THE EFFECTS OF A PYK2 KINASE INHIBITOR ON THE PROLIFERATION AND DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS

by

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

Alkaline phosphatase……..…….....	ALP
α modification of minimum essential medium……...…	α-MEM
Analysis of variance……..……..	ANOVA
Calcium hydroxide…..…….……...	$Ca(OH)_2$
Dental pulp stem cells…….…….....	DPSCs
Dentin sialoprotein……..	DSP
Dimethyl sulfoxide…….	DMSO
Ethylenediaminetetraacetic acid……...…...…….....	EDTA
External cervical resorption……	ECR
Fetal bovine serum……..	FBS
Focal adhesion kinase…….	FAK
Half-maximal inhibitory concentration……...	IC50
Mineral trioxide aggregate…….…….....	MTA
Modified radioimmunoprecipitation assay…….	mRIPA
PF-4618433…….	PF-46
Proline-rich tyrosine kinase 2…….	Pyk2
Regenerative endodontic procedures……..……..…..	REPs
Standard error of the mean……..	SEM
Sodium hypochlorite……...	NaOCl
Stem cells from the apical papilla……..…….	SCAPs

INTRODUCTION

Pulp necrosis of immature permanent teeth can occur from a variety of causes, and this condition presents challenges from both an endodontic and restorative perspective. Affecting up to 35% of the global population, untreated dental caries in permanent teeth is the most prevalent modern health condition.[1] If the carious lesion progresses to involve the pulp, bacteria and bacterial products cause pulp inflammation and eventually infect the pulp space and cause pulp necrosis.[2] Similarly, certain dental anomalies or trauma to the permanent dentition, which has been estimated to occur in 17.5% of children and adolescents, can also cause pulp necrosis of immature permanent teeth.[3, 4] Immature teeth with necrotic pulps present difficulties with cleaning and shaping due to thin dentinal walls, and obturation can be difficult due to large canals and incompletely formed apices.[5] These teeth are also at higher risk of fracture or tooth loss due to incomplete dentin deposition and root formation.[6]

Infected immature permanent teeth diagnosed with pulp necrosis were historically treated by apexification with long-term application calcium hydroxide ($Ca(OH)_2$) to induce formation of a hard tissue barrier at the tooth apex.[7] This procedure has a high reported success rate, but it also has significant disadvantages.[8] The long-term placement of calcium hydroxide has been reported to significantly weaken radicular dentin, and the procedure also requires high patient compliance and recall ability.[9, 10] A more modern approach to apexification is the technique of using bioceramics. This technique also has a high reported success and is advantageous compared to calcium hydroxide because it can be performed in as few as a single

visit; however, it still fails to address weakened tooth structure due to incomplete development of the pulp-dentin complex.[8, 11, 12]

Due to the disadvantages of apexification, regenerative endodontic procedures (REPs) were developed with the goal of regenerating the pulp-dentin complex and continuing development of the tooth roots in both length and width of dentin. If continued root development can occur, then the lack of tooth strength associated with apexification can be overcome. In spite of the successes of REPs, there are still challenges to ideal clinical outcomes.[13] Numerous studies have reported cases including minimal continued root development, empty root canal space after a REP procedure, lack of a normal pulp histology, or re-infection following REPs.[14-18] Due to these complications of REPs, tissue engineering with stem cells, scaffolds, and growth factors have become prominent research areas concerning REPs.[19]

REGENERATIVE ENDODONTIC PROCEDURES

The first step in endodontic regeneration is to address the intraradicular infection. Because immature teeth with necrotic pulps have thin walls, disinfection of the root canal space relies predominantly on irrigation and the placement of medicaments instead of mechanical instrumentation.[20] Calcium hydroxide is commonly used in endodontics as an intracanal medicament, especially in cases involving trauma to the dentition.[21, 22] Calcium hydroxide has been recommended as a standard medicament for regenerative endodontics due to its antibacterial activity as well as its biocompatibility with stem cells of relevance to regenerative endodontics.[14, 22] Other antibiotic mixtures such as DAP (ciprofloxacin and

metronidazole) and TAP (ciprofloxacin, metronidazole, and minocycline) have also been used as a substitute intracanal medicament for calcium hydroxide.[23]

Three general components are required for tissue regeneration: reparative cells, a scaffold that allows for cell growth, and bioactive molecules to guide and stimulate the reparative cells.[24] An induced blood clot is the most commonly used scaffold for REPs, but there are a wide variety of scaffolds that researchers have used in an attempt to optimize outcomes of REPs.[25] Collagen-based scaffolds, platelet-rich fibrin, and even nanofibrous scaffolds loaded with antibiotics have been investigated for clinical use.[25, 26] Bioactive molecules, such as growth factors and cytokines, can be released from the blood clot as well as from dentin.[24] Dentin is an important consideration in regenerative endodontics because it is a tissue to be regenerated, and it is also the surface upon which all intracanal treatments directly contact. Dentin is composed of a 70% mineral phase, 20% organic matrix, and 10% water by volume.[27] Instead of being considered as an inert structural material, dentin harbors many growth factors such as insulin-like growth factor, transforming growth factor beta, and skeletal growth factor.[28, 29]

STEM CELLS

Much research has been conducted in the application of mesenchymal dental stem cells for tissue regeneration. Dental pulp stem cells are multipotent cells that can proliferate extensively and form cells of all three germ layers, and these cells can be further divided based on their niche location and particular properties.[30, 31] Two dental stem cell lines of particular importance in endodontic regeneration are stem cells from

the apical papilla (SCAPs) and dental pulp stem cells (DPSCs).[32] Previous research has shown that DPSCs are capable of forming a pulp-like vascularized tissue surrounded by odontoblast-like cells and mineralized tissue resembling dentin in mice models.[33] For the current study, human DPSCs were used because of their relevance in endodontic regeneration, and these cells can be differentiated into the osteogenic lineage.

PYK2 ACTIVITY IN OSTEOGENESIS

The FAK family of kinases, which consist of the proline-rich tyrosine kinase 2 (Pyk2) and the focal adhesion kinase (FAK), play a critical role in integrin-mediated cell adhesion and cytoskeletal dynamics.[34] Pyk2 and FAK are cytoplasmic non-receptor tyrosine kinases that function in cell migration, polarization, proliferation and cell survival.[34-36] FAK and Pyk2 signaling can be activated by cytokines (interleukins), growth factors (TNF-α, TGF-β), and microRNAs.[37] Increased activation and expression of FAK and Pyk2 has been found in a variety of human cancers, and overexpression of Pyk2 in cancerous tissues has been correlated with poorer outcomes.[34, 37]

Pyk2 is expressed in a wide variety of cell types including the central nervous system, epithelial cells, hematopoietic cells, osteoblasts, and osteoclasts.[37] Pyk2 has been demonstrated *in vitro* to be a positive modulator of osteoclast activity.[38] Studies have demonstrated that Pyk2 is necessary for both osteoclast function and bone resorption.[39] Mice lacking the PTK2β gene (Pyk2 -/-) show impairment of osteoclast function and high bone mass.[39] Additionally, Pyk2 has been demonstrated to play a

role in megakaryocyte mediated osteoblast proliferation.[40] More recent studies have shown Pyk2 functions as a negative regulator of osteoblast mineralization, proliferation, and differentiation.[41] Buckbinder and colleagues demonstrated that Pyk2-/- mice exhibit enhanced osteoblast activity, which contributes to bone gain in these mice.[38, 42] In addition, Bruzzaniti and colleagues demonstrated that osteoblasts lacking Pyk2 show increased ALP activity, mineral deposition and migration.[41]

PF-431396, a chemical inhibitor against the tyrosine kinases FAK and Pyk2, was found to increase bone mass in ovariectomized mice, suggesting inhibition of these molecules have bone anabolic applications. The half-maximal inhibitory concentration (IC50) of PF-431396 to FAK and Pyk2 are 2 nM and 11 nM, respectively.[43] Oral administration of PF-562271, an inhibitor of both FAK (IC50 of 1.5 nM) and Pyk2 (IC50 of 14 nM), was shown to restore tumor related bone loss in murine models.[44, 45] Published studies from Dr. Bruzzaniti's laboratory demonstrated that a newer Pyk2-targeted inhibitor, PF-4618433 (PF-46), promoted the proliferation and osteogenic differentiation of murine bone marrow-derived stromal osteoblasts.[46] PF-46 is a Pyk2-targeted inhibitor without significant inhibitory effect against FAK.[46] PF-46 is a synthetic organic compound, and its chemical structure is shown in Figure 1.[47]

One of the main challenges with REPs is predictable continued growth of root dentin in both length and width.[14] PF-46, an inhibitor of Pyk2, has already been shown to promote osteoblast function and inhibit osteoclasts, thereby increasing mineralized bone tissue.[46] Due to the need for formation of mineralized hard tissue in both osteogenesis and dentinogenesis, it is possible that PF-46 can also promote the

formation of mineralized tissue from DPSCs, which can be useful for REPs.[48, 49] Therefore, the aims of this study were to evaluate the effects of PF-46 on the proliferation and osteogenic differentiation of human DPSCs. The results of these studies will give insight into whether a Pyk2 inhibitor could have potential applications for endodontic regeneration. The investigation of PF-46 could potentially lead to applications in regeneration of the pulp-dentin complex.

