figure 19. Graphical representation of relative q-PCR standardized by glyceraldehyde-3-phosphate dehydrogenase (mean ± standard deviation, n=3, significant difference: *, $p < 0.05$). Collagen Type 1 α2 gene expression normalized to GAPDH.

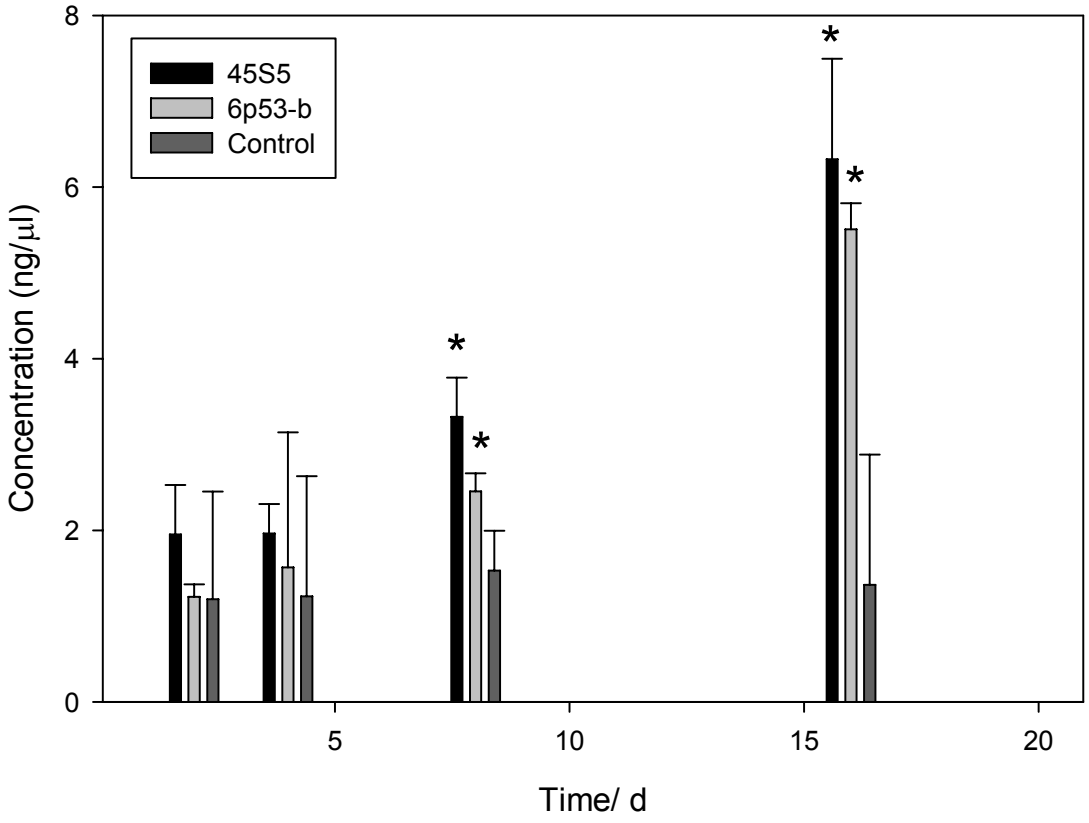

Figure 20. Intact Human Osteocalcin ELISA Expression represented by OC concentration (ng/µl). OC is over-expressed (significant difference: *, $p < 0.05$) on days 8 and 16 compared to control.

