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BOSTON UNIVERSITY

HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

DISSERTATION

SILICON, CALCIUM AND PHOSPHATE EFFECT ON HUMAN DENTAL PULP CELL CULTURES

by

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B.D.S., King Saud University, 2012
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SILICON, CALCIUM AND PHOSPHATE EFFECT ON HUMAN DENTAL PULP CELL CULTURES

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ABSTRACT

This study was designed to investigate the odontogenic effects of Silicon, Calcium and Phosphorous on human dental pulp cells (hDPCs). hDPCs derived from extracted pristine teeth were cultured in growth media with supplements of Si 25ppm, Si 25ppm+Ca 8.3ppm, Si 25ppm+Ca 8.3ppm+P 4.16ppm, Si 50ppm, Si 50ppm+Ca 16.7ppm, Si 50ppm+Ca 16.7ppm+P 8.3ppm and media without additional supplement as control, for the time intervals of 16 hours, 7, 12, and 21 days. Also, HDPCs were cultured in growth media with supplements of inorganic phosphate (Pi) in 0 ppm, 2 ppm, 4 ppm, 5 ppm, 8 ppm, for the time intervals of 16 hours, 7, 14, and 21 days. Cell proliferation rates were measured by the optical density of crystal violet dye stained cells. ALP activity was measured by fluorometric assay. Expression of Dentin Sialoprotein (DSP) was measured by ELISA. Mineralization of cultures was measured by Alizarin Red staining. Culture with 50ppm supplemental Si at day 21 yield significantly higher levels of ALP activity, DSP expression and mineralization ($P<0.05$) compared to the control group and other supplemented groups. Cultures with 2, 4, 5 and 8 ppm supplemental Pi yield significantly higher levels of ALP activity ($P<0.05$) compared to the control group at day 7. Cultures

with 5 ppm Pi supplement showed significantly higher levels of DSP expression ($P<0.05$) compared to the control group and the other groups at day 7. Cultures with Si 25ppm+Ca 8.3ppm supplemental and Si 50ppm+Ca 16.7ppm supplemental displayed significantly higher cell proliferation rates compared to the control group at day 12 and at day 21 ($P<0.05$). Cultures with 2, 4 and 5 ppm supplemental Pi displayed significantly higher cell proliferation rates compared to the control group at day14 ($P<0.05$) and at day 21 ($P<0.05$). Supplemental silicon in concentration of 50 ppm could significantly induce differentiation and mineralization of normal human dental pulp cells. Calcium has a synergetic effect in up-regulating the proliferation rates. Supplemental Pi in concentration of 5 ppm could significantly induce proliferation and odontogenesis of hDPCs. This is the first report to demonstrate Si, Ca and P induced odontogenesis of hDPCs cultures.

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LIST OF ABBREVIATIONS

BU ..... Boston University

ISO ..... International Standards Organization

ALP..……. Alkaline Phosphatase Enzyme

BIE.……..... Bioactive Inorganic Element

BMP……...… Bone Morphogenic Protein

Ca …….…….. Calcium

$Ca(OH)_2$ …….. Calcium Hydroxide

MTA……..…….…….. Mineral Trioxide Aggregate

ALP .....Alkalaine Phosphatase

ALT ..... Alanine transaminase

ANOVA ..... Analysis of variance

AST .....Aspartate aminotransferase

BMP ..... Bone morphogenetic proteins

CITI ..... Collaborative Institutional Training Initiative

CPC ..... Calcium phosphate cement

CSM ..... Calcium silicate–based material

DFPCs ..... Dental follicle precursor cells

DGP..... Dentin glycoprotein

DMP-1..... Dentin matrix phosphoprotein-1

DPP ..... Dentin phosphoprotein

DPSCs ..... Dental pulp stem cells

DSP ..... Dentin sialoprotein

DSPP ..... Dentin sialophosphoprotein

ECM ..... Extra cellular matrix

ES ..... Embryonic stem

FBS ..... Fetal bovine serum

GI ..... Glass ionomer

GMTA ..... Gray mineral trioxide aggregate

H^{o} ..... Null hypothesis

H2O2 ..... Hydrogen peroxide

hDPCs ..... Human dental pulp cells

IBC ..... Institutional biosafety committee

iPS ..... Induced pluripotent stem cells

IRB ..... Institutional review board

LD50 ..... Lethal dose

LPS .....Lipopolysaccharide

MSCs..... Mesenchymal stem cell

MTA ..... Mineral trioxide aggregate

OCN .....Osteocalcin

OD ..... Optical density

ODAM ..... Odontogenic ameloblast associated protein

OEC..... Normal oral epithelial cells

OPN..... Osteopontin

PBS .....Phosphate buffered saline solution

P ..... Phosphorous

Pi ..... Inorganic Phosphate

PC ..... Portland cement

PCNA ..... Proliferating cell nuclear antigen

PDLSC ..... Periodontal ligament stem cells

PG ..... Propylene glycol

PPM ..... Part per million

RMGIC ..... Resin modified glass ionomer cement

ROS ..... Reactive oxygen species

RT ..... Room temperature

RUNX-2 ..... Runt-related transcription factor 2

SCAP.....Stem cells from the apical papilla

SHED ..... Stem cell from human exfoliated deciduous teeth

Si..... Silicon

TG ..... Triglycerides

TGF-b ..... Transforming growth factor-beta

VPT ..... Vital pulp therapy

WMTA ..... White mineral trioxide aggregate

ZOE ..... Zinc oxide eugenol

LITERATURE REVIEW

LITERATURE REVIEW

Dentin

Dentin is mineralized tissue that forms the bulk of the crown and root of the tooth, giving the root its characteristic form; surrounds coronal and radicular pulp, forming the walls of the pulp chamber and root canals. Dentine and pulp are different types of tissues but through the life of tooth they interact closely with each other. In such a con text pulp and dentin are developmental and functional entity and best termed as endodontium. Mature dentine composed of approximately 70% of inorganic matrix, 20% organic matrix and 10% water. Dentin has 90% type I collagen and 10% extracellular matrix that includes phosphoproteins and glycoproteins. The principal inorganic component of the mature dentin is the hydroxyapatite (Ca^{10} $(PO4)^{6}$ $(OH)^{2}$). These crystals are needle shaped with a thickness of about 5 nm and length of approximately 20 nm.

Primary dentin is formed during active tooth development and it exhibits well organized pattern of tubules and cell processes. On the other hand, the secondary dentin is formed by normal pulp function after tooth formation is complete and the tubular pattern is regular, but number of tubules is less than that found in primary dentin.

Histologically the secondary dentin is separated from primary dentin by a hyperchromatic line or demarcation zone. Tertiary dentin or irritation dentin is formed in defense against any insult to the pulp. Damage can take place by external influences, including caries and attrition, and the tertiary dentin can range from regular tubular dentin that differs little from primary and secondary dentin (reactionary dentin), but also can be very dysplastic and/or irregular, and atubular when there is odontoblast loss depending on the

differentiation status of the formative cell. Finally, sclerotic dentin is characterized by calcification of the dentinal tubules as a result of injury or normal aging; it appears translucent in ground sections due to the difference in refractive indices of calcified dentinal tubules and adjacent normal tubules when examined by transmitted light (1).

Predentin

Predentin is layer of dentin situated between the odontoblast layer and the mineralized dentin. It is synthesized and secreted by odontoblasts and is composed of unmineralized organic matrix. It's mainly consist of type I and type II collagens, proteoglycans, glycoproteins, glycosaminoglycans, dentin sialoprotein and dentin phosphoprotein. Dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are important markers for cells in the odontoblast lineage. DSP is secreted from the basal end of odontoblasts; on the other hand DPP is secreted from distal end of the odontoblasts, adjacent to the Predentin region. Dentin phosphoprotein enable to bind large numbers of calcium ions which play a very important role in mineralization of dentin. (1)

Dental Pulp Biology

The dental pulp is a soft tissue of mesenchymal origin with specialized cells, the odontoblasts, arranged peripherally in direct contact with dentin matrix. The dental pulp has a unique characteristic as it is guarded by three mineralized layers: dentin, enamel, and cementum. This mineralized tissue provides a mechanical barrier to protect the pulp from pathogens present in the oral environment (2). The dental pulp population of cells are usually contains progenitor/stem cells of odontoblast lineage and react as a mechanical support system implanted within a vascular stroma (3-6).The pulp proper is the central mass of the pulp and it consists of loose connective tissue and contains nerves and the larger blood vessels. The most prominent cell in this zone is the fibroblast. This region is lined peripherally by a specialized odontogenic area, which has four layers. The pulpal core which is found in the innermost area of the dental pulp, is composed of many cells and vascular supply. (7, 8). The cell rich zone in the subodontoblastic area contains undifferentiated mesenchymal cells and fibroblasts. The outsized intercellular space in pulp is composed of type I and type III collagen fibrils (56% and 41%, respectively) (9). The cell free zone (zone of Weil), is located under the outermost odontoblastic layer, which lies next to the mature dentin and predentin. The cell free zone is rich in both nerve networks and capillaries (10). The nerve plexus of Raschkow is directed central to the cell-free zone (4). Also found in dental pulp are endothelial cells, collagen fibrils and defense cells (lymphocytes, microphages) (11). These cells offer the dental pulp to function in dentinogenesis (12) by means of nutrition and sensory (13, 14). These collections of cells usually develop from the neural crest and mesoderm, and afterwards from dental follicles.

Human dental pulp cells were assumed to be stem cells, which have the capability to differentiate into progenitor odontoblast cells, and further differentiate into odontoblast cells (15). Compared to other stem cells, dental pulp cells are much easier to obtain. Several studies indicated that dental pulp cells show multipotency, including neurogenic, pancreatic, osteogenic, odontogenic potency (16, 17). Dental pulp cells are commonly obtained from extracted third molars and primary teeth, which are usually discarded as medical waste and it can be stored for long duration without losing their multipotency (18).

Dental Pulp Disease

Several unique properties are imposed on the pulp; it is situated within a low-compliance environment that limits its ability to expand during episodes of vasodilation and increased filtration. It is mechanically protected from external stimuli by solid hard tissue such as enamel, dentin and cementum, which maintain its health and vitality if the protective layers is intact (19).

Pulp irritation occurs due to caries, cavity preparation, chemicals, cancer, and trauma. As a consequence, inflammatory reactions result in an increase in tissue pressure due to a limited increase in fluid volume. In the pulp, therefore, careful regulation of blood flow is of critical importance. As a protective mechanism, an inflammatory response can

occur by blocking the dentinal tubules or formation of secondary and tertiary dentin. Despite these strong protection mechanisms the pulp can be harmed by bacterial invasion or loss it's vitality (20). It may affect the odontoblast layer or causes dental pulp impairment. Therefore, the dental pulp component proliferates to the affected tissue to supply odontoblasts to create the reparative dentin (21, 22).

Three factors can participate in the pulp response to noxious stimuli; contingent on the host defense, the virulence of bacteria, and lymph node drainage (23). The left dentin thickness over the pulp tissue will also impact the outcome (24). As a response to inflammation, the odontoblastic layer beneath the lesion go thorough out some morphological modifications (25). When the dental pulp cannot eradicate the damaging bacteria, the defense mechanisms take place including the immune response to reduce the ability of the bacterial harm (19).

Pulp pathology can be identified as one of the following; reversible, irreversible pulpitis, pulp necrosis, hyperplastic pulpitis, internal dentin resorption and fibrous degeneration (26). Reversible pulpitis presents mild symptoms that diminish when the irritation stimulus is removed and as a result the pulp will be able to maintain its vitality. In irreversible pulpitis the pulp is severely affected to the point that it cannot keep its vitality even if the stimulus were removed (27). This kind of pulpitis is classified into symptomatic and asymptomatic; the symptomatic type occurs when inflammatory edema causes elevation of the intra- pulpal pressure, which will create spontaneous and sharp pain. If the edema is resolved through dentinal tubules then asymptomatic irreversible pulpitis occurs which is eventually the initial stage of pulpal necrosis. Pulp necrosis subsequent to

symptomatic or asymptomatic irreversible pulpitis. After the pulp becomes completely necrotic, the tooth will typically become asymptomatic until such time that symptoms develop as a result of extension of the disease process into the periradicular tissues (28).

Mesenchymal Stem Cells

Stem cells are unspecialized cells with the ability to renew themselves for long periods without significant changes in their general properties. Under certain physiological or experimental conditions, they can differentiate into various specialized cell types. Their significance in differentiation has led researchers to study their properties aiming to establish progenitor cell sources as well as the element of tissue regeneration and engineering. In dentistry, the key objective is regeneration of dental tissue. Such Mesenchymal stem cells (MSCs) involved in the regeneration of dentin are from the bone marrow (29-31), adipose tissues (32, 33), umbilical cord blood100 and from dental tissues (7). Studies indicate that the first isolated dental stem cells were the dental pulp stem cells (DPSCs) (21) followed by human exfoliated deciduous teeth (SHED) (34), periodontal ligament stem cells (PDLSC) (35) and the MSCs from apical papilla of developing teeth (SCAP) (36). Also, some studies have also used stem cells from dental follicles hence such cells are called the dental follicle precursor cells (DFPCs) (37, 38).

When grown in appropriate media, the dental tissue-derived MSCs can differentiate into different cell-restricted lineages including the osteo-odontogenic lineages (39-43). Based on earlier studies, MSCs such as DPSCs, SHED and SCAP can differentiate into the odontogenic lineage in vitro and their ability to differentiate into odontogenic lineages is of importance in dental tissue engineering. As such, they can regenerate ectopic pulp dentin-like tissue in immunocompromised mice (44-46). However, a prospect associated with the functional differences of the different dental tissue-derived MSCs where there is some degree of heterogeneity in their phenotypic features (47).

Dental Pulp Defense Cells

Pulp tissue has the ability to response to any irritant, and is well equipped with cells required foe the initiation of immune responses. These cells include macrophages, dendritic cells, mast cells and lymphocytes. Macrophages are differentiated monocytes that are active in endocytosis and phagocytosis. They are abundant around the blood vessels. Some of these cells can elicit immune reactions by processing antigen and presenting it to memory T cells and leading to process of irritant elimination. Dendritic cells are part of immune system and distributed in the pulp tissue and their function is induction of T cell dependent immunity. Mast cells are part of inflammatory reaction and are seldom found in healthy pulp. However, they are abundant in chronically inflamed pulps. Mast cells contain

granules that are rich in heparin which act as an anti-coagulant and histamine. Lymphocytes are found in the human pulp tissue as T lymphocytes and B lymphocytes. These cells elicit immune responses during pulp irritation and injury (1).

Phenotype of Human Odontoblast

Odontoblasts synthesize and secrete extracellular matrix (ECM) characterized by specific proteins. Among these are dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) both of which are dentin-specific. Others are common to both dentin and bone, such as type I collagen, alkaline phosphatase, osteocalcin, osteopontin and dentin matrix protein (DMP1) (48, 49). Specific methods are used to identify the extracellular matrix components in pulp cells. For instance, Couble et al. used immunofluorescent labeling to demonstrate the presence of type I collagen in human dental pulp cell culture and noticed strong correlation between intracellular labeling of type I collagens cell bodies with alpha 1 collagen cDNA (50). Type I collagen is the structural framework of mineralized matrix and it's considered as the first marker of dentin formation. The expression of collagen and non-collagen proteins are timely sequenced and highly regulated. During active odontogenesis, the expression of alkaline phosphatase, fibronectin and type I collagen mRNA are increased substantially. Then mRNA for osteocalcin appear just before the onset of mineralization, which is a marker of pre-odontoblast (51). Increased synthesis of

type I collagen is significant step in odontoblast differentiation process, osteocalcin is expressed in pre-odontoblasts at the same time as or shortly after deposition of the first layer of predentin, and after the first appearance of alkaline phosphatase activity which takes place before the onset of dentin mineralization (52).

Alkaline phosphatase (ALP) is an enzyme that exist in the dental pulp, and its levels are increased in the stage of dentinogenesis. Therefore, ALP is considered as a reliable indicator for determination of odontoblastic activity. Isoenzymes of ALP are found in vertebrate tissue located in liver, bone, dentin, placenta and intestinal tissues. In dentin as in bone, the main function of ALP appears to be generating phosphate ions and transporting substances from the intracellular compartment across the membrane into the extracellular region. In mineralization, ALP increase the local concentration of inorganic phosphate by carrying local phosphorous but the precise role is not yet confirmed (53). ALP may also be involved in the breakdown of pyrophosphate, a potent inhibitor of calcium phosphate deposition at the extracellular level. Elevated levels of alkaline phosphatase activity was noted to be higher in the subodontoblastic layer than in the odontoblastic layer and highest just prior to final odontoblastic specialization (58). The activity of ALP might be a prerequisite for the differentiation of pulp cells into odontoblasts (58-62).

Dentin sialoprotein (DSP) is one of the markers of mature odontoblast cells and dentin production that is produced only by these cells and has not been found in other cells (50, 59). Dentin sialophosphoprotein (DSPP) is cleaved into DSP by the terminally differentiated odontoblasts to prompt mineralization and dentinogenesis (60) However, DMP 1 revealed to be expressed by osteoblasts and other tissues (61). Dentin sialoprotein

is a 53KD protein coded for human chromosome 4q21. It's rich in aspartic acid, glutamic acid, serine and glycine and contains about 30 % carbohydrate (62). It is transcribed from a single gene and is translated into a single polypeptide chain a 387 amino acid DSP sequence and a 489 amino acid DSP sequence separated by an intervening 64 amino acid sequence. The individual components are formed by post- translational enzymatic processing (63). Further studies demonstrated DSP's presence in young and mature odontoblasts, dentin and pre-ameloblasts but not in ameloblasts, bone, cartilage, soft tissues or other components of oral tissues (61, 64). The analysis of DSP from NH2-terminal and cDNA sequence, indicate that overall composition of this protein is similar to bone sialoprotein (BSP) and osteoprotein (OPN) in bone tissue, except that DSP lacks Arg-Gly-Asp cell attachment sequence as found in other sialic acid rich protein such as OPN and BSP (65). Since DSP is expressed in both young odontoblasts and pre-ameloblasts it might play a role in epithelial-mesenchymal signaling that accompanies this phase of cellular development in teeth (62). Exact role and function of DSP is yet to be elucidated, it may be structural or modulating signal in hydroxyapatite formation similar to fibronectin or tenascin. Dentin sialoprotein might facilitates dental mesenchymal cell differentiation and dentin formation as well it may serve as a therapeutic agent for dentin-pulp complex regeneration in dental caries (66). However, the fact that it can be demonstrated in only one tissue and limited to odontoblasts allows DSP to be used as a definitive markers for odontoblastic phenotype and/or the presence of dentin.

In this laboratory, functional analysis of following expression proteins, Alkaline Phosphatase and Dentin sialoprotein as well as the detection of functional mineralization are utilized to characterize the different odontoblast stages in vitro (67).