OBJECTIVE

The purpose of this study was to investigate the effect of a Pyk2 inhibitor, PF-46, on the proliferation, differentiation, and mineralization of human DPSCs.

NULL HYPOTHESIS

DPSCs grown in the presence of PF-46 will show no difference in proliferation, differentiation, and/or mineralization of DPSCs, compared to vehicle control.

ALTERNATIVE HYPOTHESIS

DPSCs grown in the presence of PF-46 will show an increase in proliferation, differentiation, and/or mineralization of DPSCs, compared to vehicle control.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

The earliest evidence of the practice of dentistry can be seen with the Indus Valley Civilization (IVC) around the year 7,000 B.C.[50] 9 adults from the IVC civilization were discovered in a prehistoric graveyard with evidence that molar crowns had been drilled upon using a primitive drill.[50] In 5,000 B.C., the first reference of "tooth worms" can be found in a Sumerian text.[51] The idea of tooth worms has been seen in many cultures, and it was believed that these tooth worms caused caries, periodontitis, and tooth-related pain.[51] The earliest known dental practitioner was an Egyptian named Hesy-Re around 2,500 B.C., and his tomb was inscribed with "the greatest of those who dealt with teeth."[52]

Pierre Fauchard, a French surgeon considered as the "father of modern dentistry", published *The Surgeon Dentist, A Treatise on Teeth*.[52] This is considered the first book to detail the comprehensive practice of dentistry including anatomy and function, operative and restorative techniques, and denture construction.[52-55] Fauchard also described aspects of endodontics such as pus drainage from pulp chambers, pulp extirpation, leaving teeth open, and obturating teeth with a metal foil.[54, 55]

In more modern times, the focal infection theory was gaining popularity and was introduced to dentistry. In 1925, Weston Price argued that bacteria within dentinal tubules could spread from the root canal system and cause disease elsewhere in the body.[56] This work was influential in many practitioners to recommend extraction of teeth with necrotic pulps. Later work by several authors refuted the focal infection theory. In 1937, Logan argued that the mere presence of microorganisms did not automatically

mean an infection was present.[57, 58] The research of Fish, who is known for the "zones of Fish", also refuted the focal infection theory.[59]

In 1943, 20 individuals including Dr. Harry Johnston met in Chicago for a meeting, and the American Association of Endodontics (AAE) was founded.[58] At this meeting, the group discussed and formed standards of endodontic treatment.[60] The term "endodontics" was chosen at this meeting by combining two Greek words, "endon" meaning "within" and "ho dontas" meaning "a tooth."[61] A little over a decade later in 1956, the American Board of Endodontics was formed and recognized by the ADA Education Council.[61] In 1963, with over 200 American dentists limiting their practice to endodontics, the ADA officially recognized endodontics as a dental specialty.[60] In 1965, the first endodontic specialists became board certified.[61]

THEORY OF ENDODONTICS

In 1965, Kakehashi et al. published a research paper that became a bedrock of future endodontic research.[62] This study compared the pulp and periapical status of gnotobiotic rats to normal rats that had pulp chambers mechanically exposed. The gnotobiotic rats showed no evidence of disease, whereas normal rats developed pulp necrosis with apical periodontitis. This study showed that bacteria is the primary etiologic agent of periapical disease.[62]

Subsequent research supported the findings of Kakehashi. A study by Moller in 1981 compared the periapical response of pulp chambers that were maintained bacteria-free compared to pulp chambers that were allowed to be infected by the oral flora.[63] Moller's work showed that necrotic and infected pulps lead to apical periodontitis,

whereas non-infected pulps did not develop apical periodontitis. In 1976, Sundqvist's thesis also showed that teeth with necrotic pulps and apical periodontitis contained a variety of microorganisms including many anaerobes.[2]

This work that showed bacteria as the primary etiology of apical periodontitis led to the modern paradigms of endodontic treatment. Based upon these research studies, Grossman outlined his 13 fundamental principles of endodontic treatment:[64]

1. Aseptic technique
2. Instrument retention within the root canal system
3. Instruments should never be forced apically
4. Enlarging the canal space from its original size to accommodate obturation material
5. Continuous irrigation with an antiseptic throughout treatment
6. Irrigation solution should remain within the canal space
7. Fistulas do not require a special protocol
8. Negative culture should be confirmed prior to obturation of the root canal
9. Obturation should include a hermetic seal of the root canal system
10. Obturation material should not be irritating to tissues
11. Alveolar abscess should be adequately drained
12. Injections should be avoided into infectious areas
13. Surgical treatment may be required for proper healing

At the same time, Schilder introduced his obturation technique that involved the vertical compaction of warm gutta percha.[65] He advocated this obturation method, as it

creates a three-dimensional fill of the root canal system including possible accessory canals.

TREATMENT OF IMMATURE PERMANENT TEETH WITH PULP NECROSIS

The treatment of an immature permanent tooth with pulp necrosis presents multiple challenges to clinicians. These clinical challenges include the presence of an open apex, managing the root canal infection, and the presence of thin root walls.[6] The open apex makes adequate irrigation and obturation a challenge. Because of the thin root walls of immature teeth, one must rely heavily on irrigants and intracanal medicaments for adequate disinfection of the root canal space rather than mechanical debridement of the canal.[6] Furthermore, the thin root walls and shortened roots make these teeth more prone to fracture, and they can present a restorative challenge for clinicians.[6] Potential treatment options of an immature permanent tooth with pulp necrosis include no treatment, extraction, apexification, or REPs.[8, 14]

The traditional apexification procedure aimed to create a hard tissue barrier at the apical-most extent of the root canal to facilitate adequate obturation of the tooth.[66] This was done by first disinfecting the root canal system using a combination of diluted sodium hypochlorite (NaOCl) (~0.5%) and light hand filing.[67] Next, the canal is filled with calcium hydroxide paste that extends into contact with the apical soft tissue, and a well-sealed coronal restoration is placed.[12] Patients are periodically recalled at 3-month intervals to assess for washout of calcium hydroxide and to verify if a hard tissue barrier has formed. Following formation of the hard tissue barrier, the tooth can be obturated as usual.[67] The more modern approach to apexification involves the use of a bioceramic

barrier technique. Following canal disinfection, a barrier (e.g., CollaTape) is pushed beyond the apex and a bioceramic such as mineral trioxide aggregate (MTA) is packed in the apical extent of the root canal system.[68] After setting of the bioceramic, the remaining canal is obturated. Jeeruphan showed that MTA apexification technique can be performed successfully and with improved outcomes compared to calcium hydroxide apexification.[5]

An alternative treatment option to preserve immature permanent teeth with pulp necrosis is a REP. Like apexification procedures, REPs rely on initial disinfection of the canal space. In contrast to apexification, REPs aim to regenerate the pulp-dentin complex following disinfection rather than simply obturating the canal space.[20] In theory, REPs have several distinct advantages when compared to apexification. By regenerating the pulp-dentin complex, a tooth can experience continued growth of the root in both length and width.[5, 69] An outcome study compared REPs to apexification, and the authors found that REPs significantly increased root length and width compared to apexification procedures.[5]

Despite these advantages of REPs, there are still multiple challenges to be addressed. Ong published a recent systematic review and meta-analysis that included a quantitative assessment of root development in immature permanent teeth with necrotic pulps that underwent REPs.[70] The authors found that pooled rates of root lengthening, root thickening, and apical closure were 77.3%, 90.6%, and 79.1% respectively.[70] However if 20% radiographic changes were used as a cutoff point, there were only 16.1% root lengthening and 39.8% root thickening.[70] These results show that although high rates of root thickening and apical closure can be reported in studies, these changes are not

necessarily clinically significant in many cases. When a cutoff threshold of 20% change is applied, then the rates of root lengthening and thickening decrease significantly. Results of this and other studies show that the attainment of clinically significant and predictable root thickening and lengthening can be elusive to achieve with REPs.[70, 71]

REPs aim to regenerate the pulp-dentin complex, but it is unclear exactly what type of tissue is formed during REPs. A systematic review investigated the histology of REPs performed in animal studies, and this review found that none of the regeneration protocols resulted in the predictable formation of a true pulp-dentin complex.[72] Other studies have shown root formation of a combination of cementum-like tissue, bone-like tissue, and dentin-like tissue.[14, 72, 73] These studies call into question if true regeneration is occurring or if it would be better referred to as repair.[74]

CLINICAL CONSIDERATIONS FOR REPs

On 4/1/2018, the AAE published the following guidelines for clinical considerations for a REP:[75]

Case Selection

- Tooth with necrotic pulp and an immature apex.
- Pulp space not needed for post/core, final restoration.
- Compliant patient/parent.
- Patient not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2).

Informed Consent

- Two (or more) appointments.

- Use of antimicrobial(s).
- Possible adverse effects: staining of crown/root, lack of response to treatment, pain/infection.
- Alternatives: MTA apexification, no treatment, extraction (when deemed non-salvageable).
- Permission to enter information into AAE database (optional).