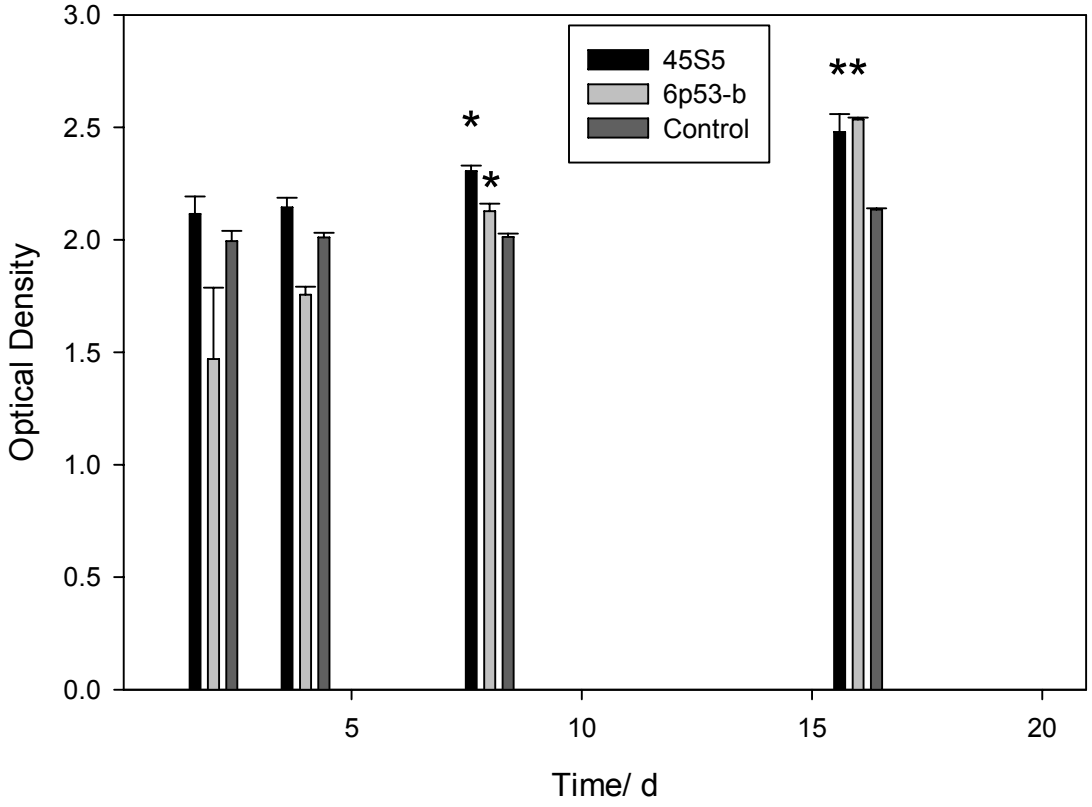

Figure 21. Alkaline phosphatase (ELISA) is represented by optical density values (absorbance 410nm). AP is over-expressed (significant difference: *, $p < 0.05$) on days 8 and 16 compared to control.