Mineralization Process of Dentin

Dentin mineralization is a complex process that starts by precipitation of calcium phosphate crystals within the matrix vesicles and upon enough growth, these vesicles rupture. These crystals aggregate in highly controlled and organized manner to form globular dentin. Subsequently, crystal deposition in form of hydroxyapatite occurs. Crystals are laid down within the fibrils themselves as mineralization matures. The crystals arranged in long axes parallel to the fibril axes and in rows conforming to striation pattern of collagen. Then, deposition occurs creating spherical shaped “calcospherites”. Dentin go under the influence of a linear type of mineralization which is reflected in the presence of incremental lines. Prior to mineralization alkaline phosphatase (ALP) levels increases substantially initiating cascade of complex interaction of molecules and leading to the mineralization. Exact role of ALP, DSP and DDP are not quite understood, but their roles are essential for mineralization.

Concept of Molecular Biocompatibility

The concept of molecular biocompatibility was initially introduced by L. Chou, where he was the first to describe the theory of substratum surface topography when getting altered it can effect cell shape and modifies the cellular matrix. He investigated the effect of different materials and found that cells produce proteins and become activated by inductive signals, which present the idea of no material is inert by nature (72-74).

L. Chou and AlBazie, studied the bioglass materials at an atomic and molecular levels. They implanted sol-gel bioglasses, perioglass and hydroxyapatite (HA) endosteally of rabbit's proximal tibias condyles for two and eight weeks. Results showed bioglass released much higher Si to surrounding tissue than other two. And this Si rich environment induced a higher expression of osteocalcin at early stage with subsequently bone formation at eight weeks (75, 76).

Silicon Importance in Cells Growth

Silicon is the fifth most abundant element in the biosphere and it is essential trace element in higher animals. It is found in osteoid and connective tissue. Silicon has been reported to be involved in early stages of bone formation and lack of it caused sever deficiency of the skull. There are interrelations with silicon, calcium and hormones in the manner of inhibition and promotion of calcification in mammals (69, 70). Silicon also was related to several human diseases (e.g: fibrotic lung disease, silicosis, pneumoconiosis, asbestosis and esophageal cancer).

Importance of Si in bone growth has been strongly established. Si is required for cell proliferation and as a regulator of DNA replication, cyclic AMP level is increased by addition of Si to Si-starved diatom culture (71). Si also stimulates DNA synthesis and proliferation in mammalian bone and fibroblast cells (72, 73). Si ions induces osteogenesis by increasing of higher proliferation sates, higher level of ALP and osteocalcin, DSP and mineralization. Modulating of cellular response to silicon has been strongly established.

Clinical applications of bioactive glasses is growing and in the dental field. These materials are used to form endosseous ridge maintenance implants for denture stability and is widely used for periodontal treatments, sinus lifts and pre-prosthodontic as perioglass with high success rates (74).

Silicon Concentration Analysis

Previously, the Si ion concentration was determined by inductively coupled plasma (ICP) technique. Lyu determined that a saturated medium containing 120 ppm Si. Lyu added the Si gel powder of 100-500 micron particle size with hollow core in amounts equal to 60, 90, 120, 150 and 180 ppm to 20 mL culture medium. These vials then were placed in a shaker/incubator at 37°C at 150 rpm's for 24 hours. After standing for 30 minutes at room temperature, it was determined that we could obtain a concentration of Si gel greater than or equal to 150 ppm but less than 180 ppm. That is, only the 180 ppm vial had any gel settle out of solution.

Phosphorous Importance in Cells Growth and Bone Tissue Regeneration

Phosphorus, does not exist by itself in nature as it exhibits a highly reactive characteristics. It is present as phosphates in the biologic systems and it occurs in both organic and inorganic forms. Organic forms include phospholipids that form the main structural components of all cellular membranes and various organic esters. The average adult has approximately 700 g of phosphorus which is distributed in the skeleton (85%), teeth (0.4%), soft tissue (14%), blood (0.3%), and extravascular fluid (0.3%). For every 1 molecule of phosphate found in the extracellular space, a 100 molecules of phosphate carries in the intracellular space. The fraction of inorganic form exists either as free

inorganic phosphate ions (85%), bound to protein (10%), or complexed with calcium, magnesium, or sodium (5%) (75).

Phosphate is greatly required for calcification, but its precise role is not yet clear. It was shown that a high-phosphate diet significantly improves bone mineralization and might play role in the formation of secondary ossification centers, since hypophosphatemia caused delayed formation of secondary ossification centers (76). In another studies, it was shown that phosphate in the culture media act as a specific signal for the induction of osteopontin gene expression and ultimately protein synthesis by osteoblast-like cells (77-79).

On the cellular activity level, the function of ALP and ATPase is also involved in the transport of phosphate and calcium by increasing the local concentration of phosphate while removing the pyrophosphate inhibitors of apatite deposition (74). There is a study that examined the effect of concentration of both calcium (Ca) and phosphorous (P) on osteogenesis. UMR 106-01 BSP cells were treated with calcium (0.5-5 mM) for 24 hours with or without an additional 5 mM phosphorous supplement. Result of the study showed a calcium-dependent increase in mineral formation. However, cultures without an additional phosphorous supplement only formed detectable mineral at a relatively high calcium concentration. Cultures treated with 1mM calcium and 5.9 mM phosphorous produced nearly twice as much calcium minerals as those exposed to 5 mM calcium and 0.9 mM phosphorous. Addition of organophosphates (typically β-glycerophosphate) to cultures of primary osteoblast cells enhanced the formation of calcium mineral. In this case, ALP is required to hydrolyze the organophosphates to release phosphorous which appears

to be the actual initiator of mineral formation. Therefore, increased phosphate seems to initiate a mineral formation that afterward enhanced by calcium level. However, we cannot exclude the possibility that a portion of the mineral formed in these cultures might be the result of a direct heterogeneous nucleation within the established extracellular matrix of the cell layer (81).

In a vitro study to identify the relationship between mineralization and supplemented phosphate, cultures were maintained in medium containing ascorbic acid after day 7 but lacking the organic phosphate source, β-glycerophosphophosphate (β-GP). Under accelerated mineralizing conditions (with β-GP), osteocalcin mRNA synthesis increased steadily beginning at day 15 in parallel with calcium accumulation in the cell layer. In contrast, when cultures were maintained in the absence of β-GP, calcium did not begin to accumulate in the cell layer until day 25. Osteocalcin was not detectable until this point. Its low level of synthesis reflects the slower rate of mineralization. However, the presence or absence of β-GP had no effect on the proliferation period or on the onset of ALP expression (82).

Also it has been reported that osteoblast apoptosis can be activated when exposed to elevated phosphate concentration potentially due to profound loss of mitochondrial membrane. When exposed to an inorganic phosphate (Pi) concentration of 1–7 mM, the osteoblast-like cells from human explants experienced a decrease in the cell viability in a time- and dose-dependent manner (83). Moreover, it was shown that Ca^{2+} modulated inorganic phosphate induced osteoblast apoptosis (84, 85). Also, a downregulation of

osteoblast gene expression was noticed with elevated phosphorous levels in the culture media (86).

Another study on rat bone marrow cells showed an inhibitory effect of phosphorous on proliferation (87). In this study, the rat bone marrow cells were cultured with various bioglasses, thus the inhibition of cell proliferation seemed to be related to high phosphate ion release or phosphate-rich precipitation.

Rationale and Clinical Significance for this Research

Preservation of pulp vitality is of prime importance, especially in immature teeth where root formation has not yet been completed (88). To achieve this in some cases, a pulp capping procedure is one of the treatment options. An ideal pulp capping material should possess sufficient physiochemical properties, stimulate the pulpal odontoblasts in formatting a dentinal bridge, and induce remineralization and healing (89-92). Over the years, there has been controversy among researchers about the pulp-capping agent used, and not the procedure itself.

Calcium hydroxide (CH) and mineral trioxide aggregate (MTA), two of the present treatment options do not exclusively stimulate dentinogenesis; instead, they induce the formation of mineralized tissue as a result of their high pH levels (93, 94). Biodentine, is a calcium silicate-based material developed in 2009. Biodentine contains tricalcium silicate,

calcium carbonate, zirconium oxide and calcium chloride (85). Some studies indicated their ability to stimulate proliferation as well as differentiation of odontoblasts (96-100).

Evidence in the literatures indicates that silicon induces bone formation, calcification, and mineralization (101-103). Yet, the mechanism behind silicon's relationship to cell behavior is not fully understood. Silicon or calcium concentration could play a dose- dependent role in increasing bone cell proliferation (104).

Thus, the aim of this study was to test the most effective concentration and combination of silicon (Si), calcium (Ca), and phosphorous (P) on normal human dental pulp cells (hDPCs), in order to establish an optimal concentration that could ultimately be used to develop a new pulp capping material which will induce an odontogenic effect.

HYPOTHESIS

HYPOTHESIS

It was hypothesized that supplemented silicon, calcium and/or phosphorus ions would up-regulate the human dental pulp cells attachment efficiency, cell proliferation and odontogenic activity, specifically cell differentiation of odontoblast cells and cell mineralization.

The null hypothesis (H^0) was that there was no difference in cell attachment efficiency, cell proliferation and odontogenic differentiation between the experimental sample groups containing supplemented silicon, calcium and phosphorus ions in different concentrations and combinations to the control group.

OBJECTIVES OF THE STUDY

OBJECTIVES OF THE STUDY

According to the hypothesis, composition of silicon, calcium, and/or phosphorous ions of bioactive glasses may play a major role to regulate the odontogenic behavior, the objective of this study was to evaluate the effect of the composition and concentration of these materials in the conditioned medium on the behavior of the human dental pulp cells.

Objective 1:

Si, Ca and P experiment: to measure cellular attachment efficiency of the human dental pulp cells with growth media containing various supplements of Si, Ca, and P in different concentrations at 16 hours after seeding the cells.

P experiment: to measure cellular attachment efficiency of the human dental pulp cells with growth media containing supplements of P in different concentrations at 16 hours after seeding the cells.

Objective 2:

Si, Ca and P experiment: to measure cellular proliferation rates of the human dental pulp cells at 3 different time points (7, 12 and 21 days) with growth media containing various supplements of Si, Ca, and P in different concentrations.

P experiment: to measure cellular proliferation rates of the human dental pulp cells at 3 different time points (7, 14 and 21 days) with growth media containing supplements of P in different concentrations.

Objective 3:

Si, Ca and P experiment: to measure the odontogenic activity and differentiation potential of human dental pulp cells cultured with differentiation media containing various supplements of Si, Ca, and P in different concentrations. This will be achieved by:

a) Measuring the tissue non-specific alkaline phosphatase activity in the collected supernatants from cell cultures collected from groups at two time intervals of 12 and 21 days.
b) Measuring the dentin sialoprotein levels expressed in the collected supernatants from cell cultures collected from groups at two time intervals of 12 and 21 days.

P experiment: to measure the odontogenic activity and differentiation potential of human dental pulp cells cultured with differentiation media containing supplements of P in different concentrations. This will be achieved by:

a) Measuring the tissue non-specific alkaline phosphatase activity in the collected supernatants from cell cultures collected from groups at three time intervals of 7, 14 and 21 days.
b) Measuring the dentin sialoprotein levels expressed in the collected supernatants from cell cultures collected from groups at three time intervals of 7, 14 and 21 days.

Objective 4:

Si, Ca and P experiment: To measure the mineralization rate of human dental pulp cells cultured with differentiation media containing various supplements of Si, Ca, and P in different concentrations. This will be achieved by measuring calcium deposits by Alizarin Red S staining on the fixed cell in the culture plate samples collected from two time interval of 12 and 21 days.

MATERIALS AND METHODS

Material	Manufacturer	Catalog number
2 mL pipettes	Fisher Scientific	13-678-11C
10 mL pipettes	Fisher Scientific	13-678-11E
25 mL pipettes	Fisher Scientific	13-675-11
1.5 mL tubes	Fisher Scientific	05-408-129
15 mL centrifuge tubes	Fisher Scientific	14-959-70C
50 mL centrifugation tubes	Fisher Scientific	14-432-22
24 wells tissue culture plates	Celltreat	229123
3x3 weighing paper	Fisher Scientific	NC9691607
Aspirating pipettes	Fisher Scientific	13-675-16
Chandler bi-bevel chisel, #7	Hu-friedy	CC
Corning 12.5 cm^2 tissue culture flasks	Fisher Scientific	08-772-1F
Corning 225 cm^2 tissue culture flasks	Fisher Scientific	1482680
Disposable masks	Kimberly-Clark	12978
EMD Millipore™ Stericup™ Sterile Vacuum Filter Units, 0.22µm	EMD Millipore	SCGVU02RE
Gloves	Fisher Scientific	19-130-1597
High speed hand-piece	Midwest	HP200

330 high speed bur	Midwest	386261
Manual pipette P20	Gilson, Pipetman	FA10003P
Manual pipette P100	Gilson, Pipetman	FA10004P
Manual pipette P200	Gilson, Pipetman	FA10005P
Manual pipette P1000	Gilson, Pipetman	FA10006P
Multi tip pipette Transferpette 20-100 µL	Brinkman	10N3297
Multi tip pipette Transferpette 50-200 µL	Brinkman	07R2380
Pipette tips (blue), 200- 1000 µL	Fisher Scientific	21-278-52
Pipette tips (yellow), 1- 200 µL	ENE Mate	1031-800-010
Pro-pette pipette aid (pipette controller)	MTC Bio	P6080
Surgical Dental Mallet, ML01. Solid Stainless Steel Mallet	Osung	ML25
Weighing dish (medium)	Fisher Scientific	02-202-101
Weighing dish (small)	Fisher Scientific	02-202-100

Table 1. List of Materials.

Item	Manufacturer	Model number
0.05% Trypsin-EDTA	Life Technologies	25300054
10% Formalin	Fisher Scientific	HT501128-4L
70% Ethanol	Fisher Scientific	S25306A
Alkaline phosphatase assay kit (Fluorometric)	abcam	ab83371
Alizarin Red S	Sigma-Aldrich	A3757
Beta Glycerophosphate	Sigma	G9422
BME (500mL)	Life Technologies	21010046
Calcium Chloride ($CaCl_2$)	Sigma	C-3306
Charcoal stripped FBS	Life Technologies	12676011
Crystal Violet, reagent	Sigma	229288-100G
Fetal bovine serum	Life Technologies	16000044
Fungizone (antifungal)	Life Technologies	15290-026
Hylcone L-Glutamine	Fisher Scientific	SH3003401
L-ascorbic acid 25g	Sigma Aldrich	A4544-25G
Menadione 25g	Sigma Aldrich	M5625-25G
Dentin Sialoprotien (DSP) Bioassay™ ELISA kit (Human)	USBiological	190989
Pen/Strep 100X	Life Technologies	15140-122

Silica Gel (Silicon Dioxide SiO_2)	Aldrich Chemical Company, Inc.	288519
Sodium Phosphate (Na_3PO_4)	Fisher Biotech	BP332-500
Vitamin D3	Sigma	1530-10UG

Table 2. List of chemicals, enzymes, growth media and assay kits.

Item	Manufacturer	Model number
360 stirrer	VWR Scientific	58935-351
Biological Safety Cabinet	LABCONCO	3430809
Centrifuge Marathon 16KM	Fisher Scientific	16KM
Hemocytometer	Reichert	26715
Incubator	Thermo ELECTRON CORPORA TION	3110
Isotemp Waterbath	Fisher Scientific	10L-M
Light microscope Leica	LEITZ DM IL	Type: 090-131.002
Microplate reader (spectrophotometer)	TECAN	Infinite 200 Pro
PH meter with electrode	CORNING	320
Roto Mix shaker	Thermolyne	M51335
Stericup® and Steritop® vacuum driven disposable filtration system	Millipore Corporation	SCGVU02RE
Stirrer	CORNING	PC-320
Table Top Centrifuge	BECKMAN	340508
Tissue culture Hood, Class II A/B3 Biological Safety Cabinet	Logic	1184
Touch mixer (tube shaker)	Fisher Scientific	231

Weight scale	DENVER INSTRUMENT	P-314
Weight scale	OHAUS	3113

Table 3. List of Equipment Used.

Approvals and Training

This study was approved by the Institutional Biosafety committee at Boston University (IBC #13-046). All necessary human subject trainings were completed through the Collaborative Institutional Training Initiative (CITI). Online lab training was provided through the Office of Research at Boston University. Exempt review (IRB# H-33173) from the human subjects was granted from the Institutional Review Board to use biological waste material of human teeth. Patient identification from waste human teeth is not possible.

Harvesting Dental Pulp Tissue Cells from Human Teeth

In this study, all human dental pulp explants used were isolated from extracted 3rd molars with incompletely formed roots of 18 and 19 years old female patients, after obtaining informed consent. These teeth were collected from the oral surgery clinic of Boston University School of Dental Medicine. Patients were in good general health and the extracted teeth had no restorations or carious lesions, also there was no evidence of pulpal disease or any other syndrome or condition affecting tooth formation. Human dental pulp cells were isolated following to the published procedure described by Stanislawski et al. with modifications (105).

Item	In total volume 500 ml	End concentration
100% FBS	50 ml	10% FBS
100x Penicillin/Streptomycin antibiotic (10,000U/ml)	5 ml	100U/ml (1x)
1000x Fungizone (250ug /ml)	0.5ml	0.25ug/ml (1x)
Eagle's Basal Medium (BME)	444.5 ml	_____

Table 4. Preparation of the growth media.

Note: Growth media was prepared to achieve 500mL total volume.

Human Dental Pulp Cell Culture

Immediately after extraction, all teeth were placed in 5 ml cold PBS-containing tubes. Teeth were transferred to the laboratory within 20 minutes and processed in less than two hours. Once the teeth reached the lab, the apical portion of roots (3-4 mm) were removed with a high-speed handpiece and # 330 bur to eliminate any periodontal fibroblasts contamination to pulp tissue, they then were sagittal scored with copious water spray to prevent heat generation without entering the pulp chamber, (2-3 mm) away from pulp tissue. Using a bi-bevel chisel, the teeth were sectioned and the dental pulp tissues were collected immediately with a sterile cotton pliers.

Then the pulp was cut into pieces using sterile surgical scissors and placed into room temperature PBS. These pulp explants were placed in 12.5 cm^2 tissue culture flasks (three to four pieces in each flask), and 6 ml of growth media was added. Culture medium consisted of 10% fetal bovine serum (FBS), 1X Penicillin antibiotic (100 U/mL), and 1X Streptomycin (100 ug /mL), Amphotericin B anti-fungal (0.25 ug/ml) in Eagle's Basal Medium (BME). The cultures were maintained in an incubator at 37° C with 5% CO_2 and saturated humidity. The culture media was changed every 3 days (Photo 1).

