THE EFFECTS OF NICOTINE ON GROWTH, SURFACE MARKER EXPRESSION, AND OSTEOGENIC CAPACITY OF STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED) AND DENTAL PULP STEM CELLS (DPSC)

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Abstract

THE EFFECTS OF NICOTINE ON GROWTH, SURFACE MARKER EXPRESSION, AND OSTEOGENIC CAPACITY OF STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED) AND DENTAL PULP STEM CELLS (DPSC)

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Background: The gaining popularity of electronic nicotine delivery systems (ENDS) and inhaling aerosols ("vaping") among young people have exposed them to unprecedented amounts of nicotine. Fourth generation ENDS manufacturers attract the adolescent demographic with a smooth vaping experience with enticing flavors such as "Piña Colada" or "Peppermint Blast." Components of e-liquids in cartridges of ENDS combine protonated nicotine, also referred as nicotine salts, with flavors in a propylene glycol or vegetable glycerin solvent. Unregulated, high amounts of nicotine in ENDS products have been associated with increased levels of nicotine in the circulating blood of users with an average concentration of about 30ng/mL. Little is known on the effects of protonated nicotine on oral health, specifically on stem cells from human exfoliated

deciduous teeth (SHED) and dental pulp stem cells (DPSC), and its implications on the repair and regeneration processes for the soft and hard tissues.

Objectives: The aim of this study is to evaluate the effects of nicotine on SHED and DPSC cell growth, surface marker expression, and osteogenic differentiation capacity.

Methods: Isolated SHED cells and DPSC cells were seeded in separate T-75 flasks with culture medium treated with 0ng/mL, 30ng/mL, and 300ng/mL of concentrated protonated nicotine. Population doublings were recorded over several passages once confluency was reached. The antigen surface marker expressions of SHED and DPSC sample populations were analyzed using flow cytometry. SHED and DPSC cell populations were cultured in osteogenic induction media, then after 7 days, collected for RNA Isolation and RT-qPCR gene expression of runt-related transcription factor 2 (*RUNX2*), secreted phosphoprotein 1 (*SPP1*), and bone gamma-carboxyglutamate protein (*BGLAP*).

Results: Population doublings showed significant decrease in growth rate of both 30ng/mL and 300ng/mL nicotine treated SHED and DPSC cell populations compared to the untreated controls. Cell surface marker expression of nicotine treated SHEDs and DPSCs were consistent with the typical presentation of human mesenchymal stem cells (MSC). Analysis of cell populations from 0ng/mL, 30ng/mL and 300ng/mL nicotinic concentrations cultured in osteoblast inducing media using qPCR revealed no changes in *RUNX2*, *SPP1* and *BGLAP* gene expression compared to undifferentiated controls.

Conclusion: Nicotine exhibits an adverse effect on the proliferation rate of SHEDs and DPSCs but shows no effect on their stem cell marker expression and osteogenic differentiation capacity.

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Chapter 1: Introduction

Background and Significance

The rise of electronic nicotine delivery systems (ENDS) in the last 10 years has been propelled by advertising from companies as a safer alternative to cigarettes and the marketing and producing of enticing flavors such as “Piña Colada” or “Peppermint Blast” (Eaton et al., 2018) (Almeida-da-Silva et al., 2021). According to the report *E-Cigarette Use Among Youth and Young Adults,* by the Surgeon General, they are characterized by being non-combustible, battery powered, the ability to deliver nicotine, flavors, and other additives through aerosolization. The evolution of ENDS started with the disposable e-cigarette, to now the 4th generation of “pod-mods”, like JUUL, that have a sleek, discrete prefilled or refillable pod cartridge and a modifiable system, appealing to youth and adolescents (Mercier et al., 2024). There are currently 10% of students or about 2.8 million youth, using some form of tobacco as reported by the 2023 Annual National Youth Tobacco Survey. Results from survey showed that among middle school and high school students that use tobacco products, ENDS were the most popular product, while cigarette and cigar use remain at an all-time low. According to the survey, more than 1 in 4 U.S. e-cigarette users use the product every day and 9 out of 10 use flavored e-cigarettes. Despite efforts from the Food and Drug Administration (FDA) to block the sale of flavored ENDS from appealing to youth and attempts to regulate the influx of unauthorized products being imported and launched, ENDS manufacturers find loopholes and ways to bring their product to market (Mercier et al. 2024). Additionally, neither strict quality control or guidelines for ENDS have been established,

leading to excess amounts of toxic substances, nanoparticles, heavy metals, and unchecked amounts of nicotine (Goniewicz et al., 2019) (Nardone et al., 2019).