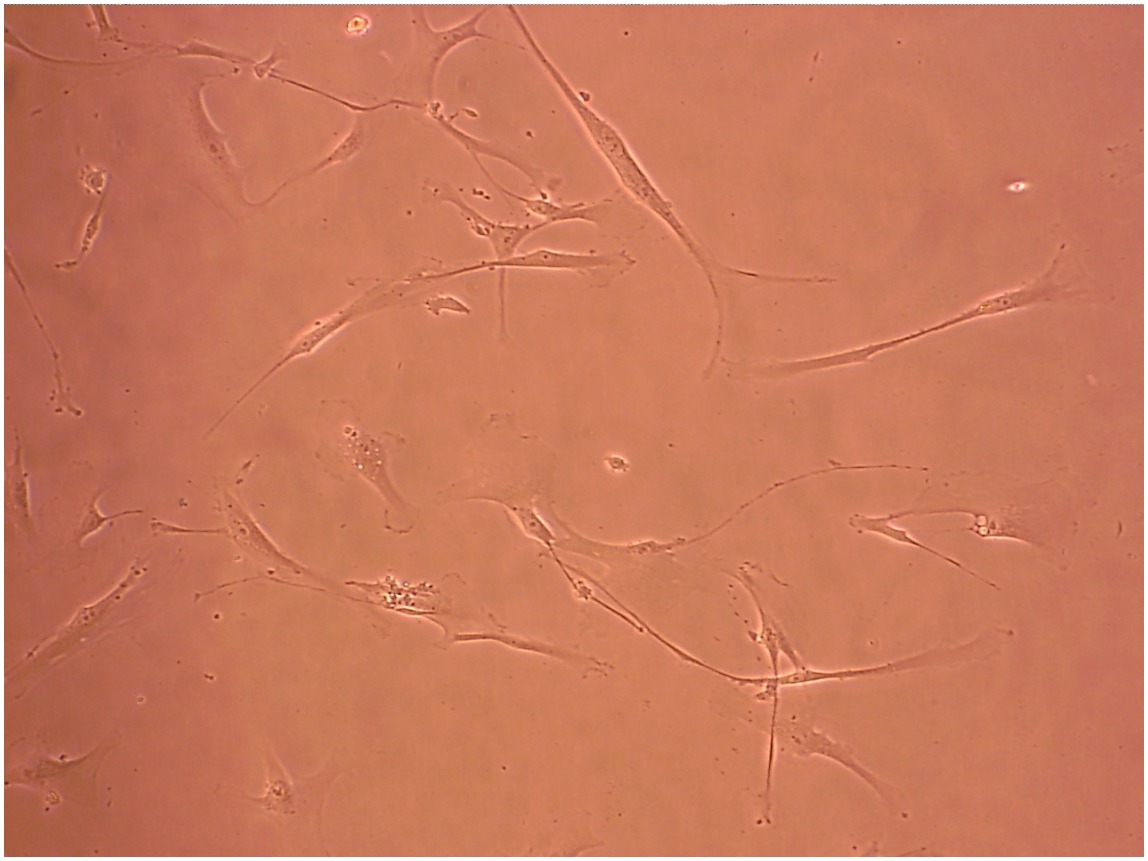

Figure 22. Primary cell culture of human Periodontal Ligament Fibroblasts (hPDLFs). Cell passages 5-9 were used for all experiments.

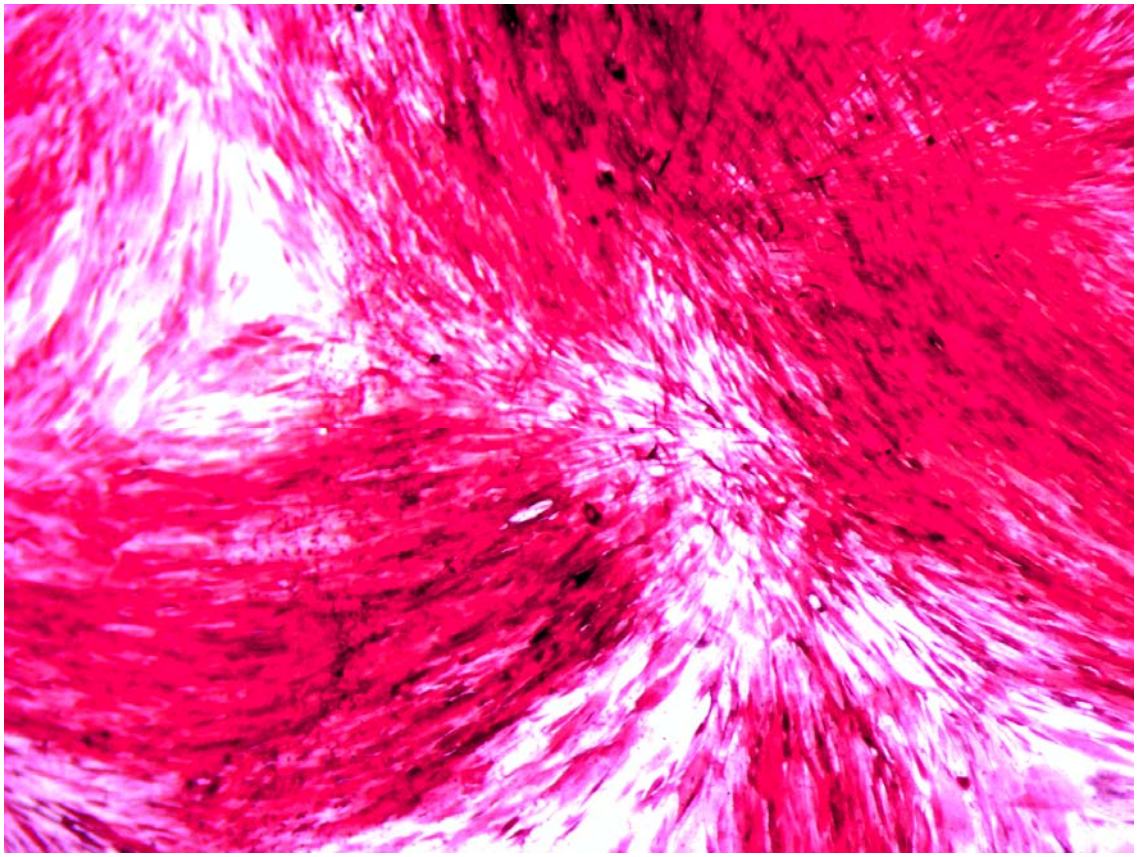

Figure 23. Alizarin Red S calcium staining (with hematoxylin) of hPDLFs grown in the presence of 45S5 glass powder ions after 16d (40x magnification).