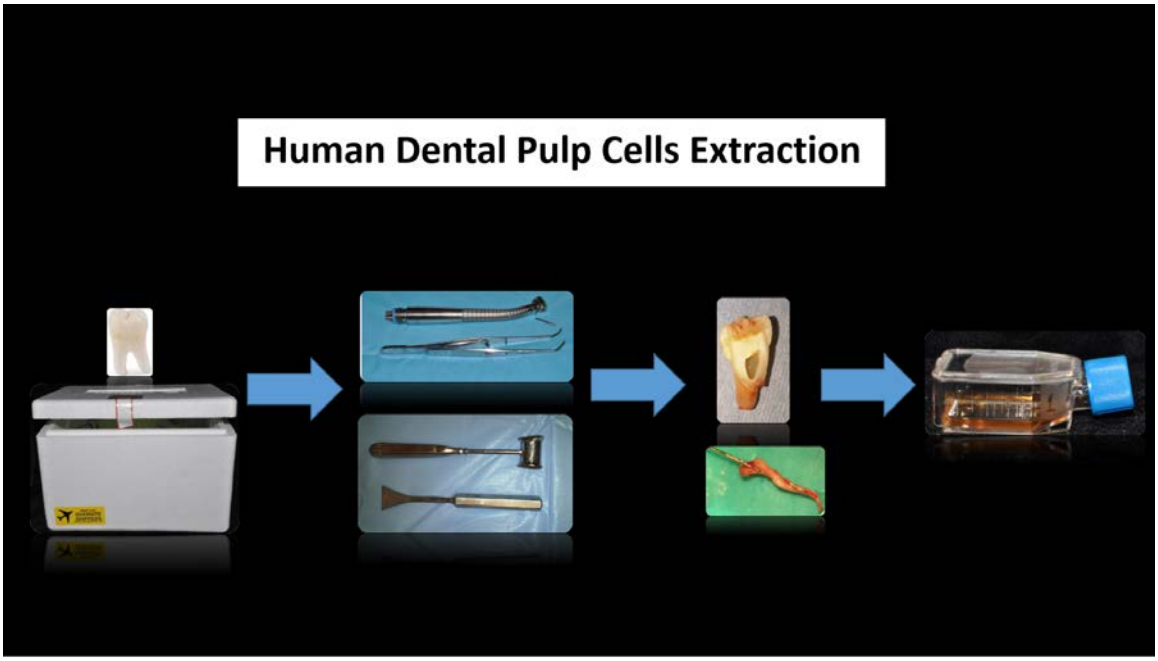


Photo 1: Steps of hDPCs extraction.

Note: The picture presents the process of extracting hDPCs from the extracted teeth and the subsequent culture of the cells in appropriate culture media.

Pulp tissue were kept in the flask for the first two weeks to allow cell migration from the pulp tissue and attaching to the flask surface. After that time, pulp tissue were removed from the flask and discarded to allow the attached cells to have more space to grow on the flask surface and reduce the risk of microbial contamination. After 4-6 weeks the cell growth reached satisfactory confluence (70-80%). Culture media was aspirated, followed by rinsing with 1X PBS then PBS was aspirated. Using 1 mL of 0.05% Trypsin EDTA for 2-3 minutes at 37°C, the attached cells in the 12.5 cm^2 flask (Photo 2) were detached in order to transfer them to a large 225 cm^2 flask for the second passage expansion.

After ensuring of complete cell detachment from the flask surface using the light microscope, 10 mL of BME media was added blocking the trypsin activity. The cells re-suspended in the media were then collected in a sterile 50 mL disposable tube for centrifugation for 5 minutes with 1000 rpm at room temperature (photo 3). When the cell pellet in the bottom of the tube was formed after centrifugation, the solution above the pellet containing the trypsin was carefully aspirated.

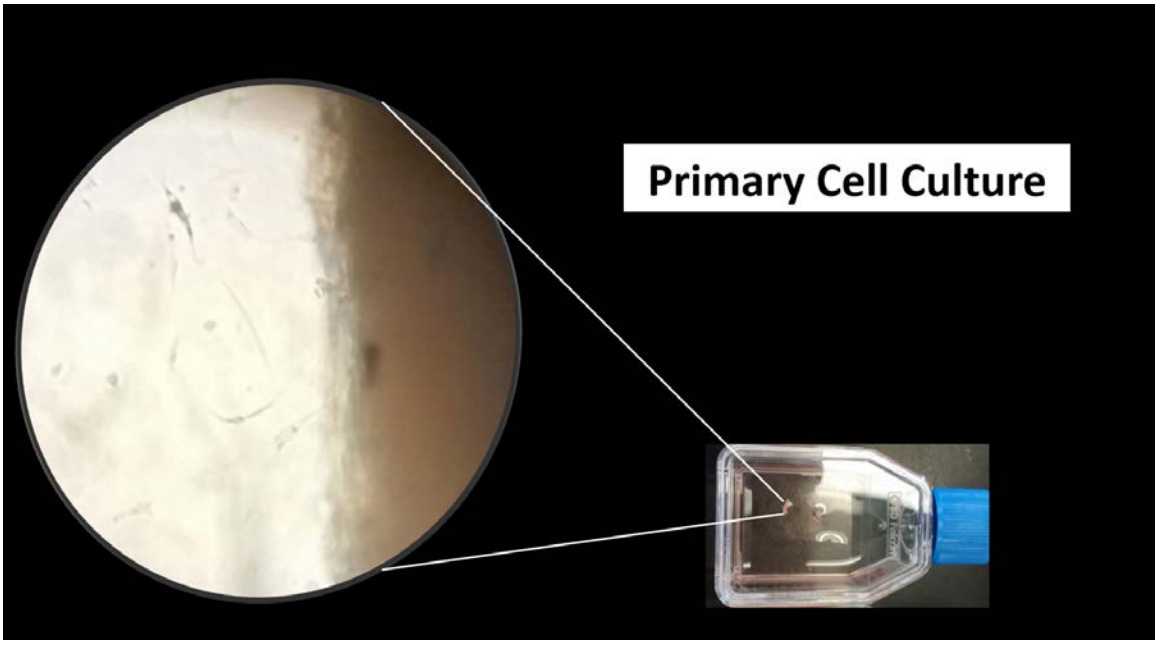


Photo 2: Display of primary cell culture in 12.5 cm^2 flask.

Note: Cells proliferated all around the pulp piece to reach 70% confluence in 3-4 weeks. Media was changed daily every day in the first week then every 3 days.

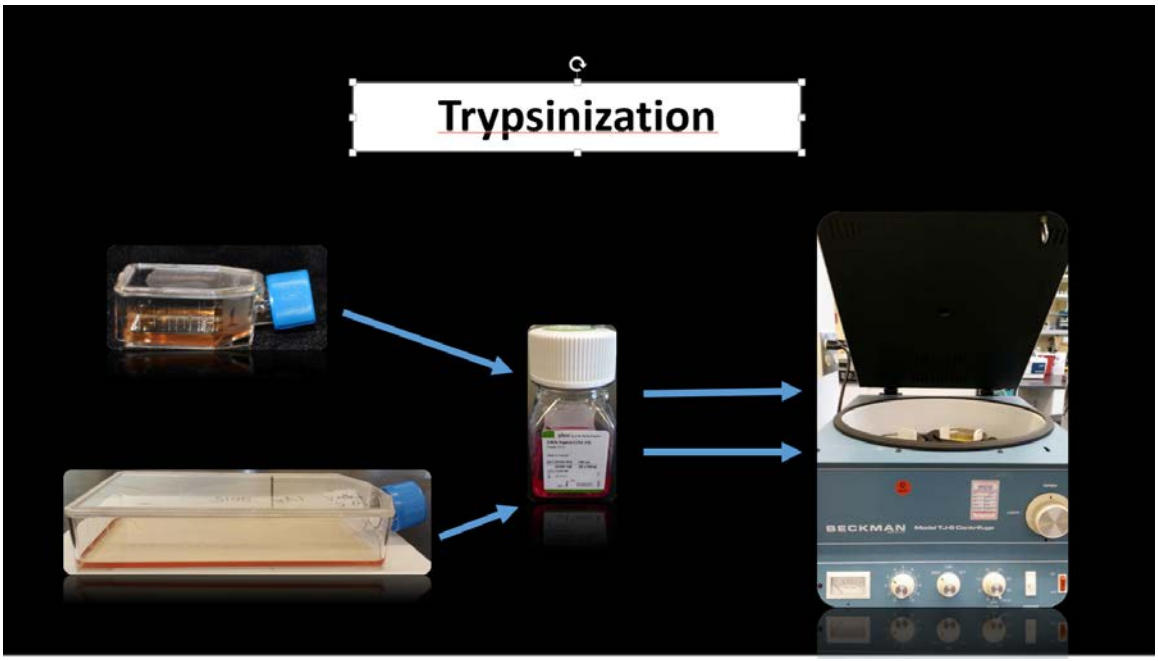


Photo 3: Trypsinization process.

Note: Trypsinization is a method to detach the cells from the flask wall where the cells float in suspention. Later on the suspended cells can be centrifuged to make a pellet of cells.

Finally cells were re-suspended in 10 mL fresh media and were then transferred in a new 225 cm^2 flask containing additional 60 mL of growth media. Same as for primary expansion cell culture, the growth media was changed every three days, until the cells reach confluence. The total volume of growth media required for the big flask is 60 mL and plates were washed with 20 mL PBS whenever changing with new media. This secondary cell culture takes about 2-3 weeks to reach a 70-80% confluence (photo 4). When cells again reached confluence, they were trypsinized and subsequently counted with hemocytometer under a microscope as described by Freshney (106). Cells suspended at 3 x 10^3 per ml and transferred to 24-well plates and cultured. At this stage, cells were immediately seeded in culture media with different biomaterial groups.

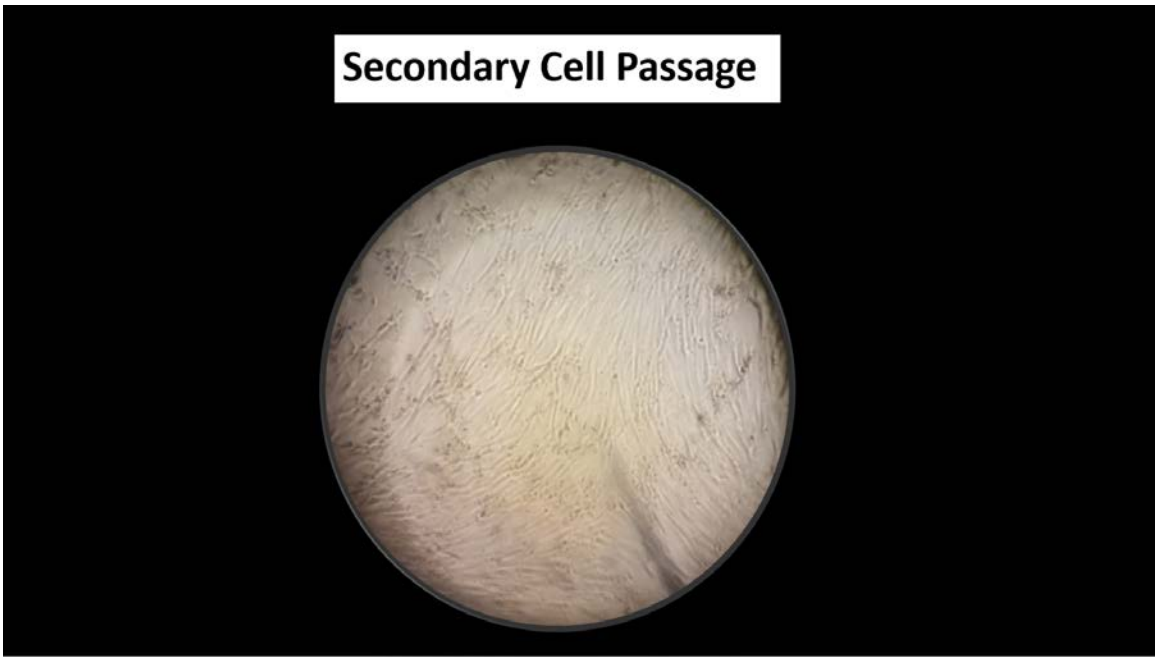


Photo 4: Secondary cell culture.

Note: Secondary cell passage was obtained after 10-14 days. Media was changed every 3 days after 7 days. This photo represents 70-80% confluence.

Determination of the Optimal Cell Seeding Concentration

A pilot study was done to determine the optimal seeding cell concentration for normal human dental pulp cells proliferation, differentiation and mineralization experiments in order to reach the results at 21 days without cell death. This was achieved through testing multiple seeding cell concentrations on 24 wells culture plates (Photo 5).

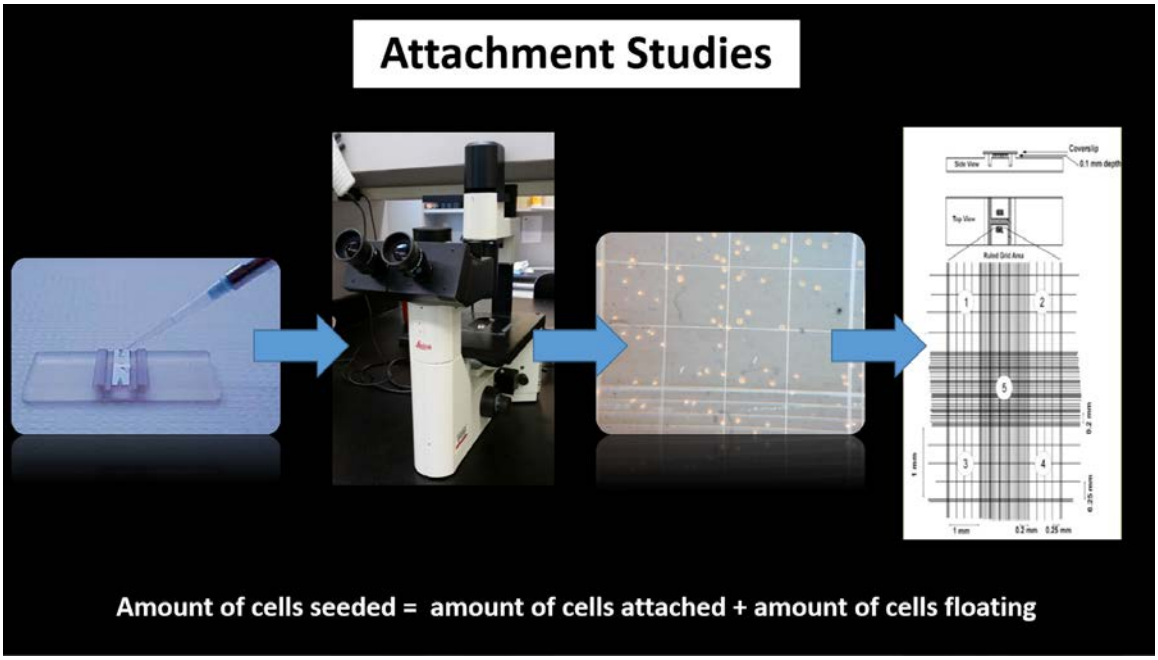


Photo 5: Hemocytometer.

Note: A hemocytometer was used to count cells under the light microscope

Groups of Materials

Pure silica gel (Silicon Dioxide SiO_2) was used to prepare the silicon supplement. Calcium chloride was used for the calcium supplement. Sodium phosphate, dibasic, anhydrous was utilized for the phosphorous supplement. Eleven containers were prepared by adding Si, Ca and/or P to 500 mL culture media (CM) each (Photo 6). Under sterile conditions, the eleven groups of materials were supplemented as follows:

1. CM with Si 25 ppm ion supplementation.
2. CM with Si 25 ppm and Ca 8.3 ppm ion supplementation.
3. CM with Si 25 ppm, Ca 8.3 ppm and P 4.16 ppm ion supplementation.
4. CM with Si 50 ppm ion supplementation.
5. CM with Si 50 ppm and Ca 16.7 ppm ion supplementation.
6. CM with Si 50 ppm, Ca 16.7 ppm and P 8.3 ppm ion supplementation.
7. CM with P 2 ppm ion supplementation.
8. CM with P 4 ppm ion supplementation.
9. CM with P 5 ppm ion supplementation.
10. CM with P 8 ppm ion supplementation.
11. Control (CM with no extra ion supplementation).

Silicon was sterilized via autoclaving prior to addition. Media containing any combination of Calcium or Phosphorus were sterilized by 0.2 µm filtration.

Photo 7 and 8 demonstrate the summary of the experimental design (Photo 7, 8).

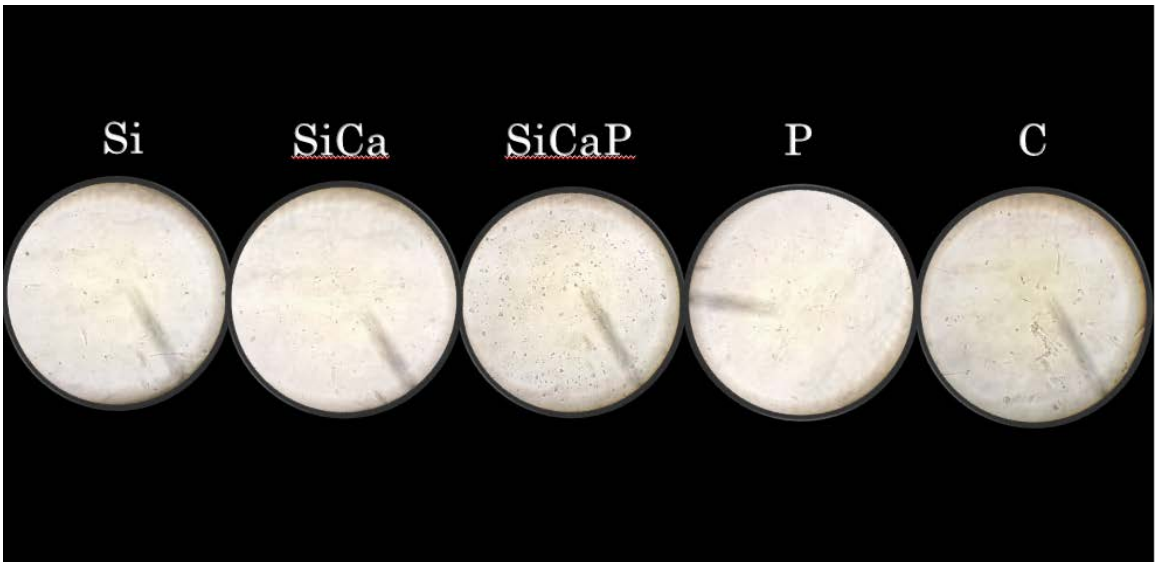
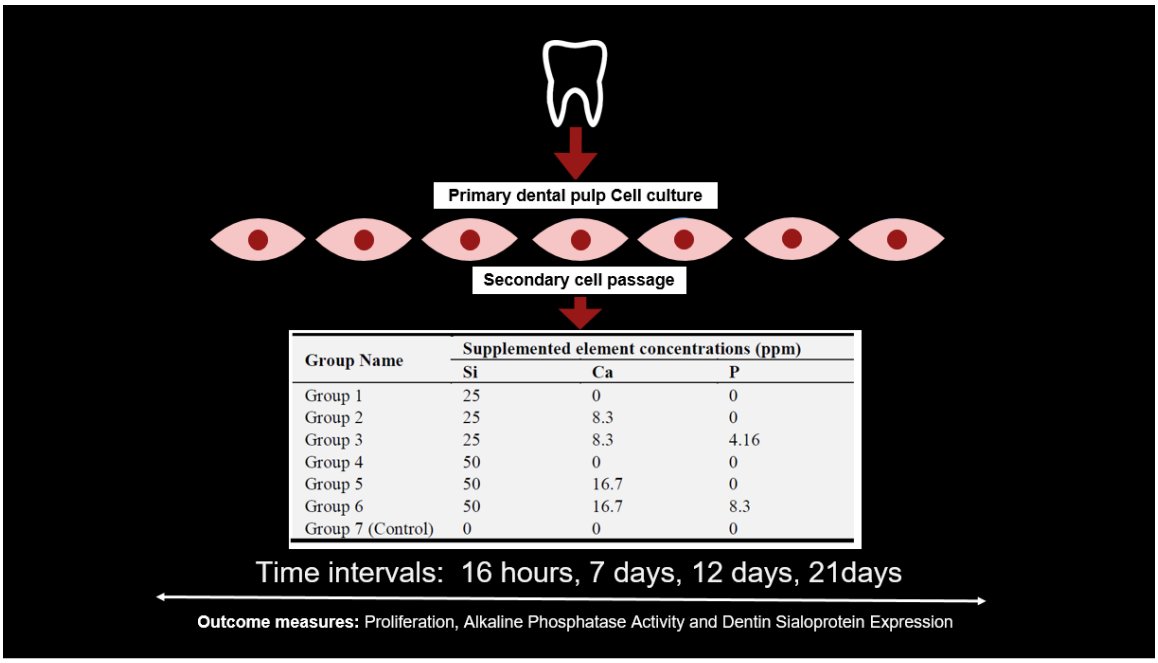


Photo 6: Cell presentation at day 5 with different material groups.