First Appointment

- Local anesthesia, dental dam isolation and access.
- Copious, gentle irrigation with 20 mL NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g., needle with closed end and side-vents, or EndoVac). Lower concentrations of NaOCl are advised (1.5% NaOCl, 20 mL/canal, 5 min) and then irrigated with saline or ethylenediaminetetraacetic acid (EDTA) (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues.
- Dry canals with paper points.
    - Place calcium hydroxide or low concentration of triple antibiotic paste. If the triple antibiotic paste is used:
        - Consider sealing pulp chamber with a dentin bonding agent (to minimize risk of staining) and
        - Mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 1-5 mg/mL. Triple antibiotic paste without minocycline paste or substitution of minocycline for other

antibiotic (e.g., clindamycin; amoxicillin; cefaclor) is another possible alternative as root canal disinfectant. Clinicians should be aware that studies have been done using higher concentrations of TAP/DAP, but a recommendation to a higher concentration can't be made at this time due to limited studies.

- Deliver into canal system via syringe.
- If triple antibiotics is used, ensure that it remains below CEJ (minimize crown staining).
- Seal with 3-4 mm of a temporary restorative material such as Cavit, IRM, glass-ionomer or another temporary material. Dismiss patient for 1-4 weeks.

Second Appointment (1-4 weeks after 1st visit)

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
- Anesthesia with 3% mepivacaine without vasoconstrictor, dental dam isolation.
- Copious, gentle irrigation with 20 mL of 17% EDTA.
- Dry with paper points.
- Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (induce by rotating a pre-curved K-file at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the cemento-enamel junction). An alternative to creating of a blood clot is the use of platelet-rich plasma, platelet-rich fibrin, or autologous fibrin matrix.
- Stop bleeding at a level that allows for 3-4 mm of restorative material.

- Place a resorbable matrix such as CollaPlug, CollaCote, or CollaTape over the blood clot if necessary and white MTA as capping material.
- A 3-4 mm layer of glass ionomer (e.g. Fuji IX, GC America, Alsip, IL) is flowed gently over the capping material and light-cured for 40 s. MTA has been associated with discoloration. Alternatives to MTA [such as bioceramics or tricalcium silicate cements (e.g., Biodentine, Septodont, Lancasted, PA, USA, EndoSequence BC RRM-Fast Set Putty, Brasseler, USA)] should be considered in teeth where there is an esthetic concern.
    - Anterior and premolar teeth: consider use of CollaTape/CollaPlug and restoring with 3 mm of a nonstaining restorative material followed by bonding a filled composite to the beveled enamel margin.
    - Molar teeth: consider use of CollaTape/CollaPlug and restoring with 3 mm of MTA, followed by RMGI, composite or alloy.

Follow-up (6-, 12-, 24-months)

- Clinical and radiographic exam:
    - No pain, soft tissue swelling or sinus tract (often observed between first and second appointments).
    - Resolution of apical radiolucency (often observed 6-12 months after treatment).
    - Increased width of root walls (this is generally observed before apparent increase in root length and often occurs 12-24 months after treatment).
    - Increased root length.

  - Positive pulp vitality test response.
  - Recommended yearly follow-up after the first 2 years.
  - CBCT is highly recommended for initial evaluation and follow-up visits.
- The degree of success of REPs is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:
  - Primary goal: the elimination of symptoms and the evidence of bony healing.
  - Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential).
  - Tertiary goal: positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue).

PYK2 INHIBITOR: PF-46

As reviewed in the introduction section, PF-46 is a proline-rich tyrosine kinase 2 inhibitor (Pyk2). Pyk2 is the second member of the FAK family of proteins, and it plays an important role in cell migration, polarization, proliferation, and cell survival.[34, 35] DPSCs and osteoblasts are both mineral-producing cells, and the Pyk2 pathway has been well-studied in both osteoblasts and osteoclasts. Buckbinder found that Pyk2 knockout mice exhibited enhanced differentiation and activity of osteoprogenitor cells, as do mice exposed to the specific Pyk2 inhibitor that was used in this study.[38, 76, 77] Increased bone mass and inhibition of osteoclast activity was also observed in Pyk2 knockout mice.[39] Furthermore, Pyk2 inhibitors have been shown to negatively modulate osteoclast function and bone resorption activity.[39, 78] Increased differentiation and mineralization of

osteoblasts has been observed when exposed to a specific Pyk2 inhibitor, PF-46.[41, 46] Because of the similarities between osteoblasts and odontoblasts, it is possible that PF-46 can also promote the formation of mineralized tissue from DPSCs. This can potentially be useful for addressing obstacles of REPs such as predictable thickening and lengthening of the root as well as to improve the healing of apical periodontitis.

MATERIALS AND METHODS

TREATMENT GROUPS

Group 1 – 0 μM PF-46

Group 2 – 0.1 μM PF-46

Group 3 – 0.3 μM PF-46

Group 4 – 0.6 μM PF-46

Groups 2, 3 and 4 represent the experimental treatment PF-46, whereas Group 1 represents the negative (vehicle) control. Previous studies with osteoblasts showed positive assay results with concentrations up to 0.3 μM PF-46, so the concentrations listed above were chosen based off those results.[46] The above groups are shown in Figure 2.

MATERIALS

The project utilized both commercially available products as well as materials prepared in the lab as described in the methodology portion. Commercially available human DPSCs, collected from the pulp of extracted teeth, have been previously purchased from ALLCells and stored in liquid nitrogen (Alameda, CA, USA).

- Cell culture 24-well plates (Alkali Scientific Inc. Cat: TP9024)
- α modification of minimal essential media (HyClone Laboratories Inc., South Logan UT, USA)
- 10% fetal bovine serum (Biowest, Kansas City, MO, USA)
- 1% penicillin-streptomycin (Lonza, Allendale, NJ, USA)
- 0.25% trypsin/EDTA (Life Technologies Corporation)
- Phosphate buffered saline (HyClone Laboratories Inc., South Logan UT, USA)

- 1% (w/v) cetylpyridinium chloride (Sigma: C0732)
- PF-4618433 (Adooq Bioscience: A13461-10)
- ALP assay kit (Sigma Aldrich)
- Alizarin Red-S (Sigma Aldrich)
- Pierce BCA protein assay kit (Thermo Scientific: 23225)
- CellTiter 96 aqueous non-radioactive cell MTS proliferation assay kit (Promega: G5421)

METHODOLOGY

PREPARATION OF PF-46 TREATMENT GROUPS

PF-46 treatment groups are prepared from a stock solution in dimethyl sulfoxide (DMSO) stored in -80°C. The stock solution is at a concentration of 44.9 mM. PF-46 was diluted in DMSO. For ease of manipulation 200 µl of 2 mM PF-46 solution was created using 191.1 µL of DMSO and 8.9 µL of 44.9 mM PF-46 stock. This 2 mM stock solution was used to create treatment groups.

HUMAN DENTAL PULP STEM CELLS CULTURE CONDITIONS

Human DPSCs were passaged once and stored in liquid nitrogen for later use. During each experiment, DPSCs were thawed and seeded in 10 cm^2 culture dishes and cultured in α modification of minimum essential medium (α-MEM) combined with 10% fetal bovine serum (FBS) plus penicillin/streptomycin. Previous experiments have provided optimal seeding densities for performance of the assays that were used in this

current project.[79] Cells between 3 and 5 passages were seeded into 24-well plates at predetermined concentrations indicated below for each assay.

The day following the seeding of DPSCs, 750 μL of fresh culture media was added to the bottom of the chamber. Depending on the treatment group, either 0, 0.1, 0.3, or 0.6 μM of PF-46 was also present in the culture media. Only culture media was used as a negative control. Total media volume was 750 μL/well. Culture media was replaced every 2-3 days with plates being incubated at 37°C in an incubator with 95% CO_2/5% O_2 for the entirety of the experiment.

CELL COUNTING

DPSCs were cultured in conditions described above. Cells were plated at 2 x 10^4 cells/well. PF-46 was present in the cell culture media as described above. 4 days following exposure to the medicaments, cells were washed once in PBS and incubated for 5 minutes in the presence of trypsin. 500 μL of cold media was added to each centrifuge tube, cells were spun at 1750 rpm for 5 minutes, and liquid was removed with a pipette. Cells were resuspended in 300 μL of cold PBS, and direct cell counting was performed with a microscope and hemocytometer. Each treatment group was cultured in quadruplicates, and direct cell counting was performed once.

MTS PROLIFERATION ASSAY

DPSCs were cultured in conditions described above. Cells were plated at 2 x 10^4 cells/well. PF-46 was present in the cell culture media as described above. 1 day following exposure to the medicaments, cells were washed once in PBS and assayed

using CellTiter 96 aqueous non-radioactive cell proliferation assay kit from Promega. This assay measures the conversion of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium, inner salt; MTS] and the electron coupling reagent phenazine methosulfate (PMS) into a formazan product. This reaction occurs through dehydrogenase enzymes found in metabolically active cells. Reagent was removed at 3 hours with a read volume of 100 µL read in duplicates for every sample. Formazan product quantity was measured at 490 nm. Each treatment group was cultured in quadruplicates, and the assay was performed three times.