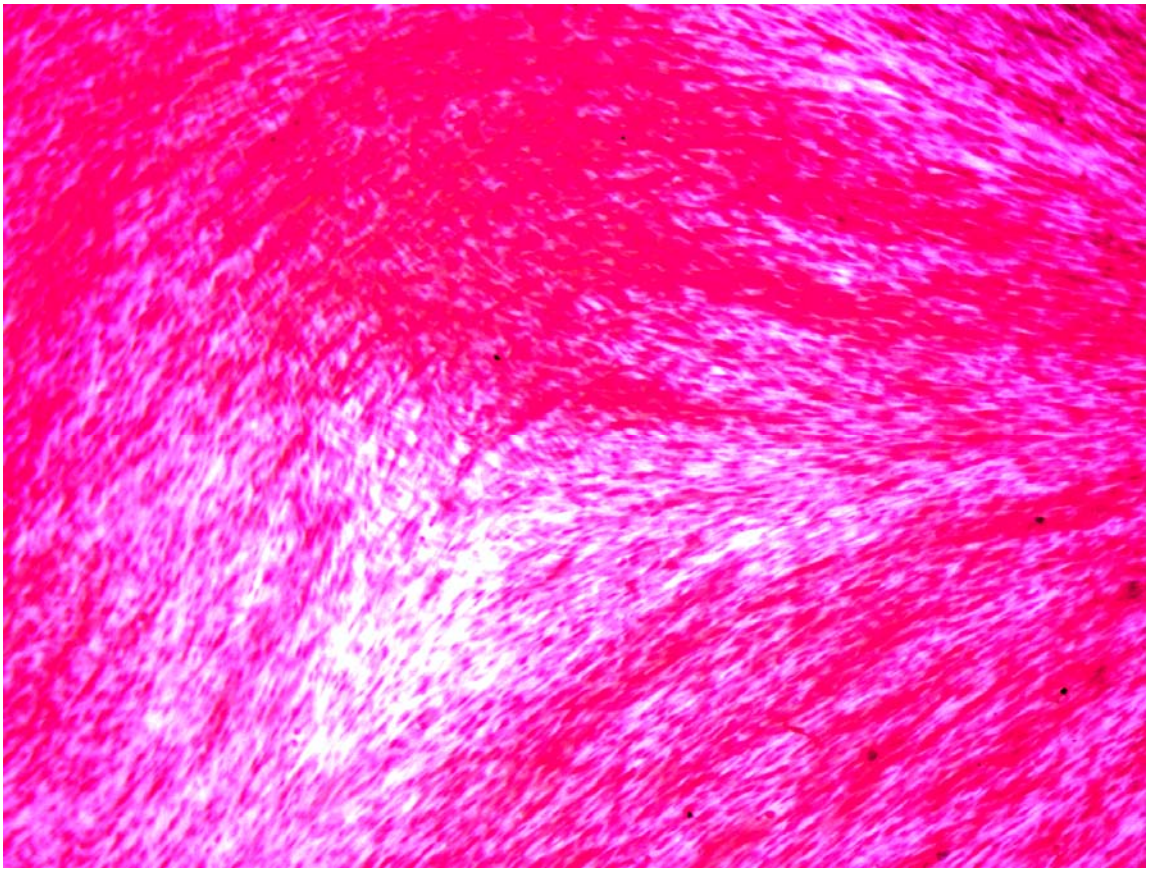

Figure 24. Alizarin Red S calcium staining (with hematoxylin) of hPDLFs grown only in DMEM after 16d (40x magnification).