Given that a large portion of patients in orthodontics are youth and adolescents, it is important for orthodontic providers to recognize the rise of ENDS in their patient population. Orthodontic care is initiated when most or all permanent teeth have been erupted or when there is a discrepancy in craniofacial growth warranting intervention, typically between 7-18 years old, a time of “vaping” experimentation. It has been reported by the Surgeon General that the developing brain of youths and adolescents are more susceptible to the negative effects of nicotine including addiction, attention disorders, cognition impairment, and mood disorders. A comprehensive review underlined heavy metals, toxic flavoring chemicals, carbonyls, and reactive oxygen species emissions contribute to the cytotoxic effects on cells of the oral epithelium and mucosa (Ebersole 2020). Research in bone metabolism have shown nicotine to induce osteoblast apoptosis and increase the activity of osteoclasts, possibly influencing the bone remodeling process of resorption and formation in orthodontic tooth movement (Mohajeri et al., 2023) (Michelogiannakis et al., 2018). A systematic review of orthodontic tooth movement in rats exposed to nicotine for 10-14 days showed increased movement, but at the expense of root resorption and decreased alveolar bone height (Michelogiannakis et al., 2018) (Lee et al., 2021). It is of clinical value for orthodontists to be mindful of balancing forces and bone turnover during the movement of teeth, and therefore an importance for being aware of patients smoking or vaping.

Nicotine in Electronic Nicotine Delivery Systems

The cartridges of ENDS contain a solution comprised of nicotine and flavoring ingredients in a carrier solution of propylene glycol, and/or vegetable glycerin (Almeida-da-Silva et al., 2021) (Nicholson et al., 2021). Fourth generation e-liquids such as in JUUL, have been shown to have the highest concentrations of nicotine on the market at about 5% per cartridge (Nardone et al., 2019). Chemical evaluation has shown that concentrations of nicotine varied dramatically among brands uncovering how refill e-liquid cartridge labels deviated considerably from the actual measured values, which would contribute to higher amounts of systemic nicotine exposure (Cheng 2014). A study examining young users of JUUL estimated nicotine blood concentration to be at around 30ng/mL by assessing metabolites of nicotine in urine samples (Nardone et al., 2019) (Goniewicz 2019). A systematic review reported nicotine concentrations in e-liquids that ranged from 0-87.2mg/mL and levels in aerosols to be 0.5-15.4mg per 300 puffs (Cheng 2014). Newer generations of e-liquids, such as the contents of JUUL pods, have converted nicotine from the free-base form primarily utilized in conventional cigarette products to the protonated form, also referred to as nicotine salts, by mixing it with acid to lower the pH and induce protonation, giving the manufacturers a way to increase nicotine concentrations in their products (Talih et al., 2020) (Gholap et al., 2020). A study found individual JUUL pods contained an average of 61mg/mL of nicotine, more than a pack of cigarettes at about 40 mg/mL (Omaiye et al., 2019). Additionally, the inhalation of the protonated form of nicotine ENDS aerosols has been attributed to a smoother inhalation versus the harshness of free base nicotine in cigarettes, a sensory characteristic that may contribute to habits of deep inhalation and frequency of puffs in users (Gholap et al., 2020) (Mercier et al., 2024). The rise in the popularity of ENDS in youth coupled

with unregulated amounts of protonated nicotine can lead to a future of public health problems and individual damaging systemic health effects in our next generation.