ALKALINE PHOSPHATASE ACTIVITY ASSAY

Alkaline phosphatase (ALP) is an enzyme that plays an important role in bone mineralization, and the ALP assay is a useful quantitative assessment tool for differentiation of cells into mineral-forming cells.[79] For the quantification of ALP activity, cells were plated at 4 x 10^4 cells/well in a 24-well plate. They were cultured in α-MEM plus 10% FBS and containing 50 mg/mL ascorbic acid and 10 mM β-glycerol phosphate to promote osteogenic differentiation for 14 days, with media changes every 2-3 days. Following 14 days, cells were washed and lysed to be assayed for ALP activity. The ALP quantification protocol has been previously described.[41] Cells were lysed with 100 µL of modified radioimmunoprecipitation assay (mRIPA) buffer consisting of 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate. The buffer was supplemented with a protease inhibitor cocktail containing 10 µg/mL leupeptin hydrochloride, 10 µg/mL aprotinin, 10 µg/mL pepstatin, 1 mM PMSF, 1mM sodium fluoride, and 1mM sodium orthovanadate. Lysates were sonicated in 1.5 mL

centrifuge tubes for 5 minutes then spun at 13,000 rpm for 5 minutes prior to collecting the supernatant. 5 µL of cell lysate was assayed in triplicates in a 96-well plate. ALP substrate containing 2 mg/mL p-nitrophenyl phosphate in 1.5 M alkaline buffer was added to the lysates and then the mixture was incubated for 45 minutes at 37°C in a foil to protect the reaction from light. Lysis buffer was also assayed with the substrate to be used for background measurements. The reaction was stopped after 45 minutes by adding 20 mM NaOH for a final volume of 200 µL. The ALP standard curve was calculated concurrently with the assay using serial dilutions of 4-nitrophenol and 20 mM NaOH to determine the ALP activity. Absorbance for the plate was read at 405 nm. ALP activity per individual sample was normalized through quantification of total protein content in each sample. Protein quantification was performed using the Pierce BCA protein assay kit and following the manufacture specifications. The final results were expressed as ALP activity per µg protein. Each treatment group was cultured in quadruplicates, and the assay was performed three times.

QUANTITATIVE MINERAL DEPOSITION ASSAY

The quantitative mineral deposition assay is proportional to the amount of extracellular mineralized deposits.[79] DPSCs were plated at 4 x 10^4 cells/well and cultured in α-MEM plus 10% FBS and containing 50 mg/mL ascorbic acid and 10 mM β-glycerol phosphate to provide an osteogenic environment, and stimulated with the PF-46 concentrations described above. After 14 days, media was removed and cells were washed in PBS and fixed in 3.7% formaldehyde in PBS for 15 minutes. Quantitative analysis of the mineral deposition was performed as previously described with Alizarin

Red-S, a calcium binding stain.[41] The cells were then washed for 15 minutes in PBS. Following aspiration of PBS, 500 µL of 40 mM Alizarin Red-S Solution at a pH 4.2 was added to each well on the plate. The plate was then placed on a shaker for 30 minutes. Following treatment with the stain, cells were washed a total of 3 times with tap water to remove any residual stain and again with PBS for 15 minutes on the shaker. Then 500 µL of 1% (w/v) cetylpyridinium chloride (CPC) was added to each well to extract extracellularly bound Alizarin Red-S stain. A standard curve was created using a serial dilution by using 1% CPC and 40 mM Alizarin Red-S and the calcium concentrations were determined based on Alizarin binding capabilities to calcium (2 mol calcium per 1 mol dye). Samples were read in triplicate at 200 µL in a 96-well plate in a spectrophotometer at 562 nm. Each treatment group was cultured in quadruplicates, and the assay was performed three times.

SAMPLE SIZE AND STATISTICAL ANALYSIS

Experiments were performed in triplicate or quadruplicate, and repeated 2-4 times, with similar results. Graphs demonstrate mean ± SEM for representative experiments for each assay. Differences between treatment groups were analyzed by a one-way analysis of variance (ANOVA) followed by pair-wise tests conducted using Tukey's multiple comparisons procedure. The ANOVAs did not assume homogeneous variances. A 5% significance level was used for all tests. Analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). George Eckert assisted with the statistical analysis (Department of Biostatistics and Health Data Science, Indiana University School of Medicine).

RESULTS

THE EFFECT OF PF-46 ON DPSC NUMBER

Direct cell counting of DPSCs was performed after cells were exposed to various concentrations of PF-46 for 4 days. The 0.6 μM PF-46 group had a statistically significant higher cell count when compared to 0 μM PF-46. Although not statistically significant, the 0.1 μM PF-46 and 0.3 μM PF-46 groups also displayed a higher cell count than 0 μM PF-46. The graph of results, shown in Figure 6, showed a trend of increasing cell count as the concentration of PF-46 increased up to 0.6 μM. There were no statistically significant differences between the groups that contained PF-46. The cell counting results are summarized in Tables 1 and 2.

THE EFFECT OF PF-46 ON MTS PROLIFERATION ACTIVITY OF DPSCs

The MTS proliferation assay measures the metabolic activity of cells, and previous studies have shown that results of this assay are proportional to live number of cells.[46] After culturing for 1 day and measuring MTS proliferation activity, no statistical differences were present when comparing the groups that contained PF-46 to the 0 μM PF-46 group. Although no significant differences were present, proliferation activity was generally higher in the groups that contained PF-46 compared to the 0 μM PF-46 group (Figure 7). There was also a trend of decreased proliferation activity as the concentration of PF-46 increased from 0.1 μM to 0.6 μM. The MTS proliferation activity results are summarized in Tables 1 and 3.

THE EFFECT OF PF-46 ON ALP ACTIVITY OF DPSCs

The ALP assay measures ALP enzymatic activity per microgram of protein, which is an indicator of early differentiation of osteogenic cells.[80] ALP activity was measured after culturing cells for 14 days with various concentrations of PF-46. All 3 groups that contained PF-46 (0.1, 0.3 and 0.6 μM) had ALP activity levels that were higher than 0 μM PF-46, and these differences were statistically significant. Although there were no significant differences between groups that contained PF-46, ALP activity increased as PF-46 concentration increased from 0.1 μM to 0.6 μM (Figure 8). The ALP activity results are summarized in Tables 1 and 4.

THE EFFECT OF PF-46 ON MINERAL DEPOSITION BY DPSCs

Mineral deposition of DPSCs was measured after cells were exposed to various concentrations of PF-46 for 14 days. The 0.6 μM PF-46 group had significantly higher mineral deposition when compared to 0 μM PF-46. Although not statistically significant, the 0.1 μM PF-46 and 0.3 μM PF-46 groups also displayed higher mineral deposition than 0 μM PF-46. The graph of results in Figure 9 showed a trend of increasing mineral deposition as the concentration of PF-46 increased up to 0.6 μM. There were no statistically significant differences between the groups that contained PF-46. The mineral deposition results are summarized in Tables 1 and 5.

FIGURES AND TABLES

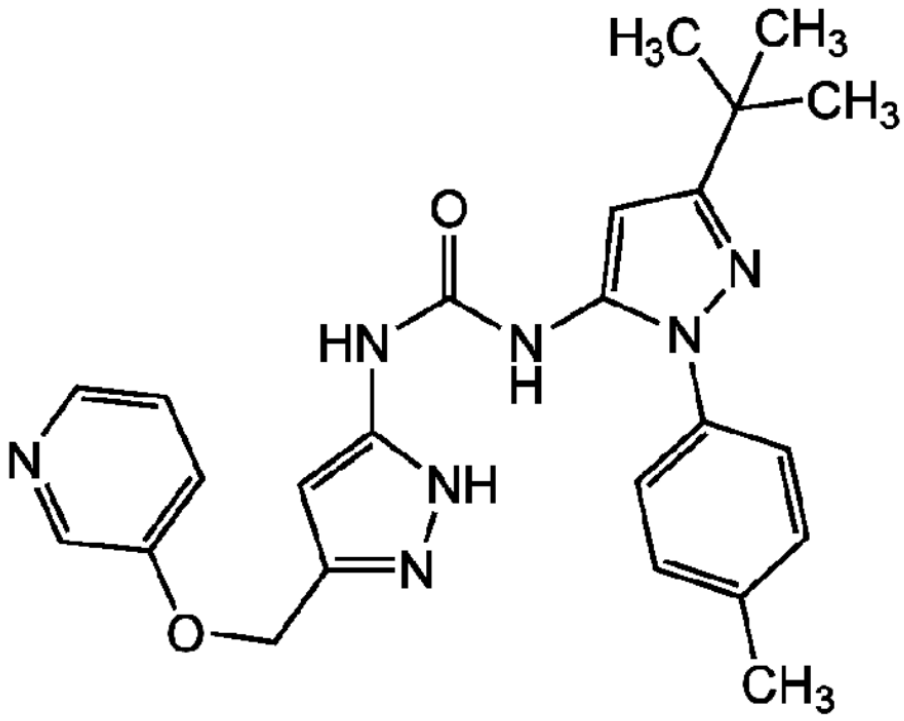

FIGURE 1: Chemical structure of PF-46.[47]

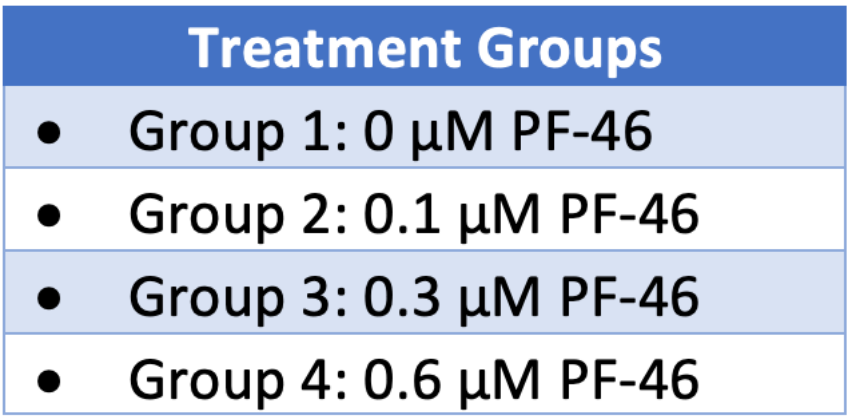

FIGURE 2: Treatment groups.