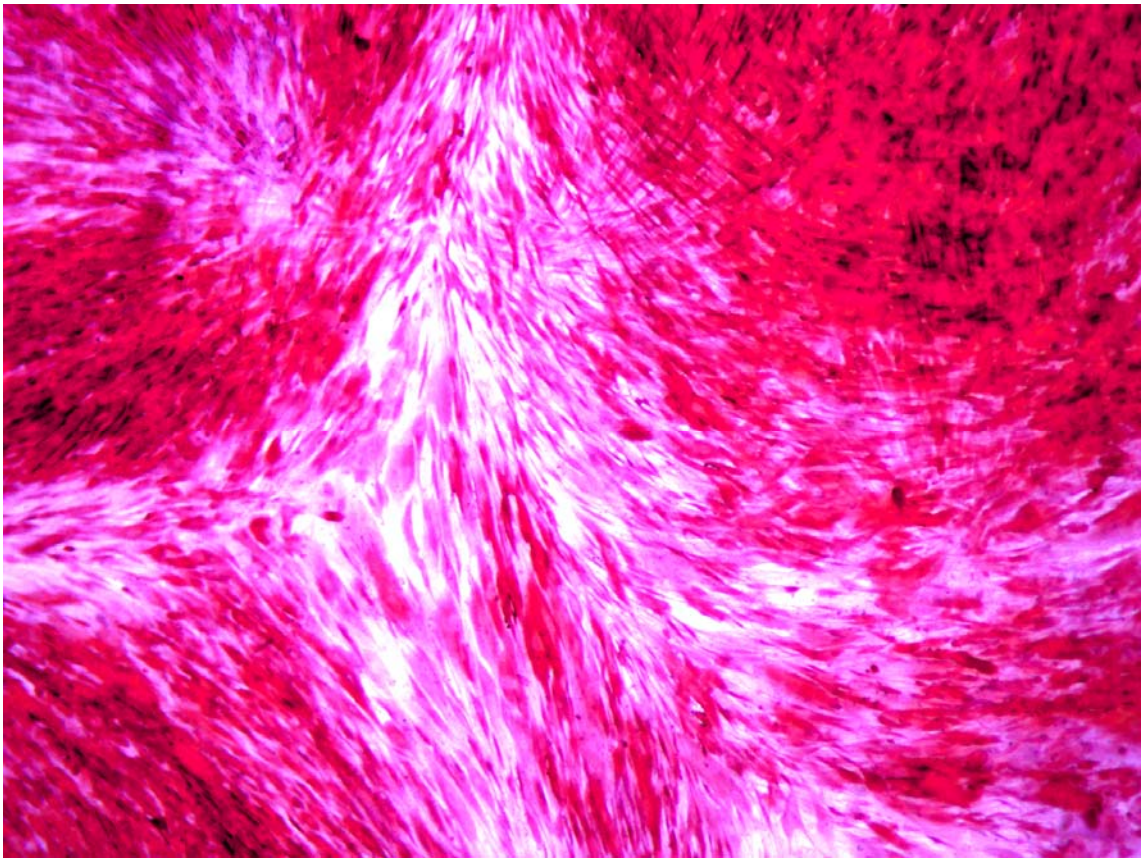

Figure 25. Alizarin Red S calcium staining (with hematoxylin) of hPDLFs grown in the presence of 6p53-b glass powder ions after 16d (40x magnification).