Smoking, Nicotine and Oral Health

Nicotine is a major component of both tobacco and ENDS products and is a selective agonist to nicotinic acetylcholine receptors (nAChRs) which are found at the neuromuscular junction, central nervous system, and many other tissues (Yanagita et al., 2008). This results in acute and long-term effects on organ systems, cell multiplication, and apoptosis (Mishra et al., 2015). Smoking and vaping first introduces nicotine into the oral cavity and therefore is first exposed to saliva. Recent reports show that salivary nicotine measured to be almost 87 times higher than in the blood plasma, which in turn increases systemic and oral nicotine absorption (Greenberg et al., 2017). Oral research studies have also found that smoking and nicotine exposure have been associated with alveolar bone loss, dental implant failure, and periodontal disease contributed by nicotine's negative effects on blood flow, cytokine production, inflammation, and connective tissue turnover in the oral cavity (Kim et al., 2012) (Nguyen et al., 2021) (Mohajeri et al., 2023).-Root resorption has been observed with accelerated orthodontic tooth movement due to nicotine by changing RANKL/RANK signaling and increasing osteoclastogenic differentiation and therefore bone resorption (Michelogiannakis 2018) (Ebersole 2020). On a cellular level, nicotine can increase oxidative stress, neuronal apoptosis, DNA damage, and reactive oxygen species (Mishra et al. 2015). Oral tissues not directly exposed to the aerosols of ENDS such as deeper tissues of the periodontium and dental pulp can also be adversely affected. Local and systemic vascular changes have been shown to affect oral tissues in

the dentin-pulp complex by exhibiting altered pulpal blood flow and permeability, an influence on the nutrient supply to the dental pulp, and an impedance of repair and regeneration (Drsquosouza et al., 2021) (Sloan & Smith 2007). Protonated nicotine in e-liquids have been shown to pass though the alveolar-capillaries more rapidly than free base nicotine, entering the systemic circulatory system at a faster rate (Mercier et al., 2024). Recent studies have investigated protonated nicotine effects on systemic inflammation and pulmonary and cardiovascular health, but knowledge of the effects of nicotine on oral health is lacking. Given the rapidly evolving production of ENDS within the past 5-10 years and an increased risk of periodontal and oral disease with age, long term epidemiologic studies have yet to be investigated (Holliday et al., 2021).

MSCs, DPSCs and SHED cells

Maintenance of bone health involves the constant formation and break-down of bone also known as bone remodeling (Liu, H. et al., 2024). Formation of new bone relies on the recruitment of mesenchymal stem cells (MSCs), which are characterized by their self-renewal and multilineage differentiation potential into adipocytes, chondrocytes, and osteocytes (Baksh et al., 2004). Commitment of MSCs to the osteoblastic lineage under specific conditions are influenced by growth factors, receptors, intracellular signaling, and transcription factors, all of which drives the beginning of osteogenesis (Liu, H. et al., 2024). MSCs are widely distributed throughout the body and have been successfully sourced not only from bone marrow, but also tissues from exfoliated deciduous teeth and dental pulp (Baksh et al., 2004). In animal transplantation studies, MSCs were shown to differentiate into the cells of local tissues and repair damaged tissues showing

their potential in tissue regeneration (Baksh et al., 2004). Due to being easily accessible and retrievable, dental derived MSCs such as human dental pulp stem cells (DPSCs) and stem cells from human exfoliated deciduous teeth (SHED), are an abundant source for oral health research (Liu, J. et al., 2015) (Gronthos et al., 2000) (Miura et al., 2003). Their capability to differentiate towards odontogenic, osteoblastic, adipogenic, and neurogenic lineages *in vitro* makes them highly valued in a wide variety of therapeutic clinical applications (Shi et al., 2020) (Miura et al., 2003). Shi et al. suggested DPSCs and SHEDs exemplify undifferentiated odontogenic capability due to the expression of dentin sialophosphoprotein (DSPP), a specific gene of odontoblasts, *in vivo* (Shi et al., 2020). Gronthos et al. demonstrated that DPSCs transplanted in mice have the ability to differentiate to odontoblast-like cells forming an ordered dentin matrix and tubular structures *in vivo* (Gronthos et al., 2000). Similarly, Miura et al. showed in a study that SHEDs transplanted into mice differentiated into odontoblasts and exhibited an osteoinductive capacity (Miura et al., 2003). After primary and secondary odontogenesis, odontoblasts respond to negative external stimuli and begin to secrete tertiary dentin matrices, supporting a protective barrier to the pulp, and therefore aiding in the regeneration of the dentin-pulp complex (Sloan & Smith 2007).