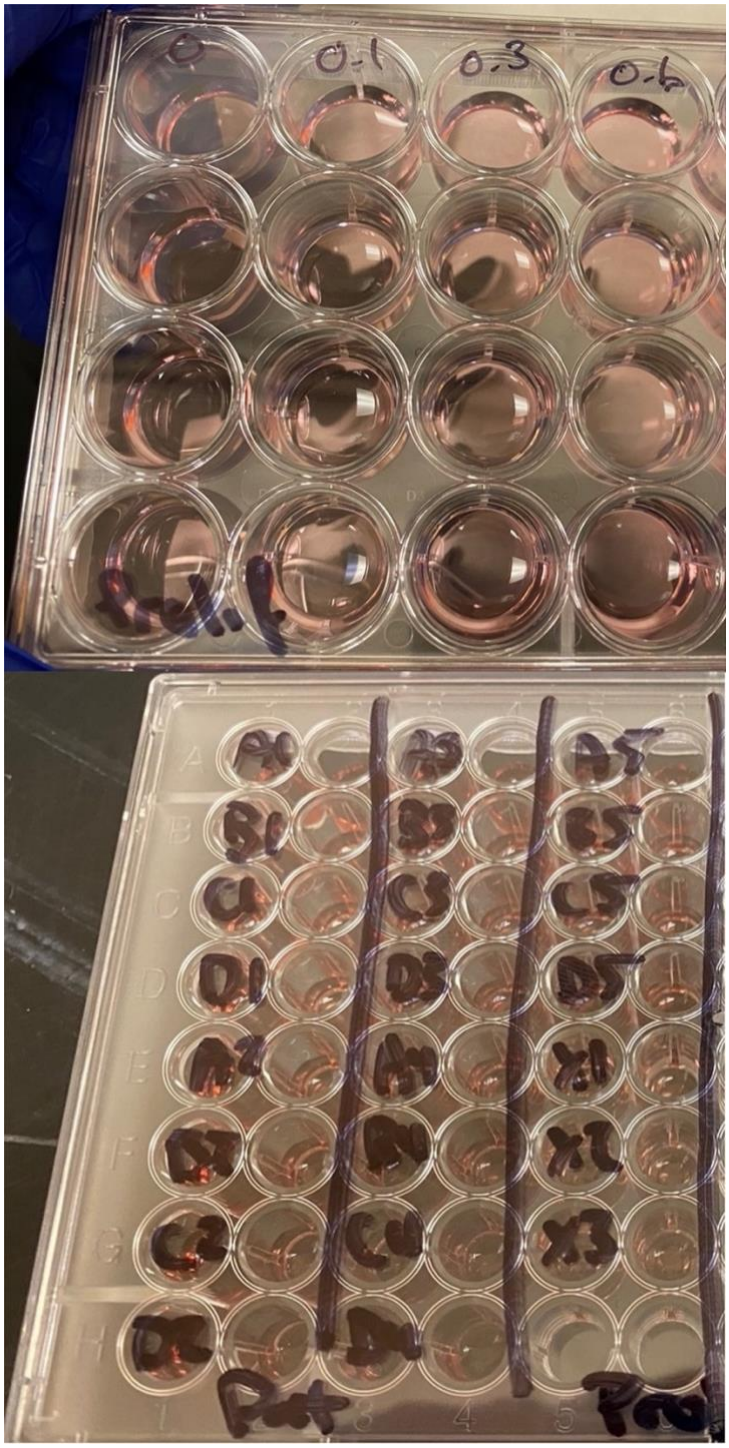

FIGURE 3: MTS proliferation images.

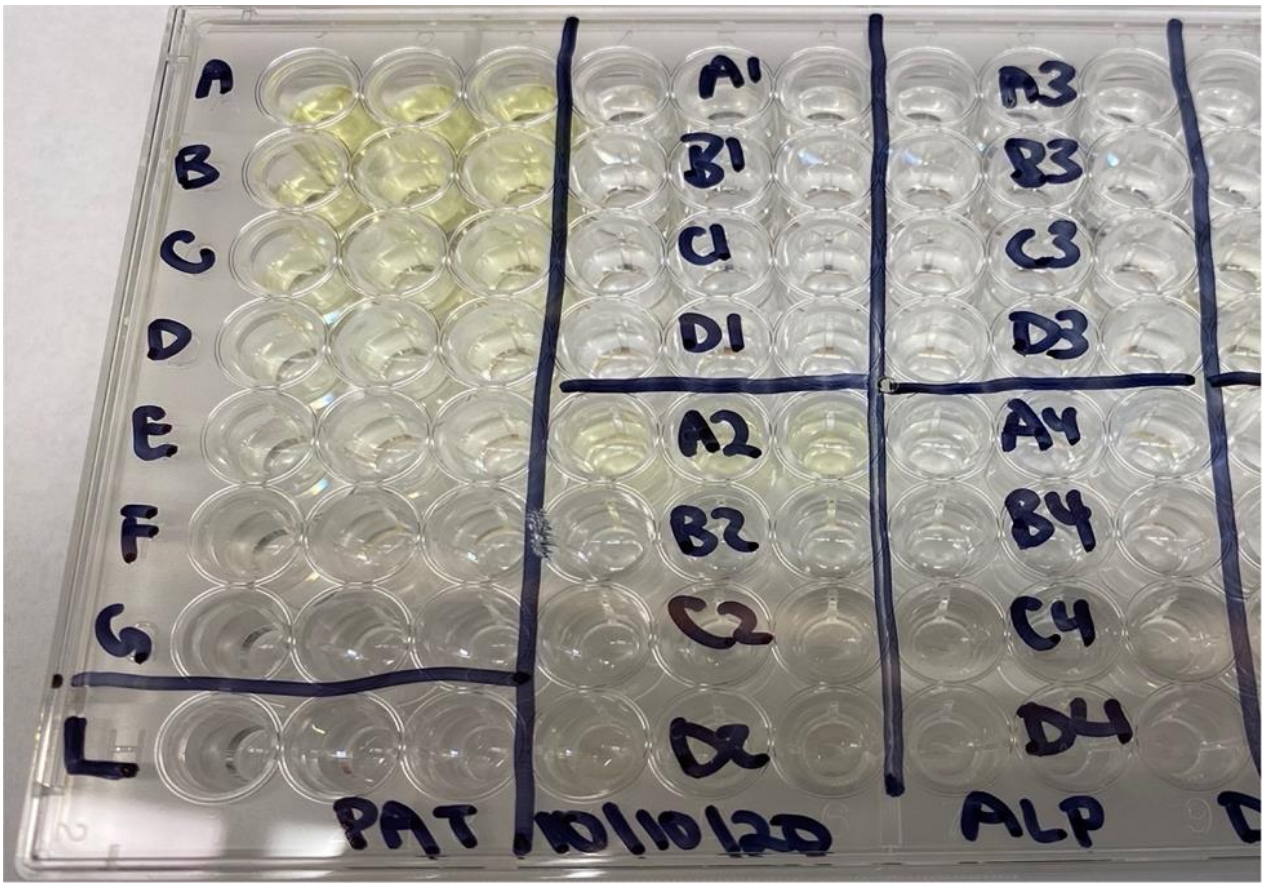

FIGURE 4: ALP image.