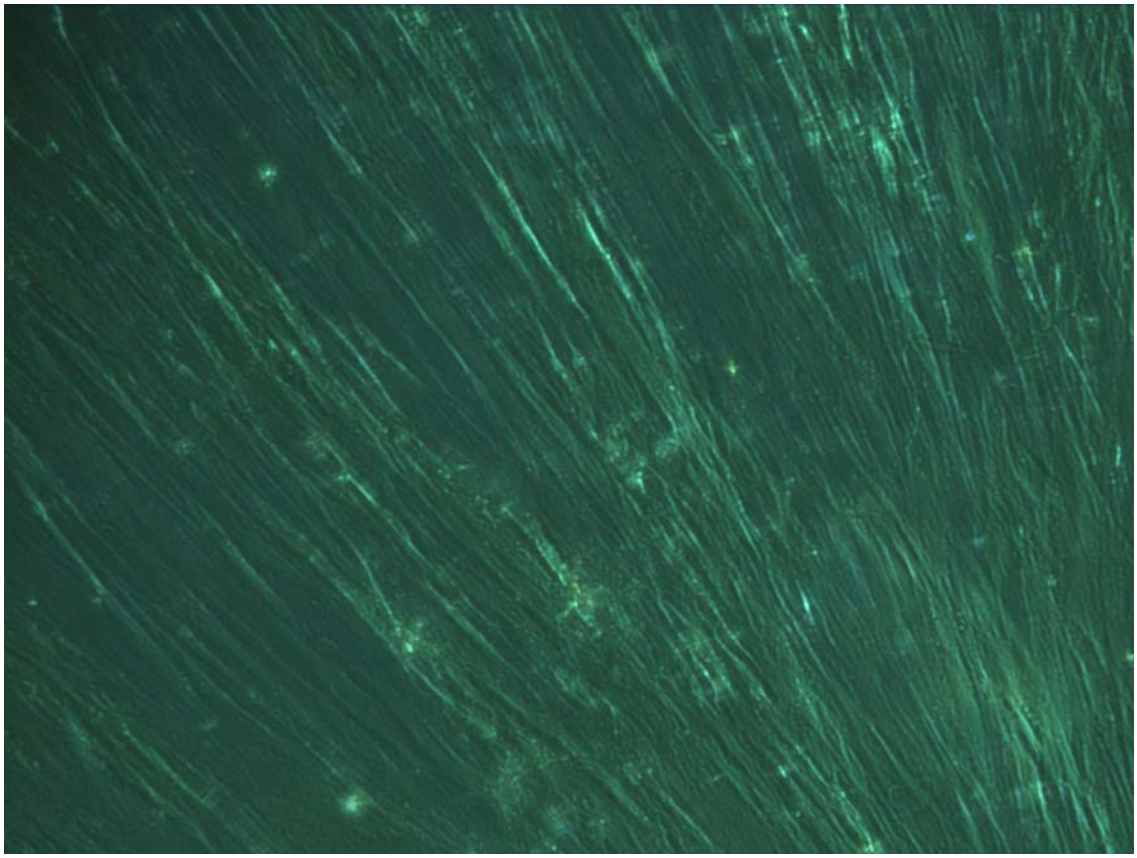

Figure 26. Optical micrograph showing extracellular matrix collagen as a result of hPDLF exposure to 45S5 GCM and ascorbic acid treatments at d2.