Note: This is the cell presentation under the microscope after addition of different material groups to the culture media.



Group Name	Supplemented element concentrations (ppm)		
	Si	Ca	P
Group 1	25	0	0
Group 2	25	8.3	0
Group 3	25	8.3	4.16
Group 4	50	0	0
Group 5	50	16.7	0
Group 6	50	16.7	8.3
Group 7 (Control)	0	0	0

Photo 7: Si, Ca and P experimental design.

Note: This is a summary of the experimental design used in the study.

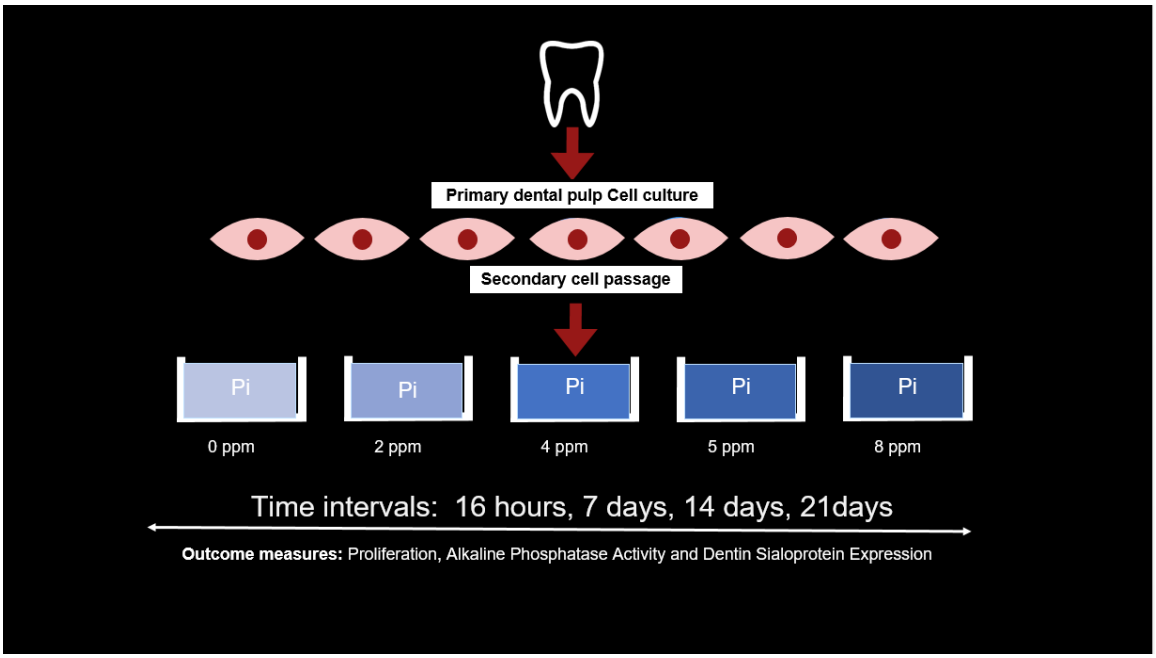


Photo 8: P experimental design.

Note: This is a summary of the experimental design used in the study.

Proliferation Rates by Crystal Violet Stain

Crystal violet stain has been applied to determine proliferation rates. Crystal violet dye is a toxic staining material and known in histological applications for staining bacteria, as well as staining cells to determine attachment to various surfaces. This dye stains the cell's DNA, and depending on the solubilization amount of dye taken up by the monolayer of cells attached on a surface, a relative optical density is quantified using a spectrophotometer. The dye is prepared by dissolving 0.2 g of crystal violet in 100 mL deionized water; the subsequent concentration is 0.2% (w/v) (107).

Three thousand cells were seeded in 24-wells containing 1 mL of growth medium with various groups as described previously. Each condition has been triplicated and growth medium was changed every 3 days. The culture plates were incubated at 37°C, 5% CO_2. For the silicon, calcium and phosphorus experiment, the cell proliferation was determined at time points of 7, 12 and 21 days. And again the same conditions applied for the phosphorus experiment except that cell proliferation rates were determined at time points of 7, 14 and 21 days.

At each predetermined time point, growth medium was removed and cells were fixed with 10% neutral buffered formalin for 1 hour at room temperature. Fixer solution was then removed, plates were washed twice with 1x PBS. Later, plates were stained with 1 mL of 0.2% (w/v) crystal violet stain for 1 hour at room temperature. Unbound stain was removed by rinsing plates thoroughly with deionized water until clear.

Then optical density value was measured by microplate reader (TECAN, Infinite 200 Pro) at 590 nm wavelength. Absorbance of crystal violet is directly proportional to cell

numbers (Photo 9). Two measurements were performed and mean cell numbers were calculated by a standard curve.

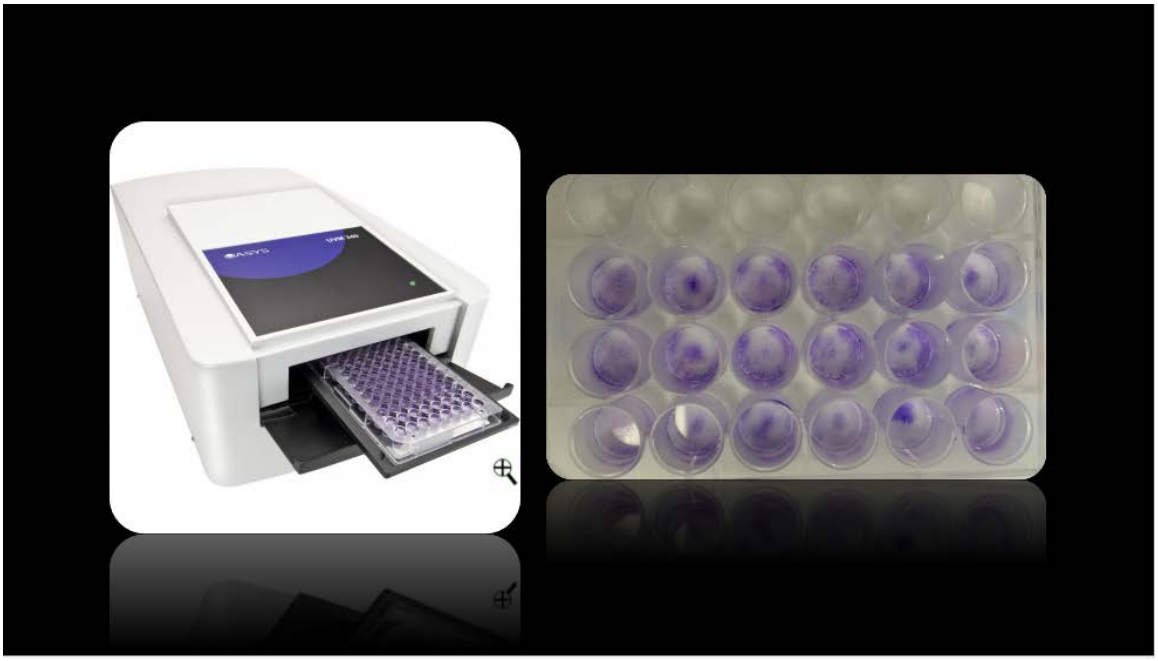


Photo 9: Microplate reader (TECAN, Infinite 200 Pro).

Note: Microplate reader measures the optical density value by absorbance of crystal violet dyed cells at a 590 nm wavelength.

Screening of Odontoblast Phenotypic Markers

For differentiation and mineralization studies, growth media were replaced with pre-odontogenic inductive medium for a total of 48-hours before each predetermined time intervals: for Si, Ca and P experiment it was at 12 and 21 days, and for the P experiment it was at 7, 14 and 21 days.

Pre-odontogenic media consists of 10% charcoal stripped fetal bovine serum FBS, 100 U/mL Penicillin G, 100 mg/mL Streptomycin, 10^{-8} M Menadione, 10 mM β-Glycerophosphate, 1.5 mg/mL L-ascorbic acid, and 2 mM L-glutamine all in a calculated volume of BME media. Under sterile conditions Si, Ca and P were added to the pre-odontogenic inductive medium of different material groups as described previously. At 24-hours before each predetermined time interval, cell culture medium were replaced by fresh pre-odontogenic inductive medium to which was added 10 nM VitD3 (Table 8). The supernatant fluid was collected at the predetermined days.

The ALP activity and DSP production were measured from the collected supernatants. The remaining fixed cells in culture plates were used to perform the mineralization assay. The odontogenesis phenotype was confirmed by adding VitD3.

Total volume (30ml)	Working solution	End concentration
Charcoal stripped FBS	3ml	10% (v/v)
Penicillin/Streptomycin antibiotic	0.3ml of stock (300uL) (100x, 10000U/ml)	1:100 (1x, 100 U/ml)
Menadione (vitamin K)	0.3ml of stock (300uL) (10^{-6} M, 1mM)	10^{-8} M
β-Glycerophosphate	3ml of stock (100mM)	10mM
L-ascorbic acid	1ml of stock (15mg in 10ml water)	1.5mg
L-glutamine	0.3ml (200uL)	2mM
Eagle's Basal Medium (BME)	22.1 ml	_____
1 , 25-Dihydroxyvitamin D3 (Vitamin D3)	_____	10 nM (1:100)

Table 5: Preparation of odontogenic media in total volume 30mL.

Note: Odontogenic media for day 4 and 18 was prepared without vitamin D3 and the same media for day 5 and 19 was prepared with vitamin D3.

Alkaline Phosphatase Assay Kit

The fluorometric alkaline phosphatase assay kit (Abcam) was employed to measure alkaline phosphatase activity in cell culture supernatants. This kit is an ultra-sensitive, direct and HTS-ready assay designed to measure ALP activity in serum and bio-samples with detection sensitivity ~1μU, which is more sensitive than colorimetric assays.

In the early stages of differentiation, the alkaline phosphatase enzyme get secreted in media upon stimulation with well-defined differentiation media. In its active form, the enzyme catalyzes the hydrolysis of phosphate esters in alkaline conditions. Secreted active functional alkaline phosphatase enzyme in the supernatants cleaved the phosphate group of the non-fluorescent 4-methylumbelliferyl phosphate disodium salt (MUP) substrate provided by the kit resulting in an intense fluorescent signal.

The ALP activity was measured according to the manufacturer's instructions. For each condition three samples were employed. In each of a 96 well black plate with clear bottom, an exactly 100 μL culture supernates were incubated with 20 μL of the non-fluorescent 4-methylumbelliferone phosphatase disodium salt (MUP) substrate; MUP was converted to the fluorescent 4-methylumbelliferone when dephospholated by active secreted ALP. After incubation for 30 minutes at 25°C in the dark, the reaction was stopped with 20 μL stop solution by gentle shaking of the plate. The emission was measured at 440 nm by excitation at 360 nm on a microplate reader (TECAN, Infinite 200 Pro). Three measurements for each value were performed for averaging and alkaline phosphatase activities were calculated by a standard curve and normalized to ALP activity on a per million cell basis.

Standard curve was created as follows:

A 50 µM MUP standard solution was prepared by diluting 5 µL of the 5 mM MUP substrate (standard) with 495 µL Assay Buffer. Standard curve dilutions as described in the table 4. were prepared and added duplicated into the 96 wells plate, each dilution has enough amount of standard to set up duplicate readings (2 x 120 µL).:

Standard #	Volume of Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End [MUP] in well
1	0	360	120	0nmol/well
2	6	354	120	0.1nmol/well
3	12	348	120	0.2nmol/well
4	18	342	120	0.3nmol/well
5	24	336	120	0.4nmol/well
6	30	330	120	0.5nmol/well

Table 6. Preparation volumes of standard curve dilutions for ALP assay

After triplicated reading of absorbance for all the standard curve dilution and subtraction of the mean of the absorbance value of the blank, the corrected bsorbance values for each standard as a function of the final concentration of 4-MU was plotted. The best smooth curve through these points to construct the standard curve was drawn with an R value higher than 0.99. Same protocol was utilized later to the sample readings to achieve the corrected absorbance. Sample readings were applied to the standard curve to get the amount of 4-MU generated by ALP sample.

Concentration (mU/mL) of alkaline phosphatase in the test samples is calculated as:

ALP activity = B/(V/T) * D, where B is amount of 4-MU generated by samples (in nmol), V is volume of sample added in the assay well (in mL), T is reaction time (in minutes) and D is the sample dilution factor.

Dentin Sialoprotein ELISA Assay

Dentin sialoprotein is a protein that is expressed and released from the odontoblasts during late differentiation. Using the competitive Dentin Sialoprotein (BioAssay™) enzyme-linked immunosorbent assay Kit Human (US Biologicals), expression levels of secreted dentin sialoprotein were measured. The kit contains a 48-well microplate pre-coated with a monoclonal antibody that is specific for human dentin sialoprotein. Supernatant samples and standards were pipetted into the wells.

To begin with the assay, we reconstituted the wash buffer by adding 10 mL of warm wash buffer concentrate to 990 mL of deionized or distilled water and gently mixing to prepare 1000 mL of wash buffer. The substrate solution was prepared by mixing equal volumes of color reagents A and B. At first, samples were centrifuged for 15 minutes with 3000 rpm to remove cellular particulates or debris. Following the manufacturer's instructions, adding 100 µL of samples and standards to the pre-coated wells, then mixed with 10 µL of balance solution. Afterward plates were incubated with 50 µL of conjugate in each well of the microplate for 1 hour at 37ºC. Duplicate samples from each condition were always included.

After the incubation period, all wells were decanted and washed five times. Then wells were further incubated with 50 µL substrate A and 50 µL substrate B for 15 min in the dark at room temperature. Finally, 50 µL of stop solution was added, which turned the solution color from blue to yellow. The intensity of the solution color was measured spectrophotometrically at 450 nm in a micro-plate reader and was inversely proportional to the concentration of the dentin sialoprotein present in the samples. Three measurements

were performed for averaging and dentin sialoprotein concentrations were calculated by a standard curve and normalized to the DSP expression levels on a per million cell basis.

The standard curve was created as follow:

First of all, the average zero standard optical density was subtracted from all values. Then a standard curve was created by plotting the mean absorbance for each standard on the x-axis against the concentration of the y-axis, the best-fit curve was drawn through the points on a logit-log linear regression curve. The data was linearized by plotting the log of the human dentin sialoprotein concentrations versus the log of the O.D. on a linear scale, and the best fit line was determined by regression analysis.

Mineralization Assay by Alizarin Red S Staining

Alizarin Red S (ARS), is anthraquinone dye, which has been widely used to evaluate calcium deposits in cell cultures. It is a sensitive tool for the recovery and semi-quantification of ARS in a stained monolayer of cells. Mineralization is assessed by extraction of calcified minerals at low pH, neutralization with ammonium hydroxide, and colorimetric detection at 405 nm. This assay is more sensitive than the cetylpyridinium chloride (CPC) extraction method, improving the detection of weakly mineralizing monolayers.

Alizarin Red S staining solution was prepared by dissolving 2 g of Alizarin Red S in 100 ml distilled water, mixing, and adjusting pH to 4.1 - 4.3 with 0.1% NH_4OH. The dark-brown solution was filtered and stored it in the dark until use within a month.

This test was utilized only in the Si, Ca and P experiment, where the attached cells in the 24-well plates on day 12 and 21 were employed. Firstly, removed the crystal violet stain from the plates by adding Triton-X 1% (v/v) (900 μL), then placed the plates on a shaker with 150 rpm for 10 minutes at RT. Afterward, washed the plates with deionized water to the point where colorless.

Later, 1 mL of a 40mM Alizarin Red S pH: 4.2 (Sigma), was added to the cellular monolayer for calcium staining. Cells were incubated at room temperature in the dark for 20 min. Then the Alizarin Red S staining solution was removed and the monolayer was washed four times with 1mL of deionized water until clear. Finally, absorbance at 405 nm was measured three times by a microplate reader and stained background was subtracted from each sample. Each condition was normalized to the amount on a per million cell basis.

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS

Data are presented in means and standard deviations, and was calculated on a per million cell basis. For the Si, Ca and P experiment the means and standard deviations (SD) of cell attachment and proliferation percentages were calculated at 16 hours, 7, 12 and 21 days. Levels of odontogenic differentiation markers (Alkaline phosphatase and Dentin sialoprotein) and mineralization were calculated at days 12 and 21.

For the P experiment the means and standard deviations (SD) of cell attachment and proliferation percentages were calculated at 16 hours, 7, 14 and 21 days. Levels of odontogenic differentiation markers (Alkaline phosphatase and Dentin sialoprotein) were calculated at days 7, 14 and 21. All experiments were performed in triplicate.

Statistical analysis was performed using software JMP Pro 12 (ver. 13.1.0) in one-way variance statistical analysis with Tukey multiple comparison post hoc test to detect the statistical differences between the groups. Differences at $P \leq 0.05$ were considered statistically significant.

RESULTS

RESULTS

Attachment Efficiency

Human dental pulp cells attachment on a standard tissue culture dish surfaces with a diameter of 60 mm was measured at different time intervals from the time of seeding. These time points were 1, 3, 6, 9, 12 and 16 hours. Each plate per timeline was measured and mean values were calculated. A hemocytometer was used to count the floating cells and that value was subtracted from the seeding cell concentration. Then, the percentage of attached cells was calculated by dividing the number of cells counted at each timeline by the total number of cells seeded. Attachment efficiency was calculated to establish a baseline (at 16 hours) on normal behavior of human dental pulp cells in second passage of cell culture (Table 7).

Time interval	Human dental pulpal cells attachment efficiency
3 hours	87%
6 hours	90%
9 hours	93%
12 hours	96%
16 hours	98%

Table 7. HDPCs attachment efficiency %.

Note: This data represents human dental pulp cell attachment efficiency percentage at different time intervals from 3 hours to16 hours cultured on tissue culture dishes. (N=5 per time intervals).

In the Si, Ca and P experiment, to detect if addition of supplemented Silicon, Calcium and Phosphorous ions in the culture medium was affecting cellular attachment, three thousands normal human dental pulp cells were cultured in 24 well plates containing culture media supplemented with different combinations and concentrations, for a time period of 16 hours. It has been observed that within this time period, no cell doubling occurs. Therefore, possible cell number changes are attributed only to the ability of human dental pulp cells to attach on culture wells when grown in culture media containing Silicon, Calcium and Phosphorous in different ion concentrations.