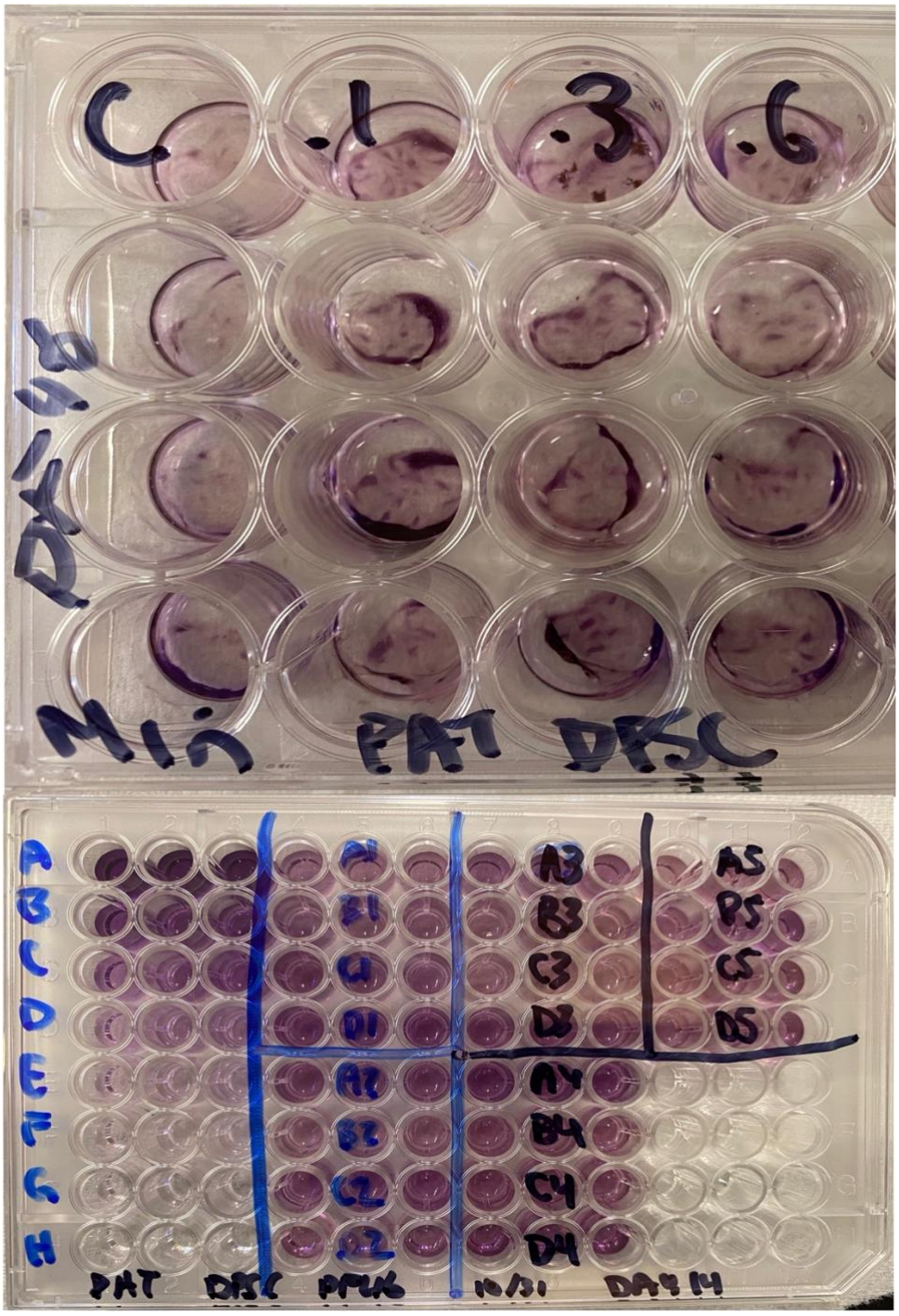

FIGURE 5: Mineral deposition images.

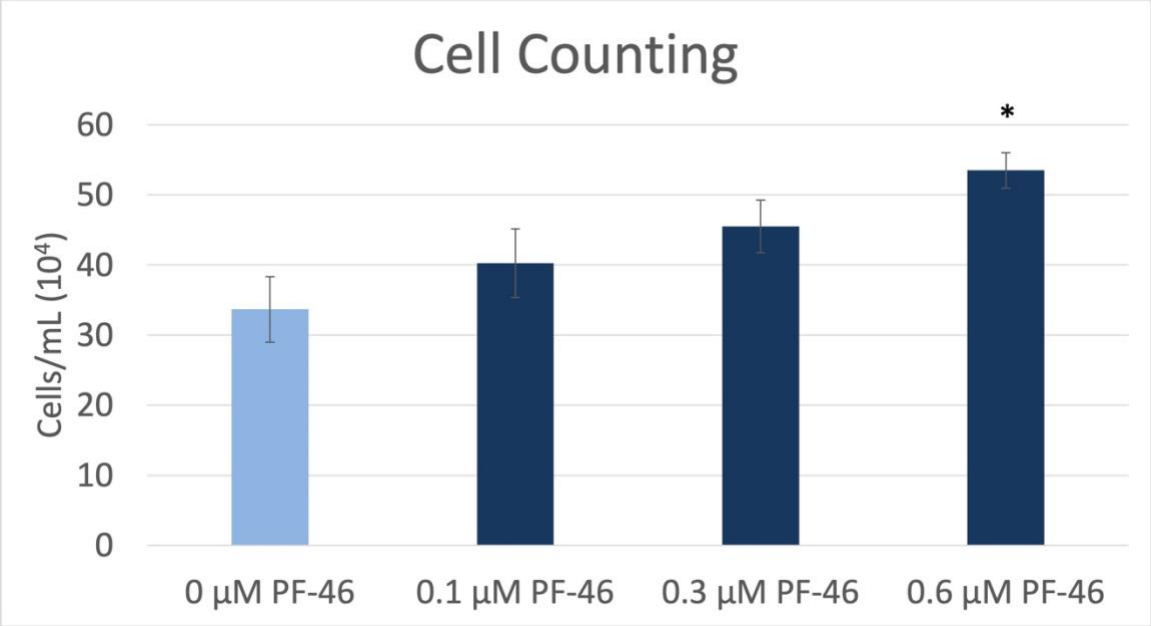

FIGURE 6: Cell counting results showing the mean of 4 wells per treatment group with standard error of the mean (SEM). An asterisk shows statistical significance compared to 0 µM PF-46.

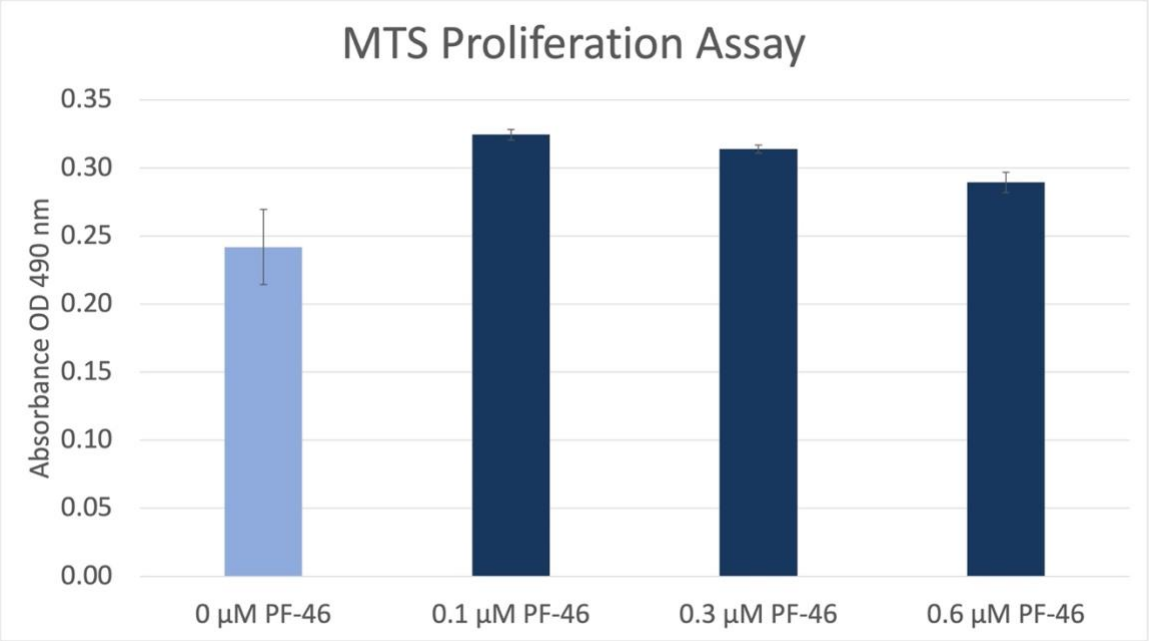

FIGURE 7: MTS proliferation assay results showing the mean of 4 wells per treatment group with SEM. There was no statistically significant difference for 0.1, 0.3 and 0.6 µM PF-46 compared to 0 µM PF-46.

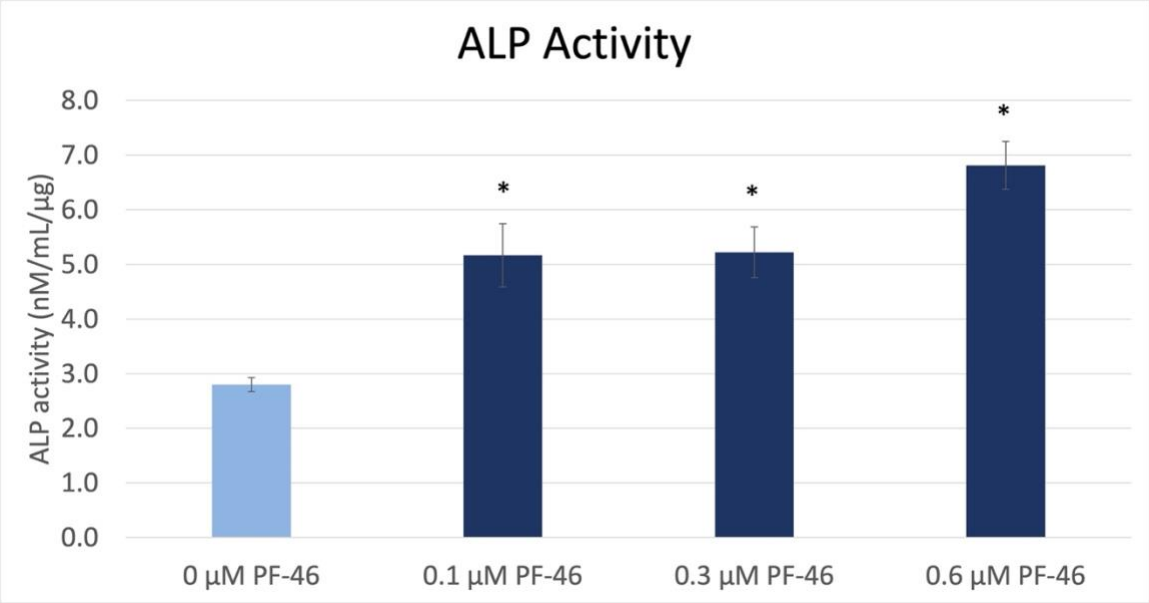

FIGURE 8: ALP assay results showing the mean of 4 wells per treatment group with SEM. An asterisk shows statistical significance compared to 0 µM PF-46.