Effect of Nicotine on MSCs

Dental derived and bone marrow derived MSCs present similar characteristics in self-renewal and multipotent differentiation capabilities. Several studies demonstrate the negative effects of smoking and nicotine exposure on both dental derived and bone marrow derived MSCs (Liu, J. et al., 2015). Yanagita et al. found that dental derived MSCs express muscular and

neuronal-type nAChR subunits and when cultured in nicotinic media, found nicotine to significantly inhibit alkaline phosphatase (ALP) activity, an enzyme expressed in bone and involved in the mineralization of hard tissues (Yanagita et al., 2008). Shaito et al. cultured MSCs following exposure of e-cigarette smoke in conditions promoting osteogenic differentiation and found decreased expression of ALP, type I collagen (COL1), and reduced mineralization capacity (Shaito et al., 2017). Similarly, Kamel et al. showed decreased proliferation and expression of ALP and osteocalcin (OCN), indicative of decreased osteoblast differentiation capability in DPSCs of smokers (Kamel et al., 2020). Studies show that the negative effects of nicotine exposure on MSCs is time and concentration dependent, reflecting the habitual use of smoking or vaping among users. Marinucci et al. found that nicotine reduces osteoblast proliferation and there was a down-regulation of the osteoblast specific gene expression, runt-related transcription factor-2 (*RUNX2*) after chronic exposure to nicotine (>13 days) (Marinucci et al., 2014). Up-regulation of *RUNX2* expression is crucial in the commitment of MSCs to the osteoblast lineage, proliferation of osteoprogenitors, and inducing the expression of major bone matrix protein genes (Komori 2010). There is existing evidence that nicotine is detrimental to the homeostasis and proliferation of bone and thus contributing to reduced bone formation and remodeling.

Research Questions

1. Do different concentrations of nicotine interfere with population doublings and proliferative potential of isolated SHED and DPSC cells?
    a. Null Hypothesis (H_0): Different concentrations of nicotine will not interfere with the populations doublings and proliferative potential of SHED and DPSC cells.

b. Alternative Hypothesis (H_A): Different concentrations of nicotine will interfere with the populations doublings and proliferative potential of SHED and DPSC cells.

2. What is the effect of nicotine on the osteogenic differentiation capacity of SHED and DPSC cells?

    a. Null Hypothesis (H_0): Nicotine has no effect on the osteogenic differentiation capacity of SHED and DPSC cells.

    b. Alternative Hypothesis (H_A): Nicotine has an effect on the osteogenic differentiation capacity of SHED and DPSC cells.

Approvals

Isolated SHED cells were a kind gift by Professor Songato Shi (Miura et al., 2003). DPSCs were isolated from extracted permanent, non-carious premolar teeth as part of routine comprehensive treatment from the dental clinic at the University of Nevada Las Vegas, School of Dental Medicine. The collection of data from human subjects for this research proposal was approved by the University of Nevada Las Vegas Institutional Review Board and was based on a protocol designed with minimum risk.

Chapter 2: Materials and Methods

Concentration of Nicotine

Protonated nicotine salt, (S)-(-)-Nicotine, 99%, Alfa Aesar™ (Thermo Fisher Scientific, Inc.) was utilized to create the nicotine stock solution containing Phosphate-Buffered Saline (PBS, Thermo Fisher Scientific, Inc) stock with 10μg/μL concentration of nicotine. Experimental nicotinic cell media for dosing was formulated using 30ng/mL and 300ng/mL concentrations of nicotine from the nicotine stock solution. Nicotine stock preparation was established based on nicotine C_{max} of fourth generation e-liquids such as those manufactured by JUUL (Bowen & Xing 2015). The 30ng/mL nicotine concentration was chosen based on a study with vape users that showed a blood nicotine level as high as 35ng/mL at a rate of one puff every 20 seconds for 10 minutes (Yingst 2019). The 300ng/mL nicotine concentration represents an extremely high blood nicotine level not seen in the average smoker (Benowitz 2019).