Cell attachment efficiency of human dental pulp cells was not significantly affected by any of the supplemented ions concentration tested in this experiment when compared to the control containing culture media without additional supplemental ions. The differences are not statistically significant indicating that the seeded cell number is invariably similar between the different groups and the control (Figure 1.).

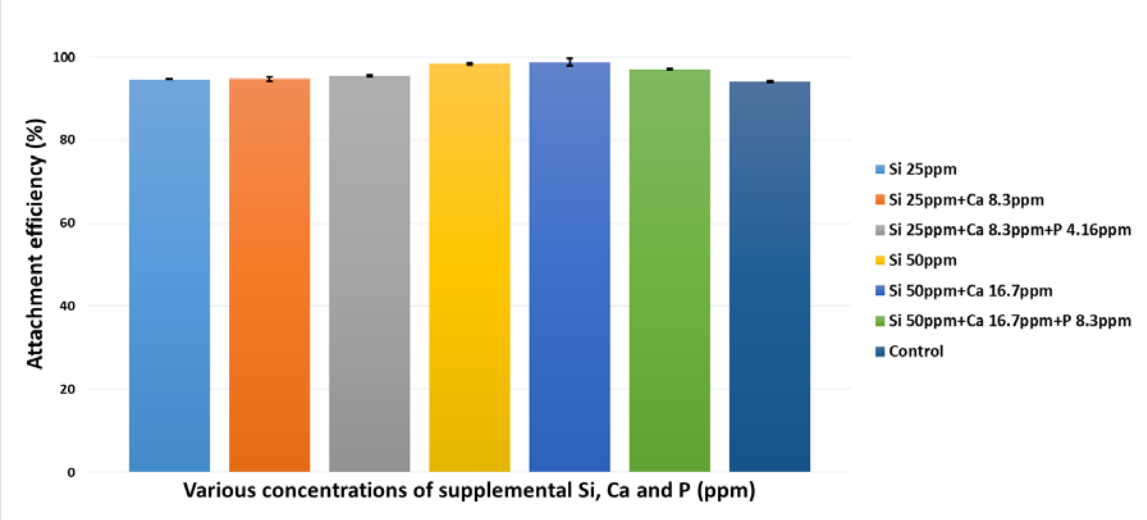


Figure 1. Human dental pulpal cells were cultured for 16 hours in media supplemented with various concentrations and combinations of Si, Ca and P. The control group treated with growth media without additional ions supplements. No significant difference was observed compared to the control in each condition (P=0.05).

*Error bars indicate the standard deviations of three replicates.

In the P experiment, to detect if addition of supplemented Phosphorous ions in the culture medium was affecting cellular attachment, three thousands normal human dental pulp cells were cultured in 24 well plates containing culture media supplemented with different concentrations, for a time period of 16 hours. It has been observed that within this time period, no cell doubling occurs. Therefore, possible cell number changes are attributed only to the ability of human dental pulp cells to attach on culture wells when grown in culture media containing Phosphorous ion in different concentrations.

However, cell attachment efficiency of human dental pulp cells was significantly affected by groups supplemented with 2, 4 and 5 ppm concentrations tested in this experiment when compared to the control containing culture media without additional supplemental ions. The differences are statistically significant indicating that cell attachment efficiency was down regulated by these three groups ($P<0.05$). (Figure 2.). Group of 8 ppm displayed no significant difference in comparison to the control group. Yet, the 8 ppm group exhibited higher cell attachment efficiency in comparison to the 2, 4 and 5 ppm P groups ($P<0.05$).

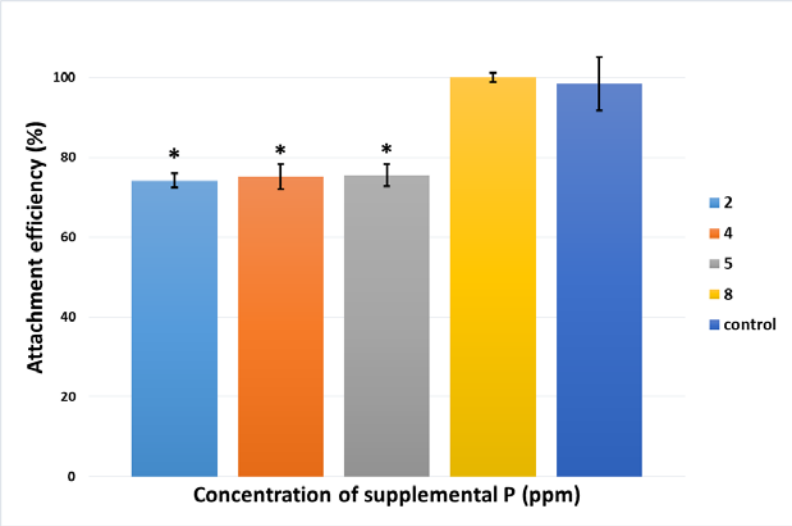


Figure 2. Human dental pulp cells were cultured for 16 hours in media supplemented with various concentrations of P. The control group treated with growth media without additional ions supplements. Significant difference was observed in groups of 2, 4 and 5 ppm supplemented groups compared to the control ($P<0.05$).

*Error bars indicate the standard deviations of three replicates.

Proliferation Rate Affected Si, Ca, and P at Various Concentrations

Number of cells grown in various supplemented media were determined by the crystal violet dye technique. In the Si, Ca and P experiment, three-thousands of human dental pulp cells were cultured in 24 well plates containing culture media supplemented with various combinations and concentrations of Silicon, Calcium and Phosphorous. Cell numbers were determined at days 7, 12 and 21 by using the optical density values measured from the spectrophotometer, values were converted to cell number. Cell proliferation rate was determined by normalizing the cell numbers at each time point with cell density at 16 hour of culture as a base line. The ratio of cellular crystal violet stain to cell number has been determined from the known seeding cell numbers in plates and measuring the corresponding dye stain from those plates two times using the same and different seeding numbers of cells. The ratio has been consistent and reproducible for all those measurements and therefore, it could be used to convert the optical density dye data to cell numbers with confidence.

Initially when the human dental pulp cells proliferated at day 7 (Figure 3.), the groups of Si 25 ppm+ Ca 8.3 ppm and Si 50 ppm+ Ca 16.7 ppm, proliferated faster than the control group ($P<0.05$), indicating that Silicon and Calcium combined could be beneficial for the human dental pulp cells growth. At day 12 (Figure 4.) the groups of Si 25 ppm+ Ca 8.3 ppm and Si 50 ppm+ Ca 16.7 ppm, again displayed a significant high results than the control group ($P<0.05$), confirming that Silicon and Calcium combined could be beneficial for the human dental pulp cells growth. At day 21 (Figure 5.), the groups of Si 25 ppm+ Ca 8.3 ppm and Si 50 ppm+ Ca 16.7 ppm still showing an enhanced

proliferation rates ($P<0.05$). At this time period, the control group showed higher proliferation rate than rest of the groups ($P<0.05$).

The trend of the affected results during the whole assay could be seen in (Figure 6.), which present the proliferation rate of normal human dental pulp cells with various supplements and concentrations of Silicon, Calcium and Phosphorus in folds, which were calculated by dividing the cell numbers at each time interval by the seeded cell numbers for each condition.

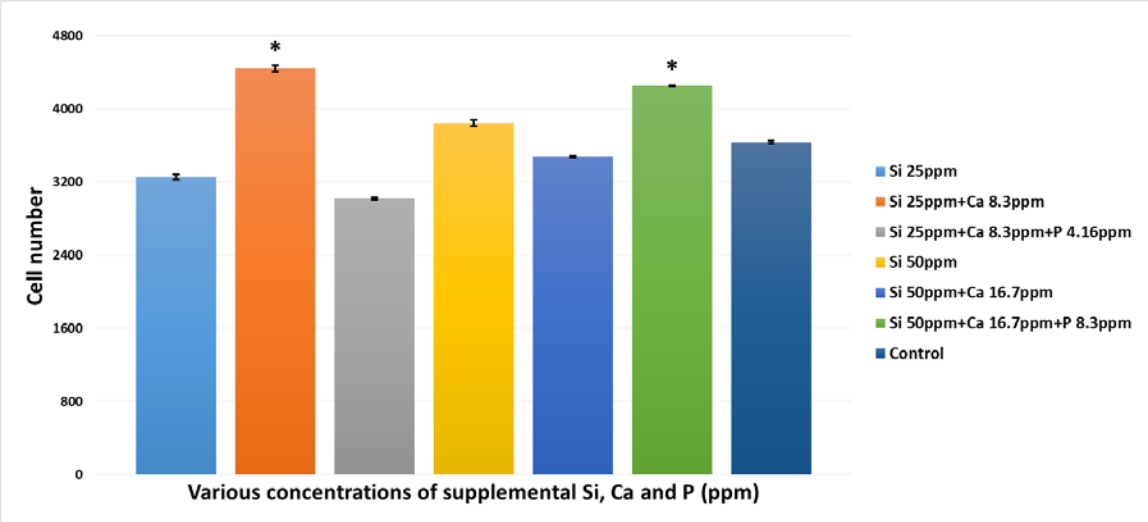


Figure 3. Proliferation of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 7 days. Cell number was calculated by using optical density measurement results dividing the constant number (per cell's optical density). The control cells were treated with growth media without additional ions supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

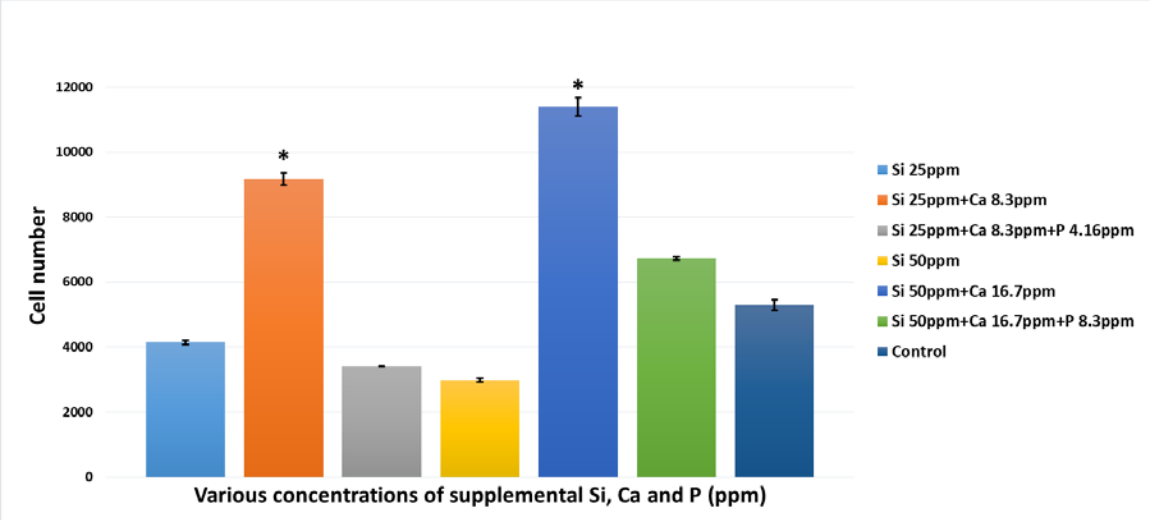


Figure 4. Proliferation of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 12 days. Cell number was calculated by using optical density measurement results dividing the constant number (per cell's optical density). The control cells were treated with growth media without additional ions supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

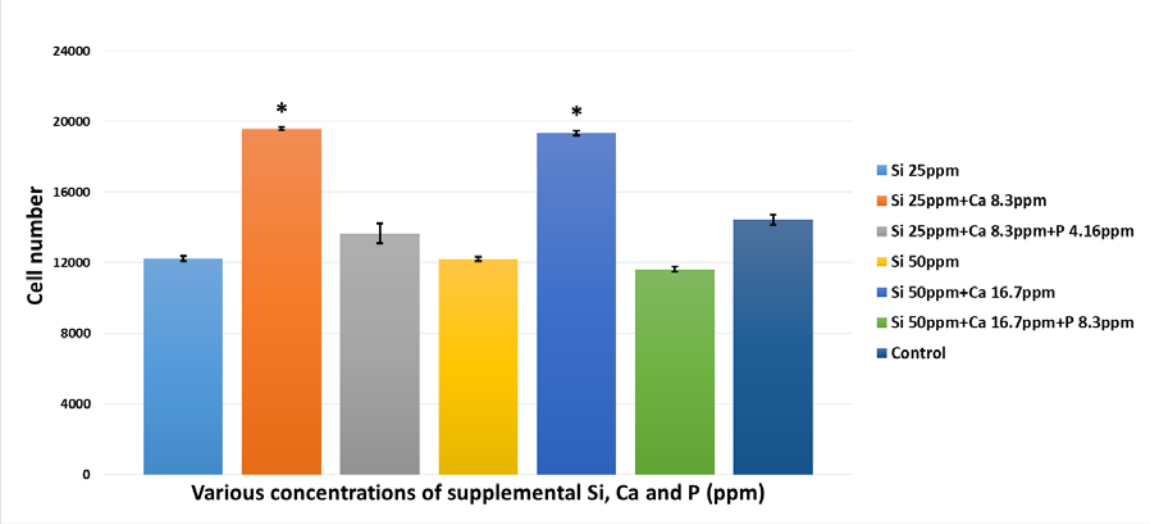


Figure 5. Proliferation of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 21 days. Cell number was calculated by using optical density measurement results dividing the constant number (per cell's optical density). The control cells were treated with growth media without additional ions supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

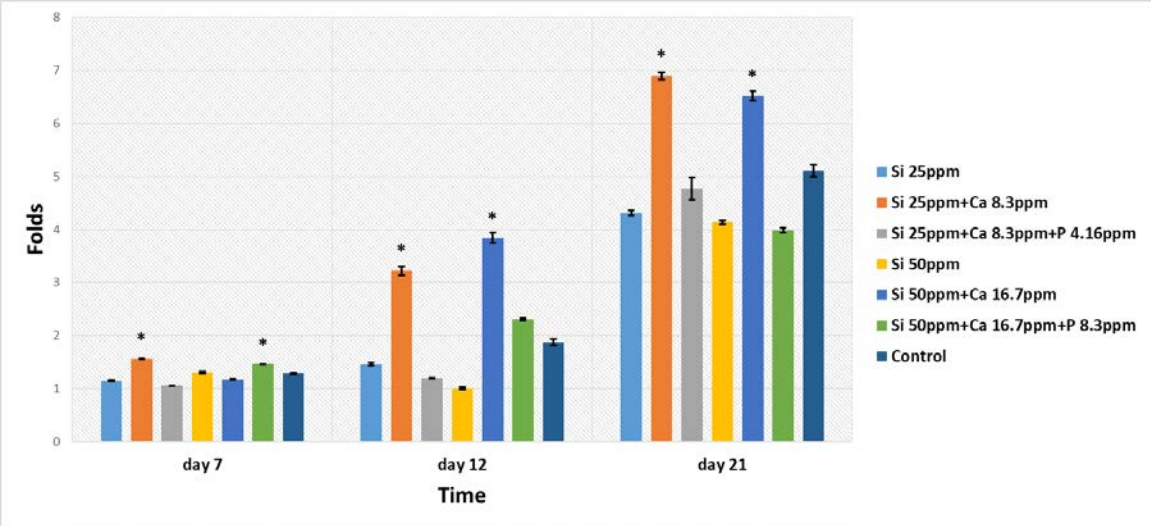


Figure 6. Proliferation of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 7, 12 and 21 days. Folds were calculated by dividing the cell numbers at each time interval by the seeded cell numbers for each condition. The control cells were treated with growth media without additional ions supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

Again in the P experiment, the same numbers of human dental pulp cells were cultured in 24 well plates containing culture media supplemented with various concentrations of Phosphorous. Cell numbers were determined at days 7, 14 and 21 by using the optical density values measured from the spectrophotometer, values were converted to cell number. Cell proliferation rate was determined by normalizing the cell numbers at each time point with cell density at 16 hour of culture as a base line. The ratio of cellular crystal violet stain to cell number has been determined from the known seeding cell numbers in plates and measuring the corresponding dye stain from those plates two times using the same and different seeding numbers of cells. The ratio has been consistent and reproducible for all those measurements and therefore, it could be used to convert the optical density dye data to cell numbers with confidence.

Initially when the human dental pulp cells proliferated at day 7 (Figure 7.), the P groups of 4 and 5 ppm, proliferated faster than the control group ($P<0.05$), indicating that Phosphorous could be beneficial for the human dental pulp cells growth. At day 14 (Figure 8.), the P groups of 2, 4 and 5 ppm, displayed a significant increase compared to the control group ($P<0.05$). The highest proliferation rate was attributed to P group of 5 ppm.

Confirming that Phosphorous could be beneficial for the human dental pulp cells growth. At day 21 (Figure 9.), the groups of 2, 4 and 5 ppm still showing an enhanced proliferation rates ($P<0.05$). And with highest proliferation rate was attributed to P group of 5 ppm. This implicate that P can be beneficial for the growth of human dental pulp cells.

The trend of the affected results during the whole assay could be seen in (Figure 10.), which present the proliferation rate of human dental pulp cells with various

concentrations of Phosphorus in folds, which were calculated by dividing the cell numbers at each time interval by the seeded cell numbers for each condition.