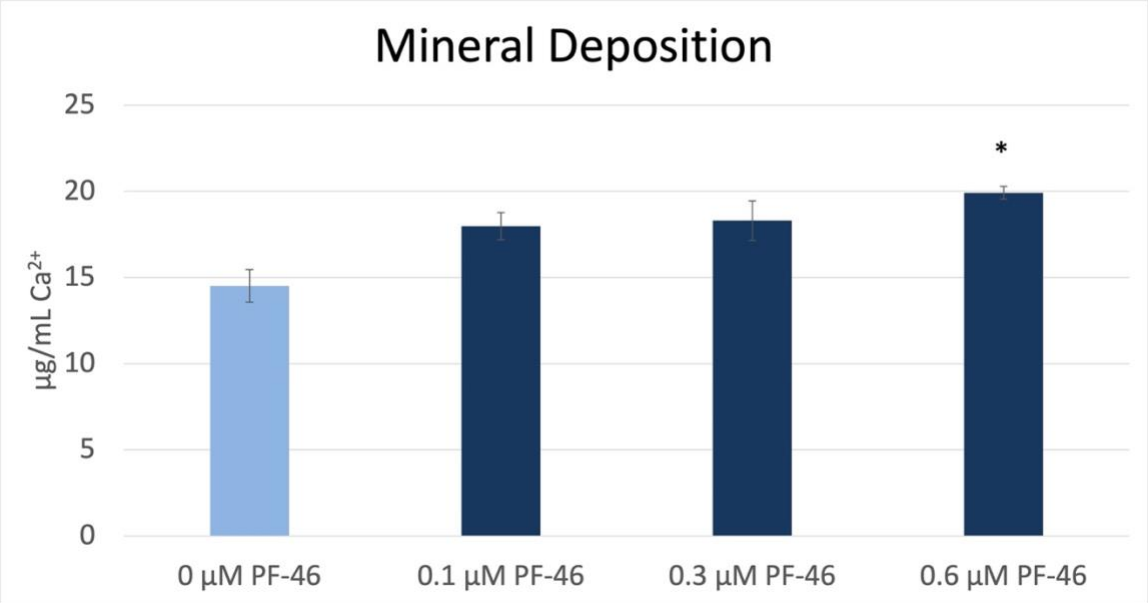

FIGURE 9: Mineral deposition assay results showing the mean of 4 wells per treatment group with SEM. An asterisk shows statistical significance compared to 0 µM PF-46.

TABLE 1

Summary of results for proliferation, ALP activity, and mineral deposition

Outcome	**Group**	**N**	**Mean**	**SD**	**SE**	**Min**	**Max**
Cell counting (cells/mL (10^4))	0 μM PF-46	3	33.67	8.10	4.68	28.50	43.00
	0.1 μM PF-46	4	40.25	9.75	4.87	26.75	49.75
	0.3 μM PF-46	2	45.50	5.30	3.75	41.75	49.25
	0.6 μM PF-46	4	53.50	5.06	2.53	49.00	60.75
MTS proliferation (absorbance OD 490 nm)	0 μM PF-46	5	0.242	0.062	0.028	0.172	0.290
	0.1 μM PF-46	4	0.324	0.008	0.004	0.318	0.335
	0.3 μM PF-46	4	0.314	0.006	0.003	0.306	0.319
	0.6 μM PF-46	4	0.289	0.015	0.007	0.267	0.300
ALP activity (nM/mL/μg)	0 μM PF-46	4	2.80	0.25	0.13	2.54	3.13
	0.1 μM PF-46	4	5.17	1.15	0.58	4.25	6.85
	0.3 μM PF-46	4	5.22	0.93	0.46	4.52	6.58
	0.6 μM PF-46	4	6.81	0.87	0.44	6.02	7.84
Mineral deposition (μg/mL Ca^{2+})	0 μM PF-46	4	14.51	1.87	0.93	12.65	16.14
	0.1 μM PF-46	4	17.99	1.58	0.79	16.43	19.62
	0.3 μM PF-46	4	18.30	2.30	1.15	15.47	20.32
	0.6 μM PF-46	4	19.91	0.74	0.37	18.89	20.50

TABLE 2

Pair-wise comparisons – Cell counting

Result	**p-value**
0 μM PF-46 & 0.1 μM PF-46 n.s.	0.861
0 μM PF-46 & 0.3 μM PF-46 n.s.	0.343
0 μM PF-46 < 0.6 μM PF-46	0.025
0.1 μM PF-46 & 0.3 μM PF-46 n.s.	0.907
0.1 μM PF-46 & 0.6 μM PF-46 n.s.	0.189
0.3 μM PF-46 & 0.6 μM PF-46 n.s.	0.440

TABLE 3

Pair-wise comparisons – MTS proliferation

Result	**p-value**
0 μM PF-46 & 0.1 μM PF-46 n.s.	0.063
0 μM PF-46 & 0.3 μM PF-46 n.s.	0.122
0 μM PF-46 & 0.6 μM PF-46 n.s.	0.485
0.1 μM PF-46 & 0.3 μM PF-46 n.s.	0.243
0.1 μM PF-46 > 0.6 μM PF-46	0.006
0.3 μM PF-46 & 0.6 μM PF-46 n.s.	0.052

TABLE 4

Pair-wise comparisons – ALP activity

Result	**p-value**
0 μM PF-46 < 0.1 μM PF-46	0.009
0 μM PF-46 < 0.3 μM PF-46	0.001
0 μM PF-46 < 0.6 μM PF-46	0.000
0.1 μM PF-46 & 0.3 μM PF-46 n.s.	1.000
0.1 μM PF-46 & 0.6 μM PF-46 n.s.	0.207
0.3 μM PF-46 & 0.6 μM PF-46 n.s.	0.144

TABLE 5

Pair-wise comparisons – Mineral deposition

Result	**p-value**
0 PF-46 & 0.1 μM PF-46 n.s.	0.079
0 μM PF-46 & 0.3 μM PF-46 n.s.	0.129
0 μM PF-46 < 0.6 μM PF-46	0.001
0.1 μM PF-46 & 0.3 μM PF-46 n.s.	0.999
0.1 μM PF-46 & 0.6 μM PF-46 n.s.	0.233
0.3 μM PF-46 & 0.6 μM PF-46 n.s.	0.676

DISCUSSION

This study investigated the proliferation, differentiation, and mineral deposition by DPSCs when exposed to various concentrations of the Pyk2 inhibitor, PF-46. Depending on the assay, cells were exposed to 0, 0.1 μM, 0.3 μM, or 0.6 μM PF-46, and cultured for either 1, 4 or 14 days, and then assayed for proliferation assay, cell growth, ALP activity and mineral deposition, respectively.

The results from direct cell counting following a 4-day incubation period with various concentrations of PF-46 are summarized in Figure 6 and Tables 1 and 2. When compared to 0 μM PF-46, the 0.6 μM PF-46 group had a statistically significant higher cell count. Although not statistically significant, the 0.1 μM and 0.3 μM PF-46 groups also had a higher cell count number compared to 0 μM PF-46. The graph in Figure 6 showed a general trend of increasing cell count as PF-46 was increased up to 0.6 μM PF-46. We also performed an MTS assay to examine DPSC proliferation. The MTS proliferation assay was performed following a 1-day incubation period with the different PF-46 concentrations, and the results of this assay are summarized in Figure 7 and Tables 1 and 3. There were no statistically significant differences when comparing the 0 μM PF-46 group to the other 3 groups, but the groups that contained PF-46 generally had an increased values for the proliferation assay. Interestingly, the only statistically significant difference between groups was a higher response for the 0.1 μM PF-46 compared to 0.6 μM PF-46. The MTS proliferation assay is a measure of cellular mitochondrial metabolic activity, and this is generally proportional to cell number. However, the 0.6 μM PF-46 group had the highest cell count from direct counting. It is possible that the 0.6 μM PF-46 group had already gone through the peak of its growth phase when the MTS proliferation

assay was performed, and this would explain its higher cell count but lower response to the MTS proliferation assay.