Culture and Propagation of SHEDS and DPSCs

DPSC cells were isolated from extracted premolar teeth of young adults undergoing orthodontic treatment in the dental clinic at the University of Nevada Las Vegas School of Dental Medicine. Teeth selected contained no caries or inflamed pulp tissue. After extraction, teeth were placed in alpha-Modified Essential Medium (α-MEM, Gibco™ Modified Essential Medium α (1x) + GlutaMAX ™ with 10% v/v FBS, 1% L-ascorbic acid, 1% v/v penicillin/streptomycin) prior to pulp removal. Any soft tissues attached were removed and teeth were placed in 70% ethanol for 10 seconds. Exposure of the pulp tissue was achieved by sectioning at the cementoenamel junction using a rotary instrument (*Dremel®*) and saline irrigation. Pulp tissue was extracted using forceps

and NiTi Endo Hand K-Files and then shredded with a scalpel. Tissue was digested with 4mg/mL collagenase/dispase and incubated at 37° C, 5% CO_2 for 1 hour, filtered through a 70mm cell strainer, washed with 10mL of α-MEM culture medium, and then centrifuged at 400 rpm for 5 minutes. Supernatant was discarded and the pelleted cells were re-suspended in serum-free α-MEM media in preparation for cell isolation.

Isolated SHED and DPSC cell lines were placed into 15mL falcon tubes with 5mL of α-MEM media and centrifuged. After the supernatant was discarded, 1mL of α-MEM was added to re-suspend the pellet. SHED and DPSC cell lines were seeded into three T-75 flasks each with 10mL of control media (no nicotine), 10mL of media with 30 ng/mL concentration of nicotine, or 10mL of media with 300 ng/mL concentration of nicotine. Flasks were incubated at 37°C and 5% CO_2. Fresh α-MEM culture media was replaced every Monday, Wednesday, and Friday. Once 80% cell confluency was reached, SHED and DPSC cell lines were sub-cultured. Previous media was removed and cells in flasks were washed with 10mL of PBS to remove debris and traces of fetal bovine serum (FBS). The PBS was then removed and cells were harvested with 3mL of Accutase™ Cell Detachment Solution (BD Biosciences™) incubated at 37°C for 5 minutes. Flasks were microscopically examined to confirm detachment of cells from the flask surface. Cells and Accutase™ contents were removed from flasks and centrifuged, the supernatant removed, cells resuspended with 1mL of α-MEM media, and 15μL of cell suspensions were then counted in a hemocytometer.

Population doublings (PDL) after each passage were recorded. PDL of SHED cells grown in 0ng/mL, 30ng/mL, and 300ng/mL concentrations of nicotine were calculated over 36 days. PDLs of adult DPSCs grown in 0ng/mL, 30ng/mL, and 300ng/mL concentrations of nicotine were

collected over 95 days. Population doubling experiments were conducted between passages 14-22 for SHEDS and 7-18 for DPSCs.

Flow Cytometry

The antigen profiles of SHED and DPSC cell surface marker expressions were evaluated by Flow Cytometry using the Attune NXT flow cytometer system (Invitrogen). Guidelines from the International Society for Cellular Therapy (ISCT) were followed to distinguish specific surface antigens (Dominici et al., 2006). Expression of the following surface antigens were quantified using the Stemflow Human MSC Analysis Kit (BD Biosciences™) for identifying human MSCs: CD73, CD90, CD105, and CD44 (Dominici et al., 2006). Surface antigens CD45, CD34, CD11b, and CD19 were utilized as negative controls, ensuring cells were not of an hematopoietic lineage (Dominici et al., 2006). UltraComp™ compensation beads (Thermo Fisher Scientific, Inc.) and antibodies conjugated with each fluorophore and the Attune NXT Software (Invitrogen) was utilized for data collection and analysis.

Cells from post-isolation passage 16 for SHEDs and passage 8 for DPSCs, were detached using Accutase™, washed, and resuspended at 1 x10^7 cell/mL in BD™ Pharmingen Stain Buffer (BD Biosciences™). Nicotine treated SHEDs and DPSCs had therefore been in culture with nicotine for several passages prior to the experiments. Manufacturer guidelines were followed to prepare antibody tubes: 100µL of cell suspension was pipetted into each antibody tube, incubated in the dark for 30 minutes at room temperature, washed twice with BD™ Pharmingen Stain Buffer, then resuspended in 300-500µL of buffer. 20,000 cells were collected from all samples. Forward scatter

(FSC) and side scatter (SSC) gating was used to narrow the cell population of interest and exclude debris and outliers, while FSC-H vs FSC-W gating was used to eliminate potential doublets.