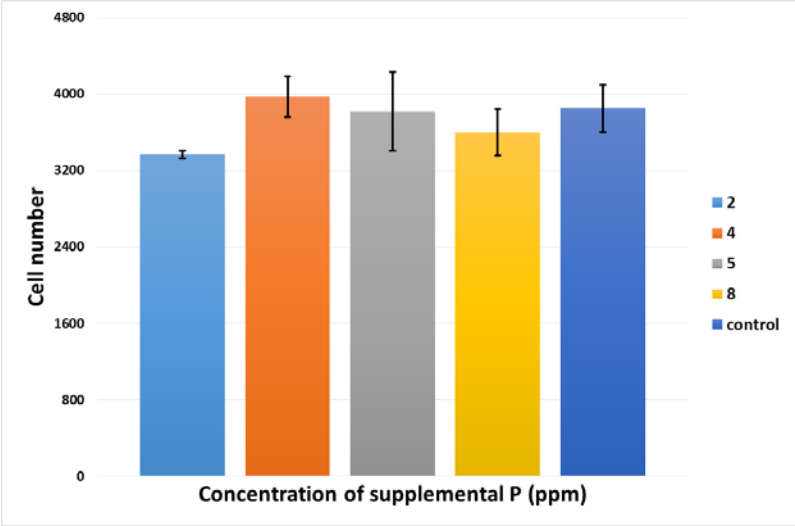


Figure 7. Proliferation of human dental pulp cells in media supplemented with various concentrations of P for a time period of 7 days. Cell number was calculated by using optical density measurement results dividing the constant number (per cell's optical density). The control cells were treated with growth media without additional ions supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

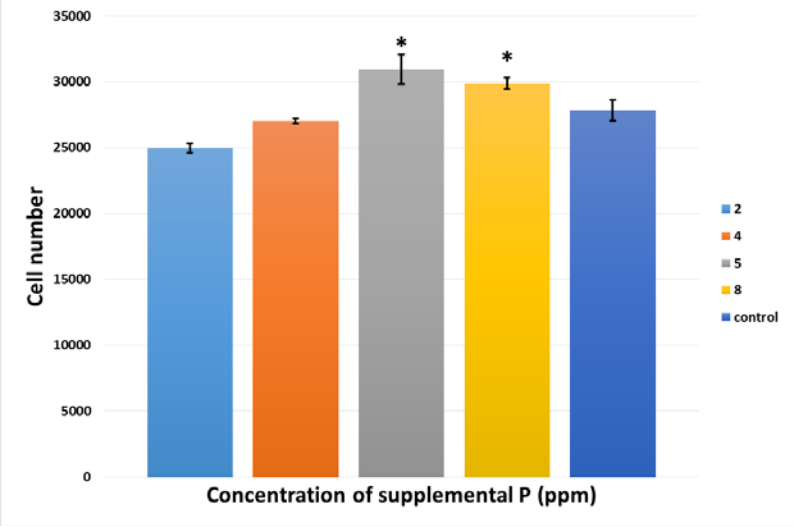


Figure 8. Proliferation of human dental pulp cells in media supplemented with various concentrations of P for a time period of 14 days. Cell number was calculated by using optical density measurement results dividing the constant number (per cell's optical density). The control cells were treated with growth media without additional ions supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

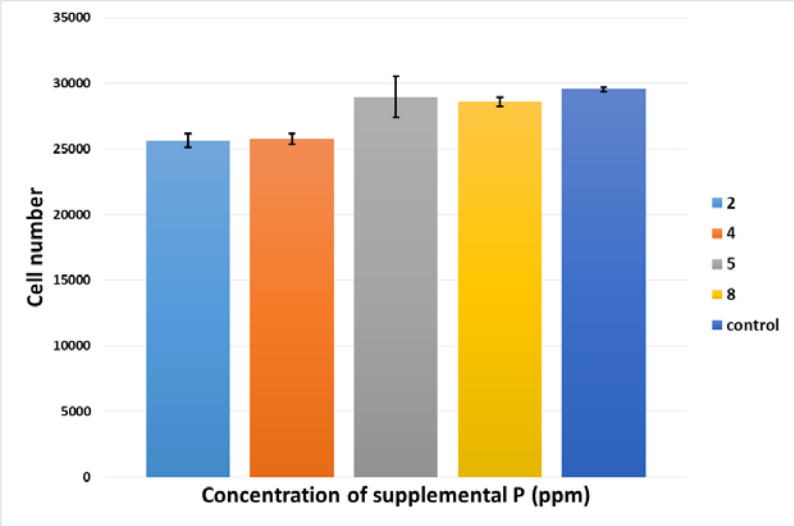


Figure 9. Proliferation of human dental pulp cells in media supplemented with various concentrations of P for a time period of 21 days. Cell number was calculated by using optical density measurement results dividing the constant number (per cell's optical density). The control cells were treated with growth media without additional ions supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

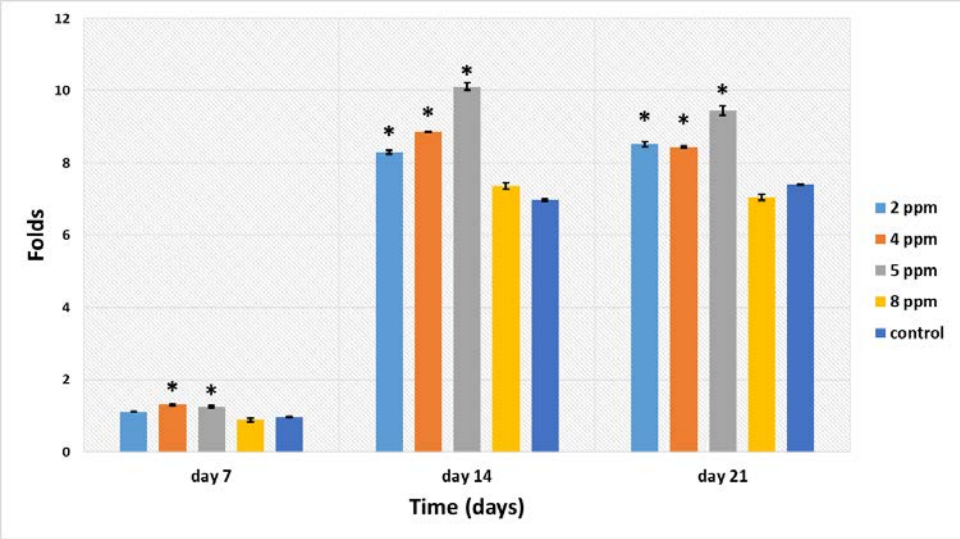


Figure 10. Proliferation of human dental pulp cells in media supplemented with various concentrations of P for a time period of 7, 14 and 21 days. Folds were calculated by dividing the cell numbers at each time interval by the seeded cell numbers for each condition. The control cells were treated with growth media without additional ions supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

ALP Activity Affected by Si, Ca, and P at Various Concentrations

Results observed from the Silicon, Calcium and Phosphorous experiment at day 12 (Figure 11.), showed a significant increase in ALP enzymatic activity when Si 50 ppm was added to the growth media. The control group displayed a higher results compared to all other groups. The same tendency was revealed at day 21 for the group of Si 50 ppm (Figure 12.), which showed significantly higher ALP activity than the other groups. And again, the control group displayed a higher results compared to all other groups.

The comparison of different time interval in the whole assay could be appreciated in (Figure 13.), which clearly showed the increasing of alkaline phosphatase activities of normal human dental pulpal cells with supplemental Si 50 ppm, However, on day 21, all groups exhibited lower activity values when compared to the values on day 12. ALP activity of Si 50 ppm group was statistically significant for day 12 and day 21 ($P<0.05$). Additionally, the control group demonstrated significantly more ALP production than the remaining other groups. The data were normalized per million cells at each time interval.

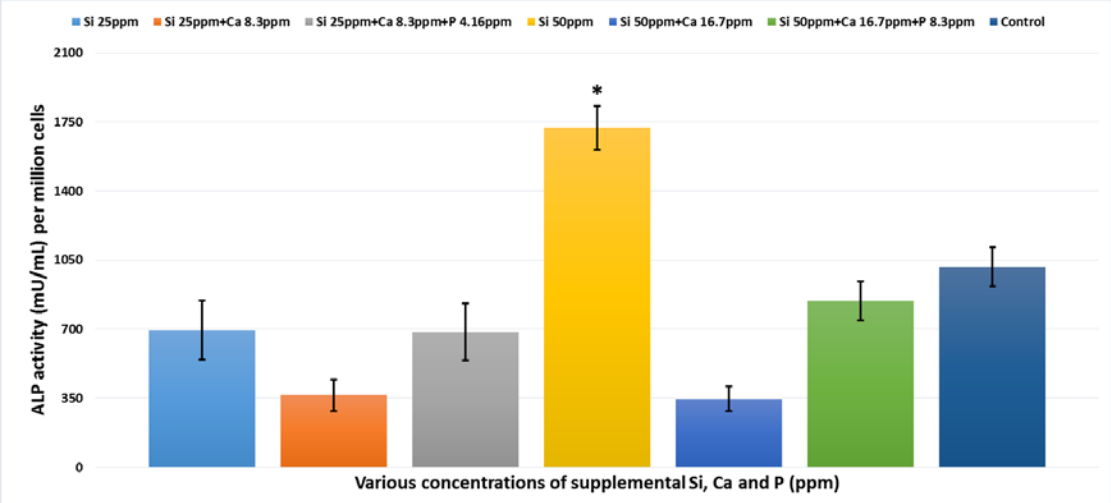


Figure 11. Alkaline phosphatase activity of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 12 days. Alkaline phosphatase activity in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

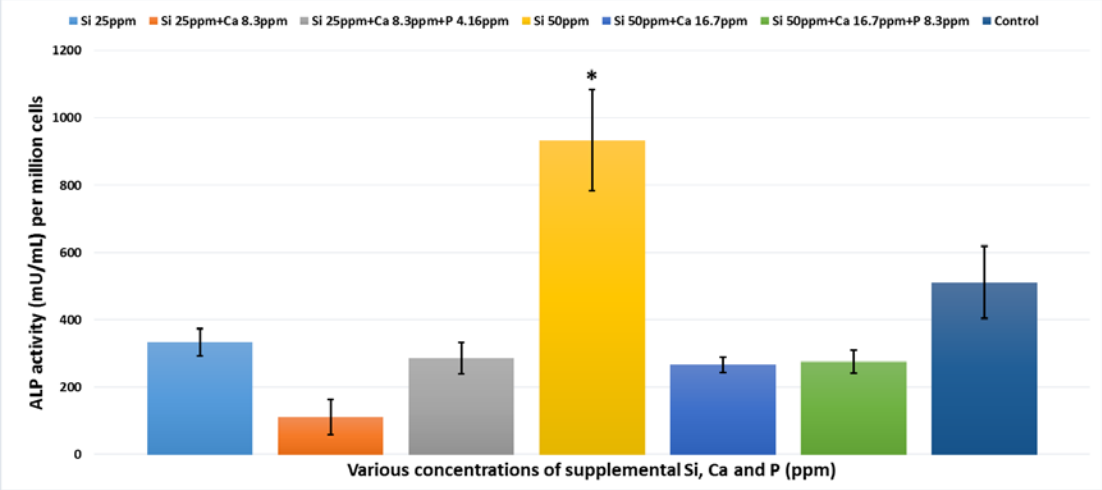


Figure 12. Alkaline phosphatase activity of normal human dental pulpal cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 21 days. Alkaline phosphatase activity in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($p<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

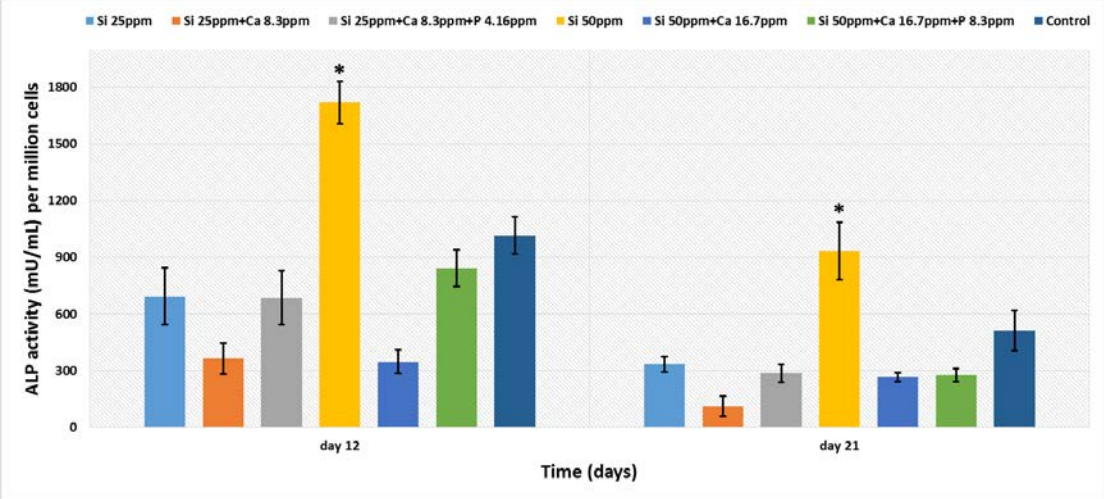


Figure 13. Alkaline phosphatase activity of normal human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time periods of 12 and 21 days. Alkaline phosphatase activity in supernatants as normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

Results observed from the Phosphorous experiment at day 7 (Figure 14.), showed significant increase in ALP enzymatic activity when P in all different concentrations were added to the growth media ($p<0.05$), compared to control group containing no supplements. The control group displayed a lower ALP activity results compared to all other groups. However, at day 14 the ALP activity in general drops to lower levels and the group of 4 ppm supplement is the only group showing significant value (Figure 15.), compared to the control group ($p<0.05$). At day 21, the ALP activity dropped even more and all groups tested were comparable to the control (Figure 16.), with no significant differences among all groups ($p>0.05$).

The comparison of different time interval in the whole assay could be appreciated in (Figure 17.), which clearly showed the increasing of alkaline phosphatase activities of normal human dental pulpal cells with supplemental phosphorous. However, on day 14 and 21, all groups exhibited lower activity values when compared to the values on day 7. ALP activity of 2, 4, 5 and 8 ppm groups were statistically significant for day 7 ($p<0.05$). The data were normalized per million cells at each time interval.

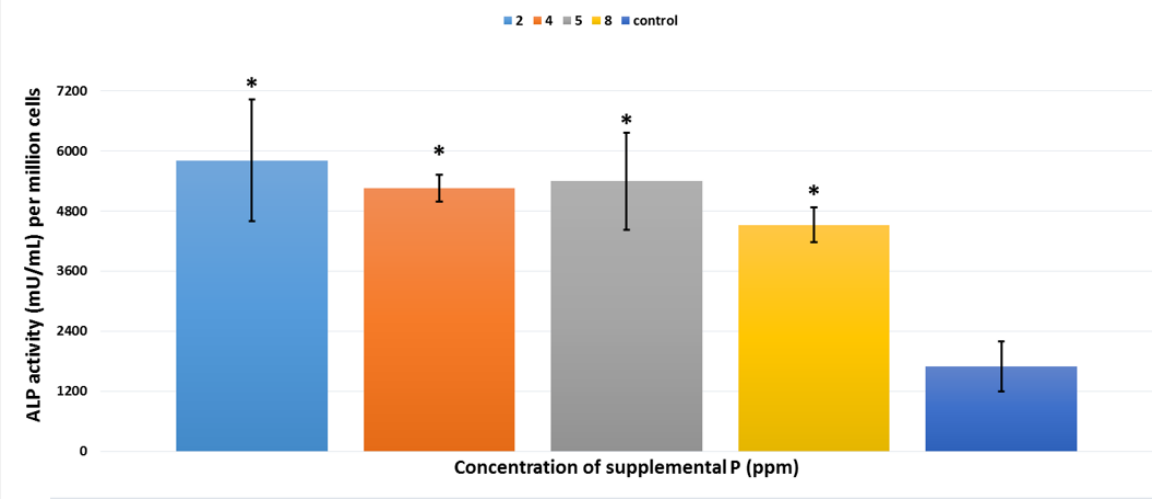


Figure 14. Alkaline phosphatase activity of normal human dental pulp cells in media supplemented with various concentrations of P for a time period of 7 days. Alkaline phosphatase activity in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

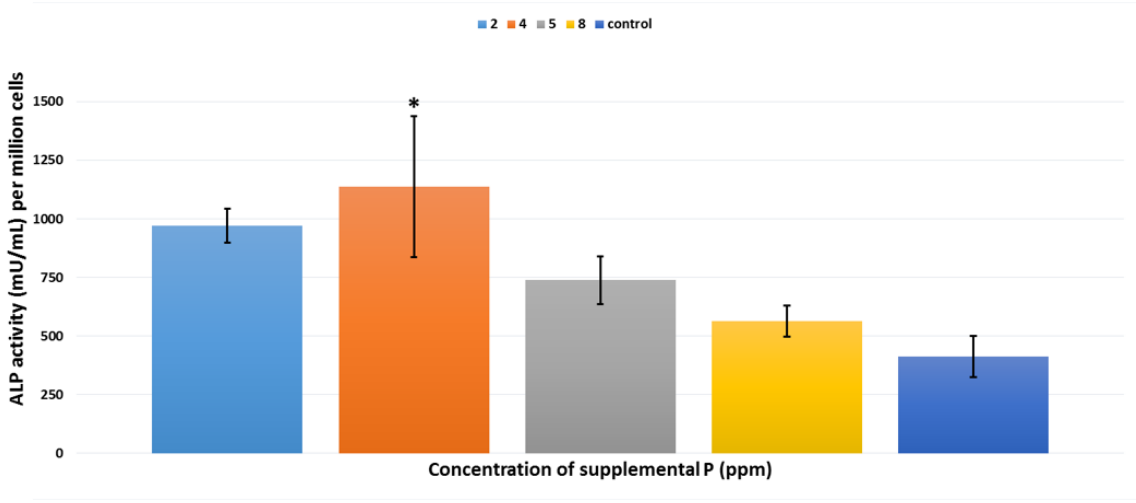


Figure 15. Alkaline phosphatase activity of normal human dental pulp cells in media supplemented with various concentrations of P for a time period of 14 days. Alkaline phosphatase activity in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

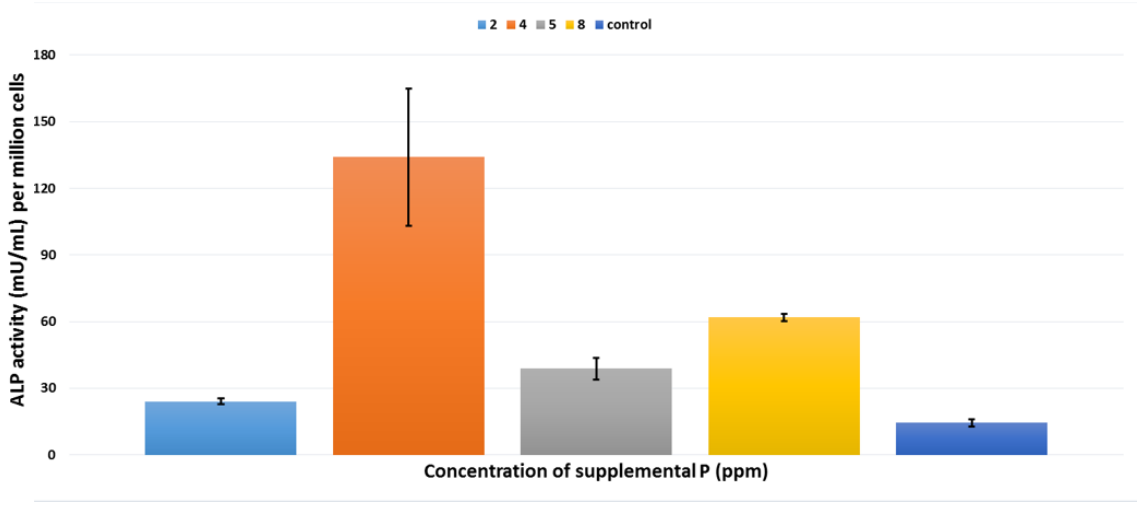


Figure 16. Alkaline phosphatase activity of normal human dental pulp cells in media supplemented with various concentrations of P for a time period of 21 days. Alkaline phosphatase activity in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

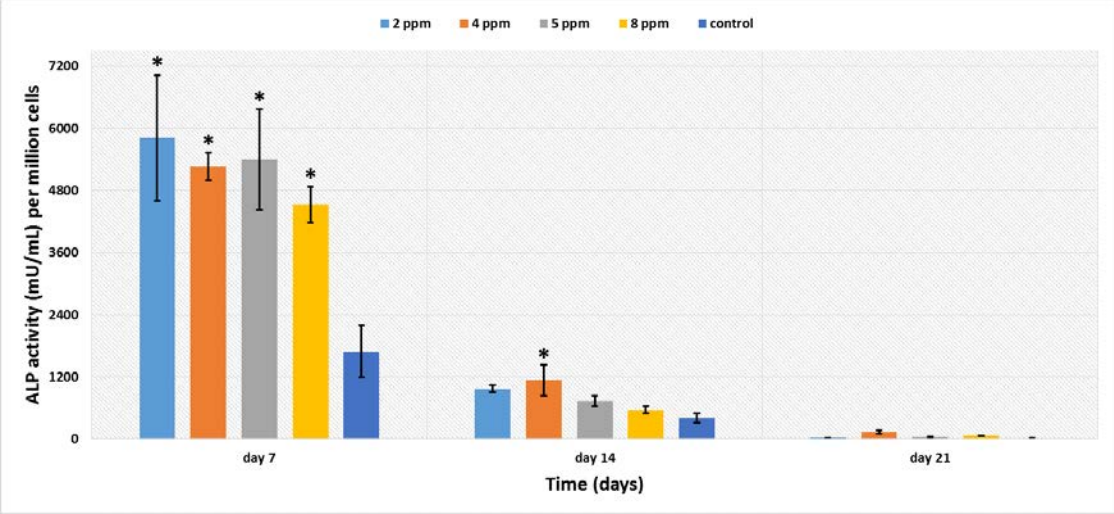


Figure 17. Alkaline phosphatase activity of normal human dental pulp cells in media supplemented with various concentrations of P for a time periods of 7, 14 and 21 days. Alkaline phosphatase activity in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

Dentin Sialoprotein Expression Affected Si, Ca, and P at Various Concentrations

Dentin sialoprotein expression is a specific indicator for odontogenic differentiation. In the Silicon, Calcium and Phosphorous experiment, the highest values of secreted DSP on day 12 were derived from cells exposed to supplemental Si 25 ppm, Si 25 ppm+ Ca 8.3 ppm+ P 4.16 ppm and Si 50 ppm ($p<0.05$) compared to the control (Figure 18.). At day 21 the highest protein production was detected in cell supernatant for the group exposed to Si 50 ppm which is the only group that displayed significant value ($p<0.05$) compared to the control (Figure 19.). All groups other than Si 50 ppm exhibit no significant differences between each other, and or to the control group. ELISA Assay was utilized to measure the secreted dentin sialoprotein in the hDPCs cultures.