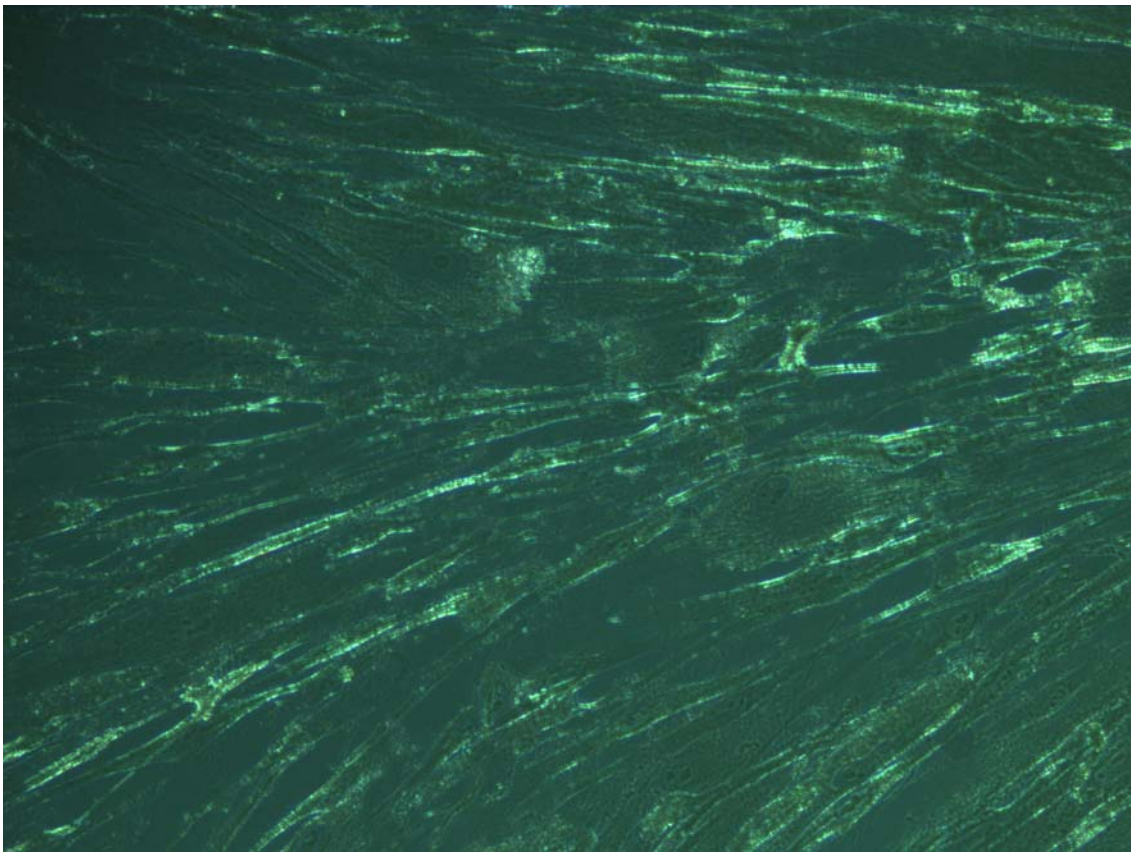

Figure 27. Optical micrograph showing extracellular matrix collagen as a result of hPDLF exposure to control media and ascorbic acid treatments at d2.

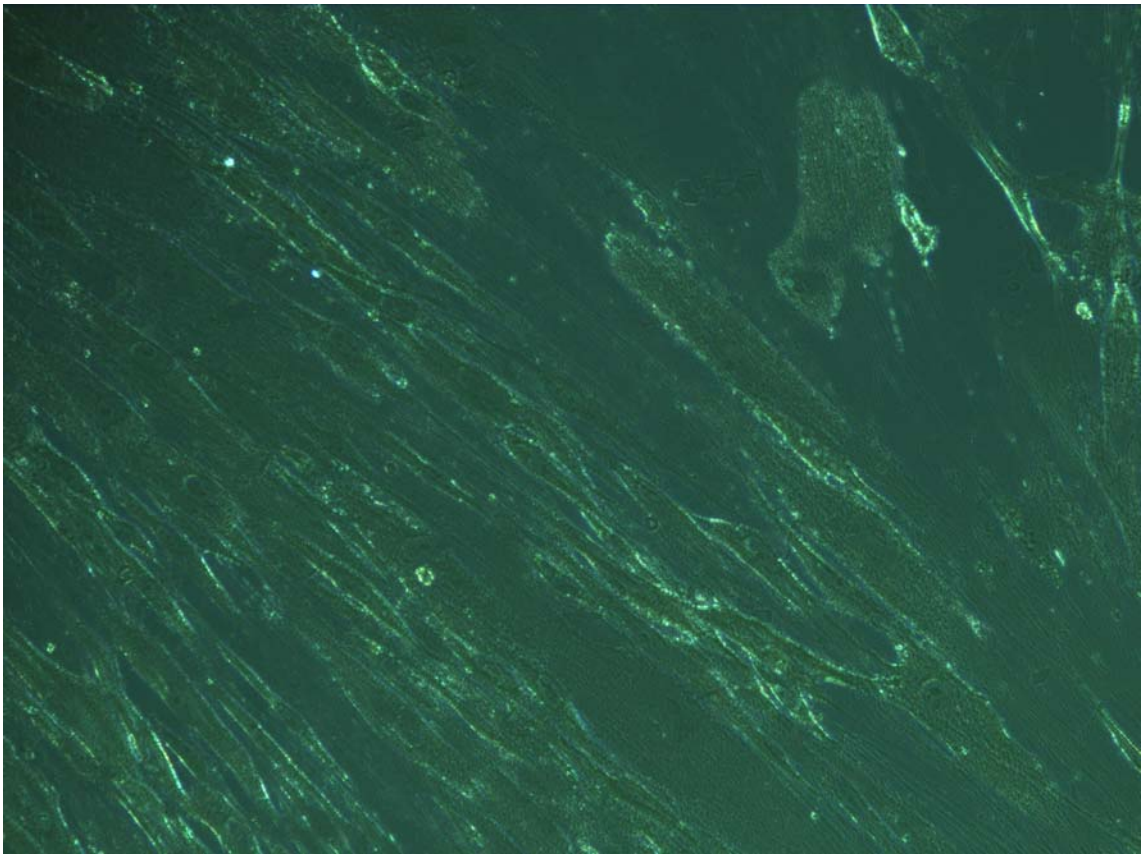

Figure 28. Optical micrograph showing extracellular matrix collagen as a result of hPDLF exposure to 6p53-b GCM and ascorbic acid treatments at d2.

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