Culture of DPSCs in osteogenic media

SHEDs and DPSCs (from post-isolation passage 12 for SHEDS and passage 6 for DPSCs) grown under each nicotinic condition, 30ng/mL and 300ng/mL, and negative controls were collected, seeded into 6 well plates, then incubated at 37°C and 5% CO_2 for 7 days in 10mL of Mesenchymal Stem Cell Osteogenic Differentiation Medium (Sigma-Aldrich). Control cells were cultured in standard α-MEM culture media supplemented with 0ng/mL, 30ng/mL, or 300ng/mL of nicotine. Nicotine treated SHEDs and DPSCs had therefore been in culture with nicotine for several passages prior to experiments. Media was refreshed every Monday, Wednesday and Friday and expended cell media was discarded.

RNA Isolation

RNA extraction from SHED and DPSC cells from each group (0, 30 and 300 ng/mL) in either osteogenic or control media was performed using the RNAeasy mini-prep kit (Qiagen) according to manufacturer's instructions. Three RNA sample replicates of SHEDs and DPSCs from each condition were collected. The total RNA concentration and purity was determined using NanoDrop^{TM} One Spectrophotometer (Thermo Fisher Scientific, Inc). RNA integrity was screened by evaluating protein absorbance at a ratio of 260 and 280nm (A260/A280). Samples recorded were within 1.95-2.17 signifying minimal protein contamination.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and cDNA Synthesis

mRNA samples served as the template for complementary DNA (cDNA) synthesis and library construction following manufacturer's protocol using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) to produce triplicates of each sample condition. RNA samples and reagents were chilled on ice. The cDNA master mix reaction reagent comprised of 6μL 10x RT buffer, 2.4μL 25x dNTP mix, 6μL 10x RT random primers, 3μL MultiScribe reverse transcriptase, and 12.6μL nuclease-free Milli-Q-water, totaling 30μL for each sample. Total RNA and nuclease free water was mixed to produce 0.15μg of RNA per reaction and the total RNA was dispensed with Master Mix in PCR tubes for a total volume of 60μL and centrifuged. The cDNA synthesis reaction was performed using Gene Amp PCR System 9700 Thermal Cycler (Applied Biosystems™). The workflow involved primer annealing at 25°C for 10 minutes, reverse transcription of RNA at 37°C for 120 minutes, enzyme deactivation at 85°C for 5 minutes, then cooling at 4°C. The collected cDNA was stored at -20°C.

Quantitative Polymerase Chain Reaction (qPCR)

Prepared genes of interest (GOI) master mix reactions by combining 10μL TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, Inc.) reagent, 1μL TaqMan® GOI primer/probe, 1μL of Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0_VIC) housekeeping primer/probe set (assay ID Hs00420895_gH), and 2μL nuclease free Milli-Q water. Transferred 14μL of GOI master mix and 6μL of respective cDNA sample in triplicate to a 96 well optical plate, then centrifuged, and placed on QuantStudio™ 3 Real-Time PCR instrument (Thermo Fisher Scientific, Inc.). Programmed instrument for fast cycling with the following conditions: enzyme activation at 95°C

for 20 seconds, denaturation at 95°C for 1 second, the annealing/extension at 60°C for 20 seconds.

The following genes of interest primers from Thermo Fisher Scientific were utilized: runt-related transcription factor 2 (RUNX2_FAM) (assay ID Hs01047973_m1), secreted phosphoprotein 1 (SPP1_FAM) (assay ID Hs00959010_m1) that encodes Osteopotin (OPN), and bone gamma-carboxyglutamate protein (BGLAP_FAM) (assay ID Hs01587814_g1) that encodes Osteocalcin (OCN) (Komori 2019) (Chan et al., 2021).

Statistical Analysis

Cumulative population doublings from all experiments were compared using repeated measures analysis of variance (ANOVA) using Prism (Graphpad Software). Statistics for gene expression experiments were performed using Mann-Whitney U test on Microsoft Excel. A p value less than 0.05 indicates statistical significance.

Chapter 3: Results

Population Doublings Assessment

Population doubling level (PDL) refers to the number of times the cell population doubled since its initial isolation. This is related to the cell's logarithmic phase of growth. The figures below show the population doubling number versus days in culture for SHED and DPSC cells grown in nicotine treated and untreated culture medium. The colored shapes on each line denotes the cell population of interests passage or when cells were sub-cultured after reaching confluency. (**Figure 1)** represents the PDL growth of the SHED cells through 36 days. There is a significant slowing in PDL rate of SHEDs grown in 30ng/mL and 300ng/mL compared to the untreated control after day 15 (p value <0.001). The control, SHED cells grown in 0ng/mL concentrations of nicotine, showed steady growth with PDL rate slowing by day 21 and reaching a plateau by day 35.