Overall, Dentin sialoprotein expression was significantly increased on day 21 compared to its expression on day 12, which could be seen in (Fig 20.). The highest DSP amount measured on day 21 was expressed by the Si 50 ppm group.

The data collected on day 21 are similar to the ones observed for the same time period for alkaline phosphatase activity, indicating once more that addition of Si 50 ppm to culture media is sufficient to induce odontogenic differentiation.

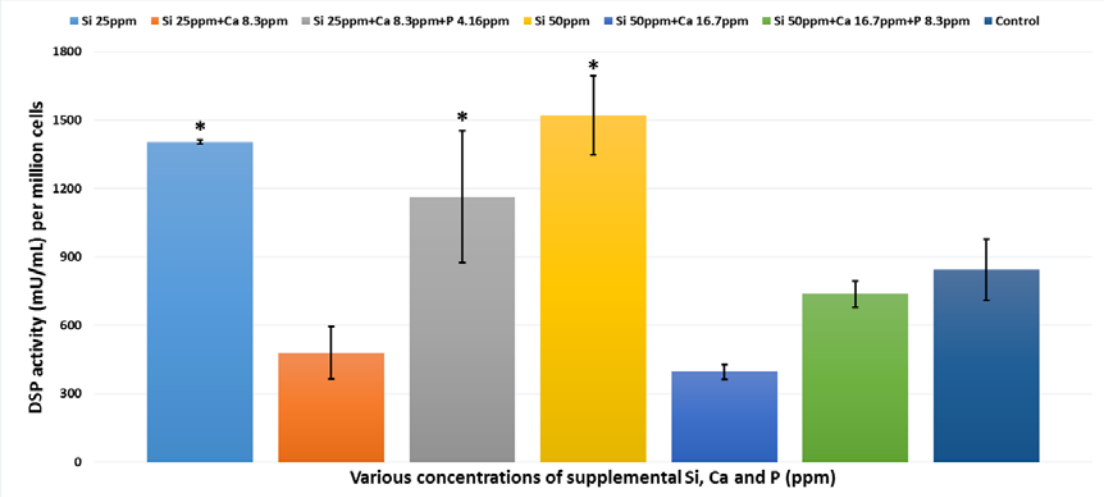


Figure 18. Dentin sialoprotein expression of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 12 days. Dentin sialoprotein expression in supernatant was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

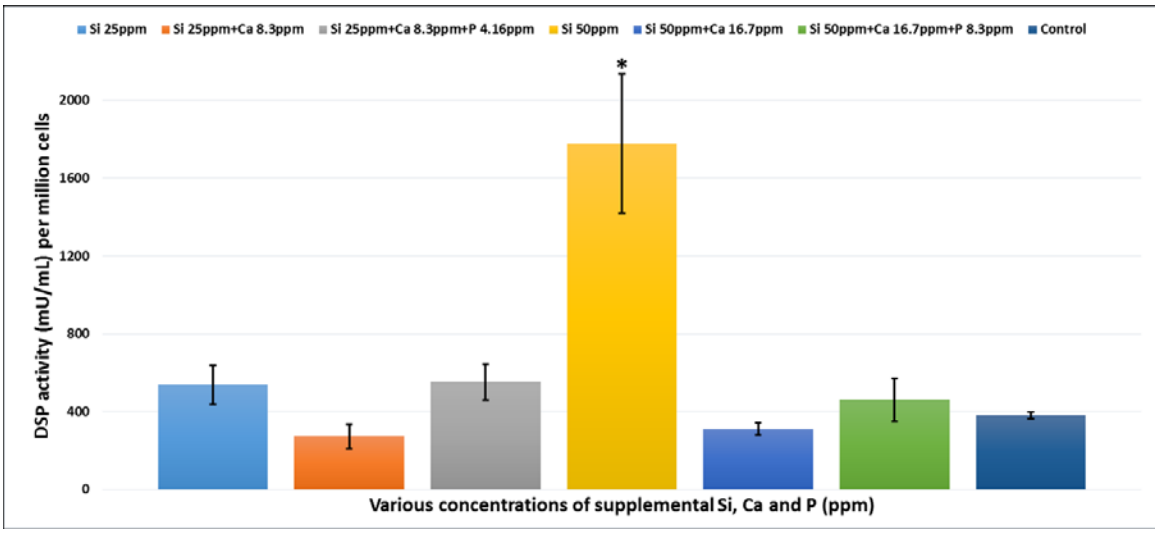


Figure 19. Dentin sialoprotein expression of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 21 days. Dentin sialoprotein expression in supernatant was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

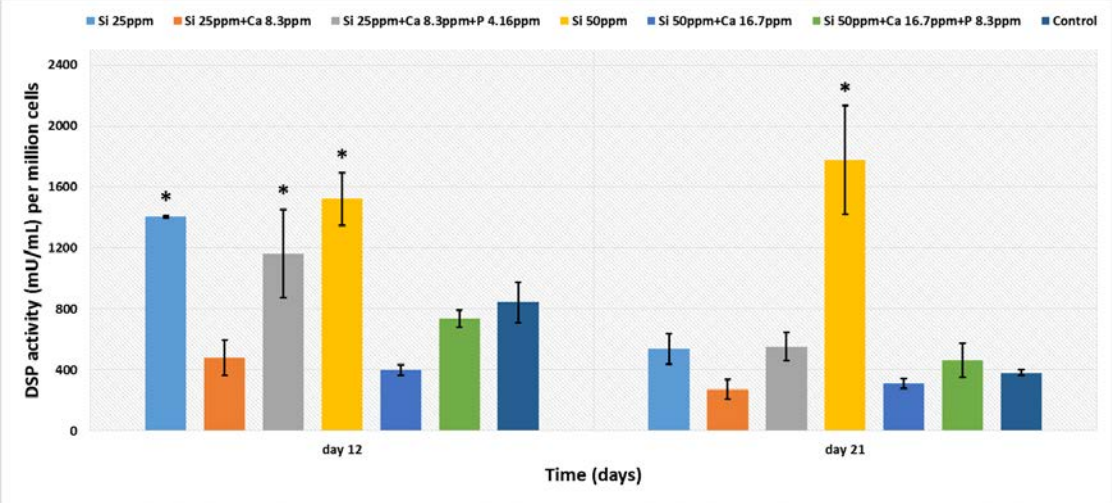


Figure 20. Dentin sialoprotein expression of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 12 and 21 days. Dentin sialoprotein expression in supernatant was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

In the Phosphorous experiment, the highest values of secreted DSP on day 7 were derived from cells exposed to supplemental P 5 ppm ($p<0.05$), compared to the control (Figure 21.). Protein production in the 2 and 4 ppm groups were also increased but displayed a comparable DSP expression levels as the control group. However, group of 8 ppm exhibited significantly lower value compared to the control group. As in the ALP activity test, at day 14 the DSP activity drops to lower levels and the groups of 2 and 4 ppm supplements are the only groups that displayed significant values (Figure 22.), compared to the control group ($p<0.05$). The DSP level dropped even more and the same trend continued also on day 21 (Figure 23.). Groups of 2 and 4 ppm supplements are the only groups that showed significant values compared to the control group ($P<0.05$).

Overall, Dentin sialoprotein expression was significantly increased on day 7 compared to its expression on day 21, which could be seen in (Figure 25.). The highest DSP amount measured on day 7 was expressed by the 5 ppm P group.

The data on day 7 are similar to the ones observed for the same time period for Alkaline phosphatase activity, indicating once more that addition of P to culture media up to 5 ppm is sufficient to increase the odontogenic differentiation and values 8 ppm inhibit the differentiation process.

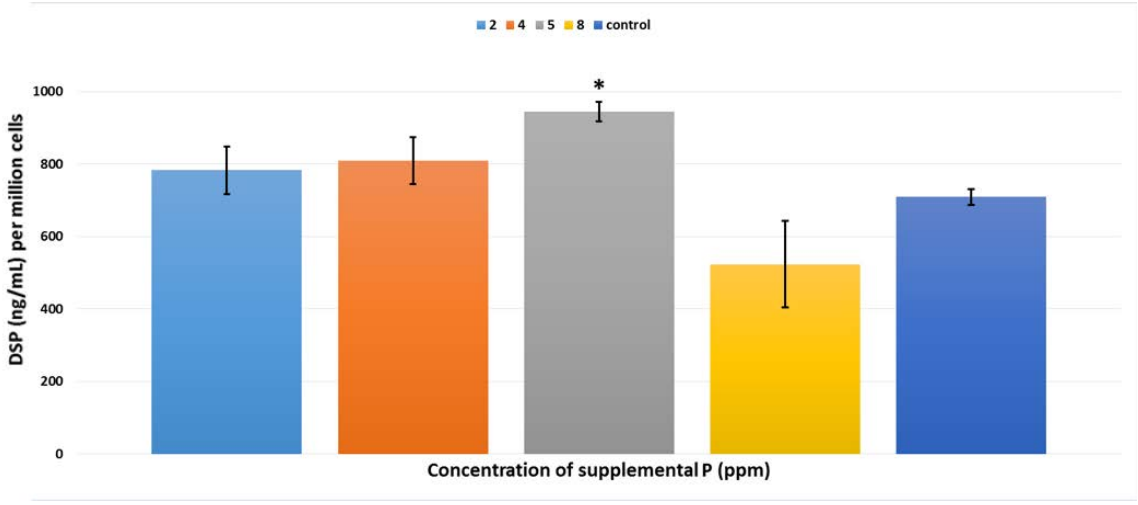


Figure 21. Dentin sialoprotein expression of human dental pulp cells in media supplemented with various P concentrations for a time period of 7 days. Dentin sialoprotein expression in supernatant was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

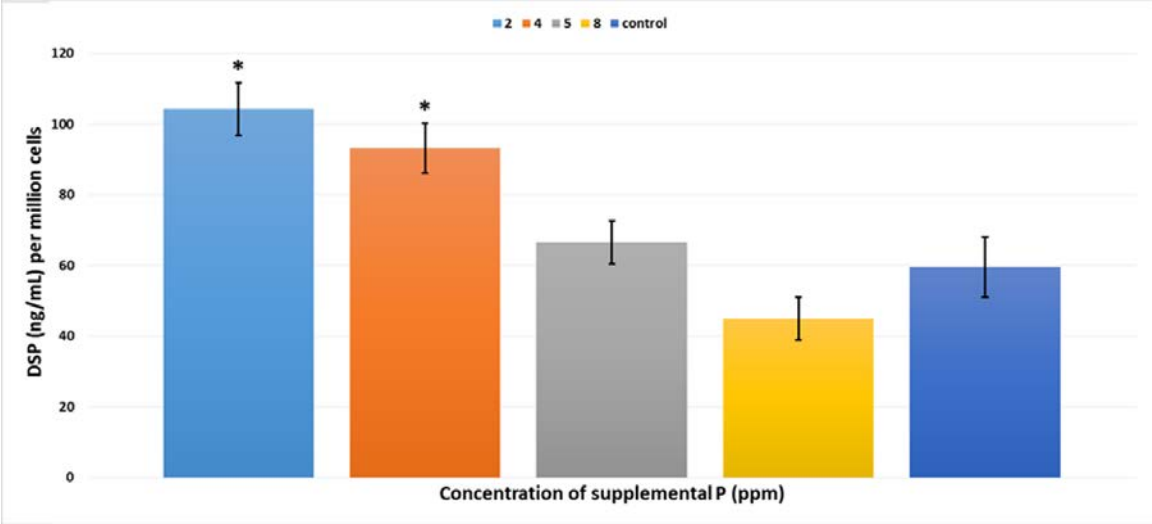


Figure 22. Dentin sialoprotein expression of human dental pulp cells in media supplemented with various P concentrations for a time period of 14 days. Dentin sialoprotein expression in supernatant was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

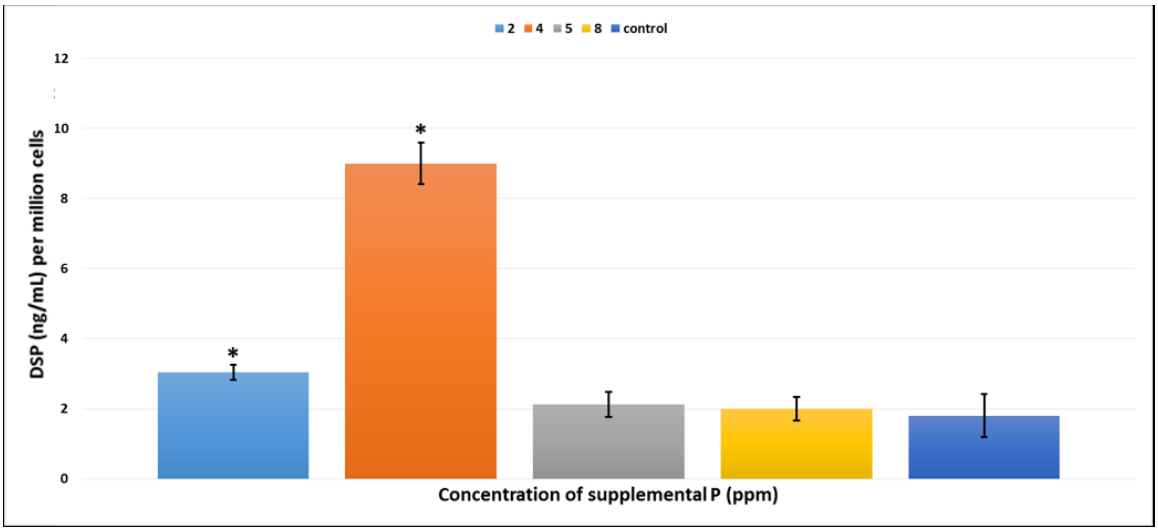


Figure 23. Dentin sialoprotein expression of human dental pulp cells in media supplemented with various P concentrations for a time period of 21 days. Dentin sialoprotein expression in supernatant was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

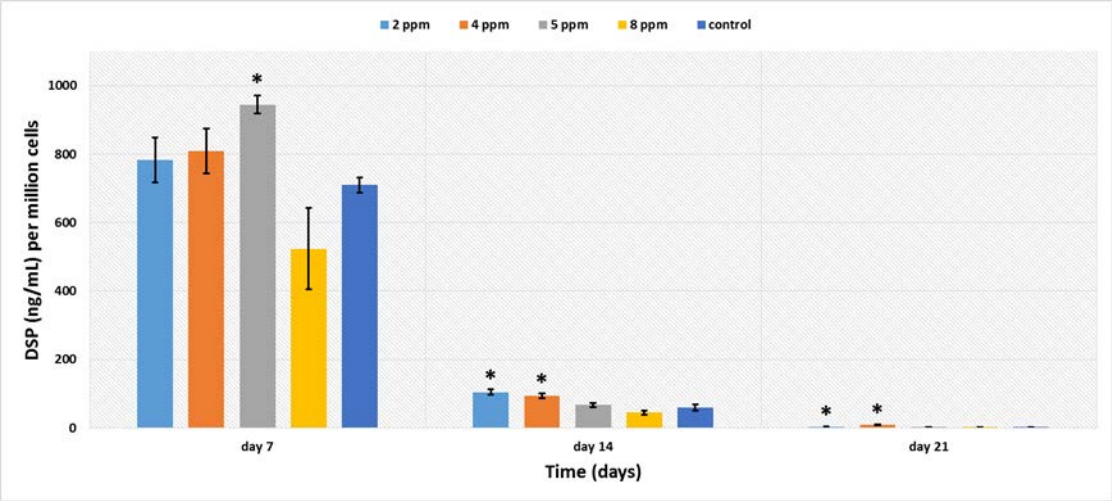


Figure 24. Dentin sialoprotein expression of human dental pulp cells in media supplemented with various P concentrations for a time period of 7, 14 and 21 days. Dentin sialoprotein expression in supernatant was normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

Mineralization Affected by Si, Ca, and P at Various Concentrations

Multiple biomarkers during the Silicon, Calcium and Phosphorous experiment had significantly increased expression of odontoblast differentiation. Mineralization of differentiating odontoblasts was measured by Alizarin Red S staining. Alizarin Red S staining showed data for bone nodule formation and calcium deposition. At day 14, the degree of mineralization recorded was highest in the groups of Si 25 ppm, Si 25 ppm+ Ca 8.3 ppm+ P 4.16 ppm, Si 50 ppm, Si 50 ppm+ Ca 16.7+ P 8.3 ppm ($p<0.05$) compared to the control group (Figure 25.). However, at day 21, data from the Si 50 ppm group presented higher mineralization when compared to the control (Figure 26).

To conclude the whole assay, (Figure 27.) illustrates the results during different time intervals. Eventually at day 21, Silicon 50 ppm clearly increased the area of calcified nodules up to 5.2-fold compared to the control group at day 21. There were no significant differences ($p>0.05$) between other groups. Again, confirming the odontogenic effect of the Si 50 ppm group on the cell culture of human dental pulp cells.