The results from the ALP assay following a 14-day incubation period with various concentrations of PF-46 are summarized in Figure 8 and Tables 1 and 4. All 3 groups that contained PF-46 (0.1, 0.3, and 0.6 μM) had a statistically significant higher ALP activity compared to 0 μM PF-46. Although there was no statistical difference among the groups that contained PF-46, Figure 8 shows a trend of increasing ALP activity as the concentration of PF-46 increased up to 0.6 μM. The ALP enzyme plays an important role in bone mineralization, and ALP activity levels correlate with differentiation into mineral-producing cells. The results of this assay show that the 0.1, 0.3, and 0.6 μM PF-46 groups had increased differentiation into a mineral-producing cell line.

The mineral deposition assay was the final assay performed, and these values were obtained after 14 days of incubation with the various concentrations of PF-46. Figure 9 and Tables 1 and 5 show a summary of the mineral deposition data. The 0.6 μM PF-46 group had a statistically significant higher mineral deposition compared to 0 μM PF-46, and there were no other statistically significant differences between groups. Also, the graph in Figure 9 demonstrates that mineral deposition had an increasing trend as the concentration of PF-46 increased up to 0.6 μM. In the formation of hard tissue, mineral deposition is one of the final steps. The results of this assay show that the addition of PF-46, specifically at a concentration of 0.6 μM, increased mineral deposition compared to no PF-46.

To summarize the results, there was a general dose-dependent increase in cell count, ALP activity, and mineral deposition as the concentration of PF-46 increased up to 0.6 μM. Furthermore, the 0.6 μM PF-46 group had a statistically significant higher cell count, ALP activity, and mineral deposition compared to 0 μM PF-46. The higher cell count with 0.6 μM PF-46 indicates that this concentration caused in increased proliferation. Similarly, the increased ALP activity and mineral deposition of 0.6 μM PF-46 indicates that this concentration increased differentiation and mineralization of DPSCs. Thus, the null hypothesis that there would be no difference in proliferation, differentiation, and/or mineralization of DPSCs grown in the presence of PF-46 compared to only cell culture media was rejected.

To the authors' knowledge, no previous study has investigated the effects of modulating the Pyk2 pathway on the function of DPSCs. However, the Pyk2 pathway has been studied previously in both osteoblasts and osteoclasts. Buckbinder and colleagues found that Pyk2 knockout mice enhanced differentiation and activity of osteoprogenitor cells, as does the Pyk2 inhibitor that was used in this study.[38] Gil-Henn et al. also investigated Pyk2 knockout mice, and they found that Pyk2 knockout osteoclast function was inhibited due to impaired microtubule-dependent podosome organization.[39, 41, 78] More recently, Posritong and colleagues researched the effects of a PF-46 loaded into a hydrogel on osteoblast activity and mineral deposition.[46] This hydrogel containing PF-46 promoted ALP activity and mineral deposition, and the response was most pronounced at a concentration of 0.1 μM PF-46.[46] In contrast to this study that was performed with murine bone marrow stromal osteoblasts, our study showed the highest assay responses with a concentration of 0.6 μM PF-46.[46]

One limitation of this study is that the highest tested concentration of PF-46, 0.6 μM, produced the most favorable assay results for cell counting, differentiation and mineral deposition. This maximum value of 0.6 μM PF-46 was chosen based on the concentrations used previously in an osteoblast study which showed maximum responses with a concentration of 0.1 μM PF-46.[46] A future experiment that included even higher concentrations of PF-46 would be beneficial to determine the optimal concentration of PF-46. Also, extending the duration of the cultures to 21 days may also show more robust differences between the treatment groups and the negative vehicle control. Several studies using osteoblasts has shown increased mineral deposition after 21 days.[76]

Another limitation of this study is that it is unclear based upon the collected data if the DPSCs differentiated into an osteoblast-like or odontoblast-like lineage. There are previous studies that have investigated the histology of the regenerated pulp-dentin complex *in vivo*, and the results are variable. One case report stated that apical closure and the increase in root length and width were due to the deposition of cementum-like tissue without dentin.[81] Other studies have shown root formation of a combination of cementum-like tissue, bone-like tissue, and dentin-like tissue.[14, 72] It is difficult to identify odontoblast-like cells *in vitro* due to their lack of unique cellular markers and overlap with osteoblast-like cells. Dentin sialoprotein (DSP) is expressed by both odontoblast-like and osteoblast-like cells, although DSP is expressed nearly 400 times greater in odontoblasts.[82] Similarly, expression of the intermediate filament protein, nestin, has been suggested to be used to identify odontoblast-like cells.[83, 84] In a future experiment, quantitative polymerase chain reaction or RNA-sequencing analyses of these and similar

genes can potentially be performed to elucidate clues as to whether the DPSCs are differentiating into more of an odontoblast-like or osteoblast-like cell type.[85]

External cervical resorption (ECR) is a challenging clinical entity to treat. Since Pyk2-deletion and Pyk2 inhibitors reduce osteoclast bone resorbing activity, Pyk2-targeted therapies such as PF-46 theoretically might have a positive effect in arresting or reversing osteoclast activity in ECR lesions.[86] Although not fully understood, it is believed that ECR develops from both an initiating factor and a stimulating factor.[87] A common initiating factor can be local destruction of the normal PDL structure, such as gap in the cementoenamel junction that could allow granulation tissue to reach the dentin.[87] For ECR to continue, a stimulating factor such as bacteria or continuous mechanical force is also required.[87] When exposed to dentin and stimulated, osteoclasts resorb dentin which can subsequently be replaced with new bone formation by osteoblasts.[88] Inhibiting Pyk2 has been shown in previous studies to promote osteoblast activity and inhibit osteoclast activity.[46] Because of this, a potential future project could investigate if Pyk2-targeted therapies such as PF-46 could be beneficial in treating ECR lesions.

SUMMARY AND CONCLUSIONS

In conclusion, the null hypothesis was rejected based on the results of this study. When compared to the vehicle control (0 μM PF-46), the 0.6 μM PF-46 group had a significantly higher cell count, ALP activity and mineral deposition. Also, the 0.1 and 0.3 μM PF-46 groups had significantly higher ALP activity compared to the 0 μM PF-46 group. There was a general concentration-dependent increase in assay results as the concentration of PF-46 increased up to 0.6 μM. A future study that includes higher concentrations of PF-46 would be beneficial to determine the optimal concentration of PF-46. Although further research is needed, these results suggest that strategies that target Pyk2 may potentially be used to improve the osteogenic differentiation of DPSCs to aid endodontic regeneration.

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ABSTRACT

THE EFFECTS OF A PYK2 KINASE INHIBITOR ON THE PROLIFERATION AND DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS

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Introduction: Regenerative endodontic procedures are an effective treatment option for immature teeth with infected necrotic pulps to allow for healing and potential continued root development, yet challenges to ideal treatment outcomes remain. Consistent development of root length and width of dentin remains a challenge, as does development of the pulp-dentin complex. Previous *in vitro* studies have assessed the role of different growth factors and bioactive molecules in combination with scaffolds to potentially facilitate continued development of the pulp-dentin complex using dental pulp stem cells (DPSCs). The proline-rich tyrosine kinase 2 (Pyk2) is linked with osteoblast activity and the regulation of bone mass. Further, the Pyk2 inhibitor PF-4618433 (PF-46) has been shown in previous studies to enhance osteoblast activity and mineral deposition *in vitro*. However, whether Pyk2 targeting promotes the osteogenic differentiation of DPSCs remains unknown.

Objective: The purpose of this study was to investigate the effect of a Pyk2 inhibitor, PF-46, on the proliferation, differentiation, and mineralization of human DPSCs.

Materials and Methods: Human DPSCs were cultured in 24-well plates with α-MEM with 10% FBS, and containing 0 μM (vehicle control) or 0.1 μM, 0.3 μM, or 0.6 μM PF-46. Fresh media and treatments were replaced every 2-3 days. After 1 day incubation, cytotoxic effects were evaluated by using an MTS proliferation assay. After 4 days of treatment, direct cell counting was performed. To induce osteogenic differentiation, ascorbic acid and β-glycerol phosphate were added to the culture media and the DPSCs were cultured with PF-46 for 14 days. Then, an alkaline phosphatase (ALP) assay and mineral deposition assay were performed. Differences between

treatment groups were analyzed by a one-way ANOVA followed by pair-wise tests conducted using Tukey's multiple comparisons procedure with a 5% significance level.

Results: The 0.6 μM PF-46 group had a significantly higher cell count, ALP activity and mineral deposition when compared to 0 μM PF-46. The 0.1 and 0.3 μM PF-46 groups also had significantly higher ALP activity compared to the 0 μM PF-46 group after 14 days of incubation. There was a general trend of increased differentiation and mineral deposition as the concentration of PF-46 increased from 0.1 μM to 0.6 μM.

Conclusion: There was a general concentration-dependent increase in cell count, differentiation, and mineral deposition by human DPSCs as the concentration of PF-46 increased from 0 μM up to 0.6 μM, with the highest activity observed with 0.6 μM PF-46. Although further research is needed, these results suggest that strategies that target Pyk2 may potentially be used to improve the osteogenic differentiation of DPSCs to aid endodontic regeneration.

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