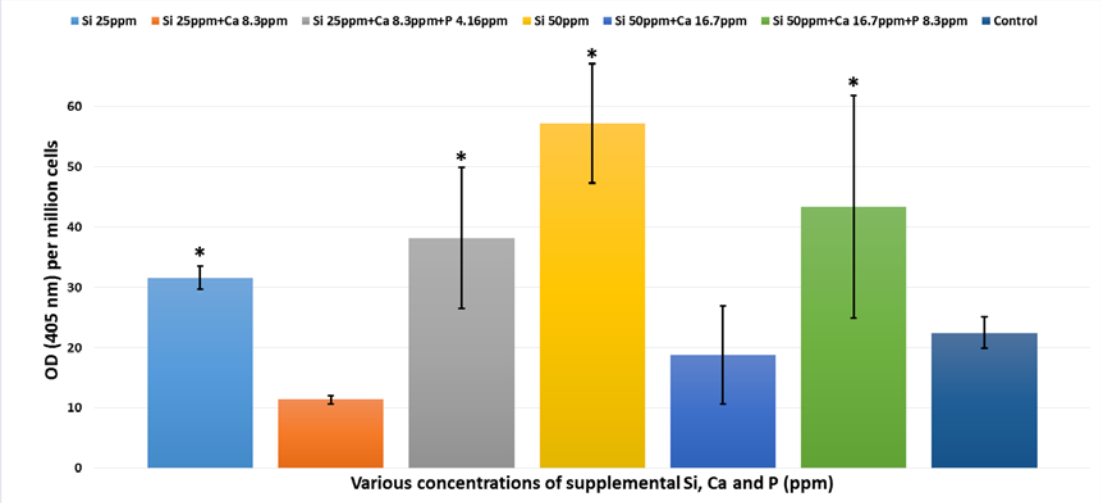


Figure 25. Mineralization of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 12 days. Mineralization of fixed cell samples was measured by spectroscopic analysis at 405 nm and normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

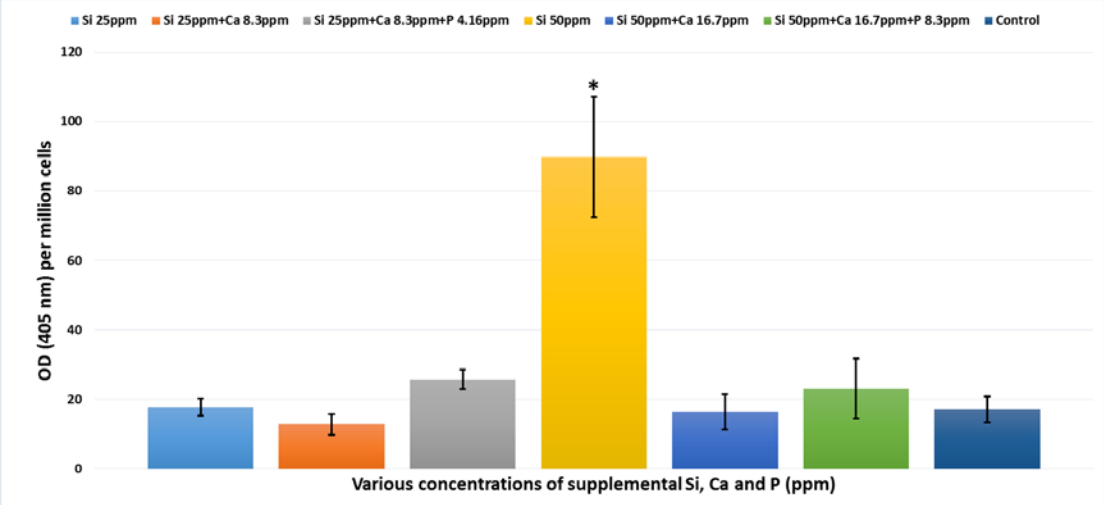


Figure 26. Mineralization of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 21 days. Mineralization of fixed cell samples was measured by spectroscopic analysis at 405nm and normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

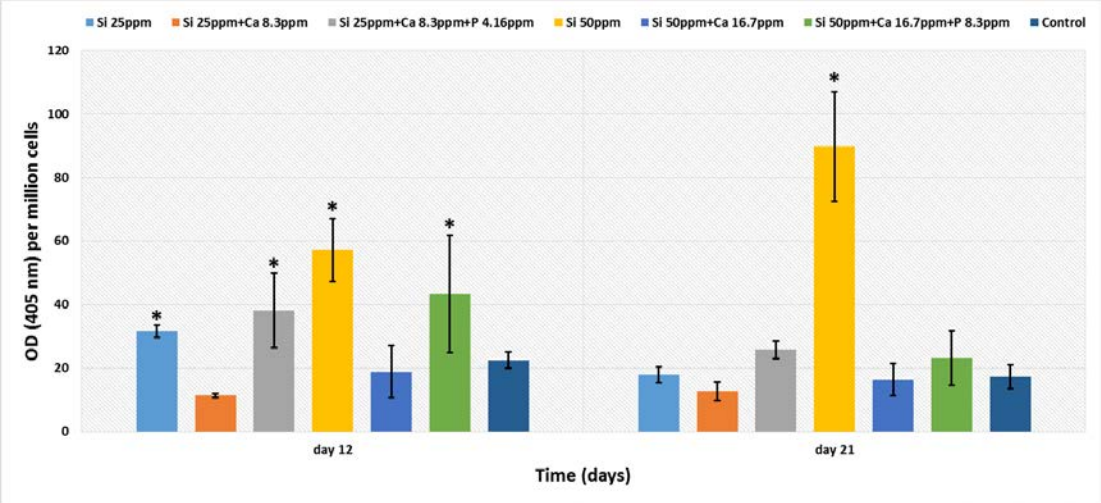


Figure 27. Mineralization of human dental pulp cells in media supplemented with various concentrations and combinations of Si, Ca and P for a time period of 12 and 21 days. Mineralization of fixed cell samples was measured by spectroscopic analysis at 405nm and normalized per million cells at each time interval. The control cells were treated with growth media without any supplements.

*Significant difference ($P<0.05$) compared to all groups at the specified time point. Error bars indicate the standard deviations of three replicates.

DISCUSSION

DISCUSSION

Dental pulp is a highly-complex sensory organ, which allows progenitor cells to partially compensate for loss of enamel or dentine caused by dental caries, iatrogenic sources or trauma. The progenitor cells will give rise to a new generation of odontoblasts that stimulate the healing process by producing reparative dentin (108). Calcium hydroxide was the gold standard pulp capping agent for several decades, due to its excellent antibacterial properties and its ability to form dentinal matrix (109-111). It facilitates formation of mineralized tissue over the pulp wound area. It does not, however, stimulate dentinogenesis per se (92, 112,113). Histologically, calcium hydroxide produces reparative dentine with tunneling defects which expose the pulp to bacterial penetration and infection over time (114-117). In addition, due to its high pH level, a foci of necrosis develops at the site of exposure (118). Mineral trioxide aggregate was initially used as a root-end filling material. Later, it was used for pulp capping, apexogenesis, restoration of root perforations, and several other procedures. In many studies, MTA has shown superior biocompatibility, decent sealing ability (90, 119,120) and exhibits a higher success rate than calcium hydroxide (121, 122). However, MTA undergoes major drawbacks in terms of -handling difficulty, long setting time, tooth discoloration and high cost (123). On the other hand, Biodentine demonstrated the same efficacy in pulp capping treatment as MTA (124, 125), but with a shorter setting time, easy handling properties and with less tooth discoloration (123). There are no studies so far that investigate the biocompatibility and mineralization of hDPCs in the presence of Si, Ca and P ions. Therefore, speculations about these materials might confer favorable

and perhaps better properties and bioactivity for pulp healing, similar to MTA/Biodentine.

In this study, individual ions and their mixtures were formulated to test their odontogenic potential on hDPCs. Alkaline phosphatase activity, dentin sialoprotein, and mineralization were selected as specific markers of hDPCs differentiation. Silicon studies on osteoblasts revealed enhanced proliferation rates and gene expression by influencing metabolism, bone formation and mineralization of normal human osteoblasts (126-128). In other osteoblast studies, Si promoted proliferation as measured by DNA and alkaline phosphatase activity (73, 129). Also, calcium ions in the extracellular area of bone tissue demonstrated significant effect on proliferation and differentiation (130). However, so far there are no reports that display the individual effect of Si and Ca ions on odontogenic cell cultures. In this study, the groups containing Si 25 ppm+ Ca 8.3 ppm and Si 50 ppm+ Ca 16.7 ppm demonstrated significant upregulation in cell proliferation when compared to the control group ($P<0.05$). Additionally, groups supplemented with Si alone or in addition with Ca and P decreased the proliferation rates compared to the controls.

Various studies demonstrated that the osteogenic activity in osteoblast cultures was improved upon by bioactive glass treatment (73, 129, 131- 134). The presence of increased levels of Si led to increased levels of ALP expression in human osteoblast cells (73). The results of this study, coincide with the osteogenic studies. They show that expression of ALP was significantly highest at Si 50 ppm concentration ($P<0.05$). Interestingly in our study the peak of ALP expression was reached at day 12, and declined when it reached day 21. Detecting an inverse relationship between proliferation and differentiation supports the

theory that if cells are more proliferative, the differentiation of the cells declines, and vice versa (89). And since ALP is present in the early stage of differentiation (136) it is speculated that the highest activity was reached at day 12 and declined afterward.

DSP is one of the markers of mature odontoblast cells and dentin production that is produced only by these cells and has not been found in other cells (50, 59). Dentin sialophosphoprotein (DSPP) is cleaved into DSP by the terminally differentiated odontoblasts to prompt mineralization and dentinogenesis (60). A previous study had observed higher levels of DSP expression in MTA-treated dental pulp tissue than in tissue treated with Biodentine. These findings illustrate the ability of MTA and Biodentine to induce new reparative dentin, but with MTA, production of dentin has better characteristics (60). The result of this study shows summiting of DSP levels at day 21. Si at 50 ppm supplement released the maximum amount in comparison with other groups and controls ($P<0.05$). This reveals that Si at 50 ppm has greater effect on inducing hDPCs differentiation into odontoblast-like cells than other groups. This finding is especially valuable because it clearly shows the stimulatory effect of Si on odontogenesis.

At the late stage of odontoblast differentiation, mineral deposition accumulates to form new dentine (137). The mineralization study at day 21 become similar to the one recorded from DSP expression. This effect is possibly caused by delayed mineralization, which could be explained by the prolonged proliferation and delayed differentiation (138,139). Apparently Si 50 ppm supplement has an up-regulating proliferative and odontogenic effect on hDPCs. Taken together, it can be concluded that Si 50 ppm

supplemented concentration would produce the most proliferation, differentiation and mineralization up-regulation.

Inorganic phosphate is an essential nutritional element supporting skeletal development, metabolic processes and energy production (140). Moreover, it serves as an important component of DNA, RNA, cellular membrane phospholipids and cell-signaling intermediate. The aim of the present study was to evaluate the odontogenic effect of various concentrations of supplemented Pi on normal hDPCs cultures. Taking into consideration the 140 ppm of Pi supplemented in the BME culture media, which reflects the physiological concentration existing in the human body, any supplementary amount in this study would be in addition to what already exists in the culture media. Genetically, the odontoblast and osteoblast cell profiles are almost identical (141). However, there is a clear difference in cellular morphology between bone and dentin.

Until now, there are no reports demonstrating the effect of Pi ions on the hDPCs cultures. In this study, cell attachment efficiency, proliferation rate, alkaline phosphatase activity and dentin sialoprotein expression were investigated on the hDPCs cultures.

Several studies indicated that the surface topography of substrata was a key factor effecting cell attachment efficiency (142-144). It has been observed in the current study that within the 16 hour time period, no cell doubling occurred. Therefore possible cell number changes are solely attributed to the ability of hDPCs to attach to culture wells when grown in culture media containing different Pi concentrations. The results demonstrated a significant down regulation tendency in groups at 2, 4 and 5 ppm, but the exact mechanism and precise role of Pi is not clear.

A report on rat bone marrow cells showed an inhibitory effect of Pi on proliferation (87). In that study, rat bone marrow cells were cultured with various bioglasses and inhibition of cell proliferation seemed to be related to a high phosphate-ion release or phosphorus-rich precipitations. In the current study, hDPCs did not behave like the rat cells. Instead, a significant upregulation of cells proliferation in all groups containing supplemented Pi was observed. This indicates that supplemented Pi can be beneficial to the growth of hDPCs. An exception manifests itself with the high concentration Pi group (8 ppm), which exhibited almost an equal proliferation rates compared to the control group. This can be explained by the fact that osteoblast apoptosis can be activated when cells have been exposed to elevated phosphate concentrations, potentially due to the profound loss of mitochondrial membrane. Reports showed that when exposed to different Pi concentrations, the osteoblast-like cells from human explants exhibited reduced cell viability in a time- and dose-dependent manner (83).

One study examined the effect of concentration of both calcium and phosphorous on osteogenesis. UMR 106-01 BSP cells were treated with calcium (Ca) for 24 hours with or without the addition of Pi supplement (81). Results of the study showed a Ca-dependent increase in mineral formation. However, cultures without an additional Pi supplement only formed detectable mineral at a relatively high Ca concentration.

Cultures treated with low Ca and high Pi concentrations produced nearly twice as much calcium mineral as those exposed to high Ca and low Pi concentrations. Moreover, the addition of organophosphates (typically β-glycerophosphate) to cultures of primary

osteoblast cells enhanced the formation of calcium mineral. In this case, ALP is required to hydrolyze the organophosphates to release Pi which appears to be the actual initiator of mineral formation. Therefore, increased Pi seems to initiate a mineral formation that afterward is enhanced by Ca levels. However, we cannot exclude the possibility that a portion of the mineral formed in these cultures might be the result of a direct heterogeneous nucleation within the established extracellular matrix of the cell layer.

Pyrophosphate is present in the extracellular matrix of most tissues and is a by-product of many intracellular metabolic reactions, acting as a mineralization inhibitor in bone (145, 146). Meanwhile, the function of ALP and ATPase are also involved in the transport of Pi and Ca by increasing the local concentration of Pi while removing the pyrophosphate inhibitors of apatite deposition (80). In our study, the presence of supplemental Pi led to increased levels of ALP expression in hDPCs cultures. Alkaline phosphatase activity was significantly high in all Pi supplemented groups and the remarkable peak of ALP expression was reached at day 7, but then declined radically at 14 and 21 days. This phenomena is elucidated by the inverse relationship between proliferation and differentiation (135). This high ALP activity which was reached at day 7, is mostly attributed to the fact that ALP occurs in the early stages of differentiation (136).

Dentin sialoprotein was significantly higher in the 5 ppm supplemented Pi group in the current study. This masks the fact that supplemented Pi at 5 ppm has less effect on inducing hDPCs differentiation into odontoblast-like cells than other groups. Dentin sialophosphoprotein (DSPP) gets cleaved into DSP by the terminally differentiated odontoblasts to prompt mineralization and dentinogenesis (60). DSP is an important

marker for dentin production since it is produced only by mature odontoblast cells and has not been found in other cells (50, 59). As with the ALP study, the DSP results showed summiting of DSP levels at day 7. Subsequently the expression gets noticeably reduced at 14 and 21 days. To identify the relationship of phosphate and mineralization, in a previous in vitro study on rat bone, cell cultures were maintained in medium containing ascorbic acid after day 7 and lacking the organic phosphate source, β-glycerophosphophosphate (β-GP) (82). Under accelerated mineralizing conditions (with β-GP), osteocalcin mRNA and synthesis increased steadily beginning at day 15 in parallel with Ca accumulation in the cell layer. In contrast, when cultures were maintained in the absence of β-GP, Ca did not begin to accumulate in the cell layer until day 25. Osteocalcin was not detectable until this point. Its low level of synthesis reflects the slower rate of mineralization. However, the presence or absence of β-GP had no effect on the proliferation period or on the onset of ALP expression.

Taken together, it is concluded that Pi at 5 ppm supplemented concentration would produce the most optimal proliferation and differentiation up-regulation. This is the first report to demonstrate the optimal Pi concentration for inducing human odontogenesis, which may lead to potential development of clinical applications using dental-pulp related biomaterials. More importantly, the current study tested normal human dental pulp cells in vitro and has set a landmark for future studies. For instance, micro-molecular investigations are required to understand the cellular functions of Pi. Furthermore, the reported optimal Pi concentration needs to be verified using in vivo studies as well with optimal concentration of Pi released from implanted material having various degradation rates. The

generated results of the current study are essential when manufacturing Pi-containing biomaterials for dental-pulp complex tissue regeneration.

CONCLUSIONS AND CLINICAL CONSIDERATIONS

CONCLUSIONS

The Si, Ca and P experiment demonstrated that:

- Supplemented silicon at 50 ppm can significantly induce differentiation and mineralization of hDPCs.
- Silicon supplement combined with calcium has a synergistic effect in significantly up-regulating the proliferation rates in normal hDPCs.
- This is the first report to demonstrate that silicon and calcium induced mineralized tissue formation, pointing the way to the potential development and clinical application of a future novel dental pulp capping material.

The P experiment demonstrated that:

- Supplemented inorganic phosphate at 5 ppm could significantly induce proliferation and odontogenesis of hDPCs.
- This is the first report to demonstrate the Pi-induced mineralized tissue formation, leading to potential development and clinical application of future Pi containing dental pulp capping or root canal filling materials.

CLINICAL CONSIDERATIONS

Pulp capping is a technique used in dental restorations to preserve the health and vitality of the pulp complex. Many different materials have been used for pulp capping. Most commonly used are calcium hydroxide (Dycal®), and mineral trioxide aggregate (MTA). However, none of them has the property of inducing dentinogenesis. The present study has proposed a new alternative pulp capping material containing bioactive inorganic elements. This study investigated the effects of silicon, calcium, and phosphate ions on the growth, differentiation and mineralization of the dental pulp in vitro. Correspondingly, the observed significant increase in alkaline phosphatase activity, dentine sialoprotein expression and mineralization may translate clinically to an enhancement of human dental pulp cell differentiation and potential odontogenesis. This will result in a better healing outcome, and longer survival of the dental pulp tissue. Future implementation of silicon, calcium, and phosphate in dental pulp capping materials may help to improve direct pulp capping, pulpotomy procedures, decrease its failure rate and may be a useful therapeutic agent for pulpal repair.

FUTURE STUDY

FUTURE STUDY

This research demonstrated the important role of silicon, calcium and phosphate on odontogenic activity. The cell attachment, proliferation rate, alkaline phosphatase, dentin sialoprotein and mineralization activity were dose dependent. Also this study dealt with a narrow spectrum of ion concentrations and their combinations. Further in vitro studies could be conducted on combinations of the ions concentrations' and their effect on pulp tissue cells, cell cycle, and at molecular level, their effect on any production of inflammatory and anti-inflammatory cytokines.

Furthermore, this study demonstrated the effect of silicon, calcium and phosphate on non-infected human dental pulp cells. Additional studies are required for evaluating the effect of silicon, calcium and phosphate on contaminated human dental pulp cells. This is to evaluate the antibacterial effect and odontogenic effect of silicon, calcium and phosphate on human dental pulp cells.

Also, further studies are necessary to determine the degradation rate of silicon, calcium and phosphate -containing biomaterials to achieve the optimal in vivo concentrations of released ions, which can induce odontogenesis. Animal studies will also be required to obtain necessary data leading to future clinical applications of novel silicon, calcium and phosphate -containing odontogenic materials.

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