(Figure 2) below represents the PDL growth of the DPSCs over 95 days. PDLs in both nicotine exposed and non-exposed groups showed a gradual increase with the untreated control displaying PDL growth consistently higher than both the DPSCs grown in 30ng/mL and 300ng/mL concentrations of nicotine. Repeated Measures ANOVA between the three treatment groups showed p <0.00001 indicating significantly different population doublings among groups.

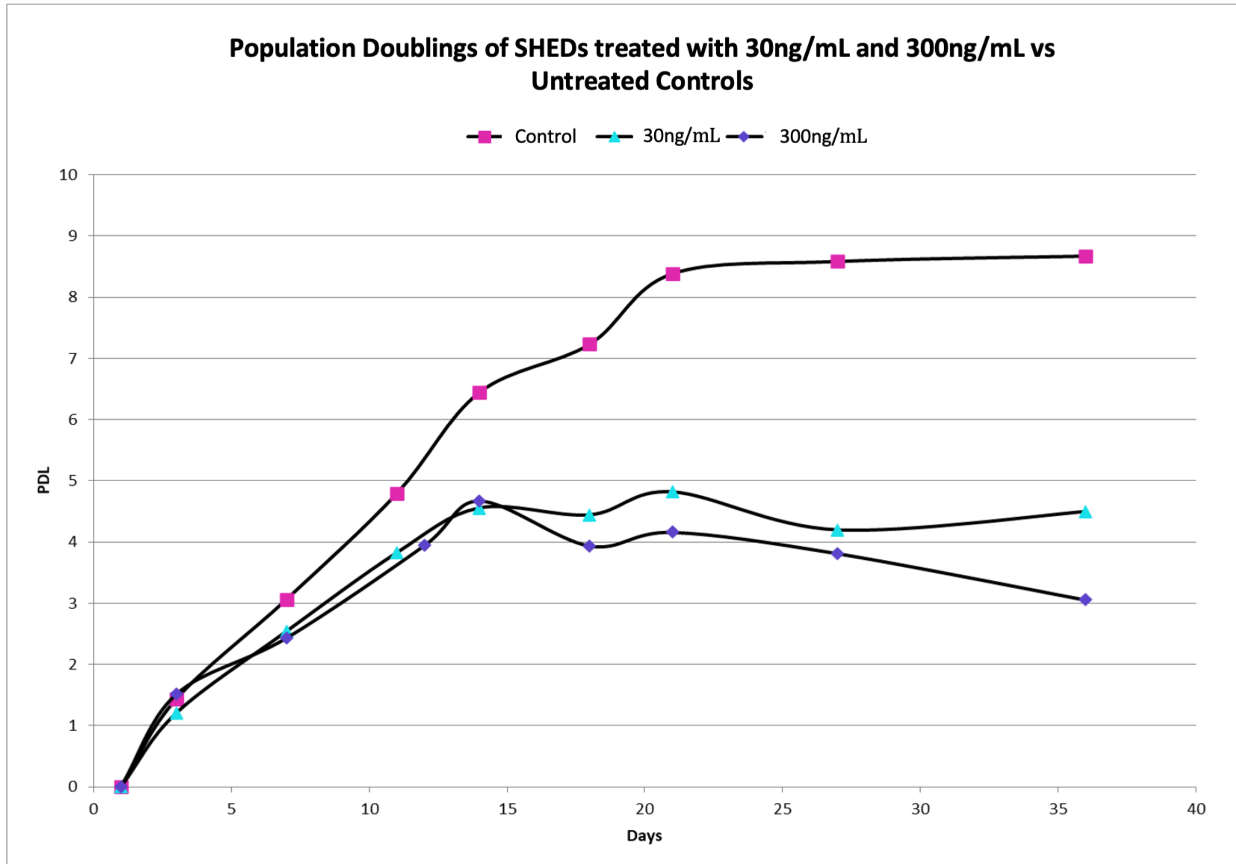

Figure 1. PDL of SHEDs treated with 30ng/mL and 300ng/mL vs Untreated Controls.

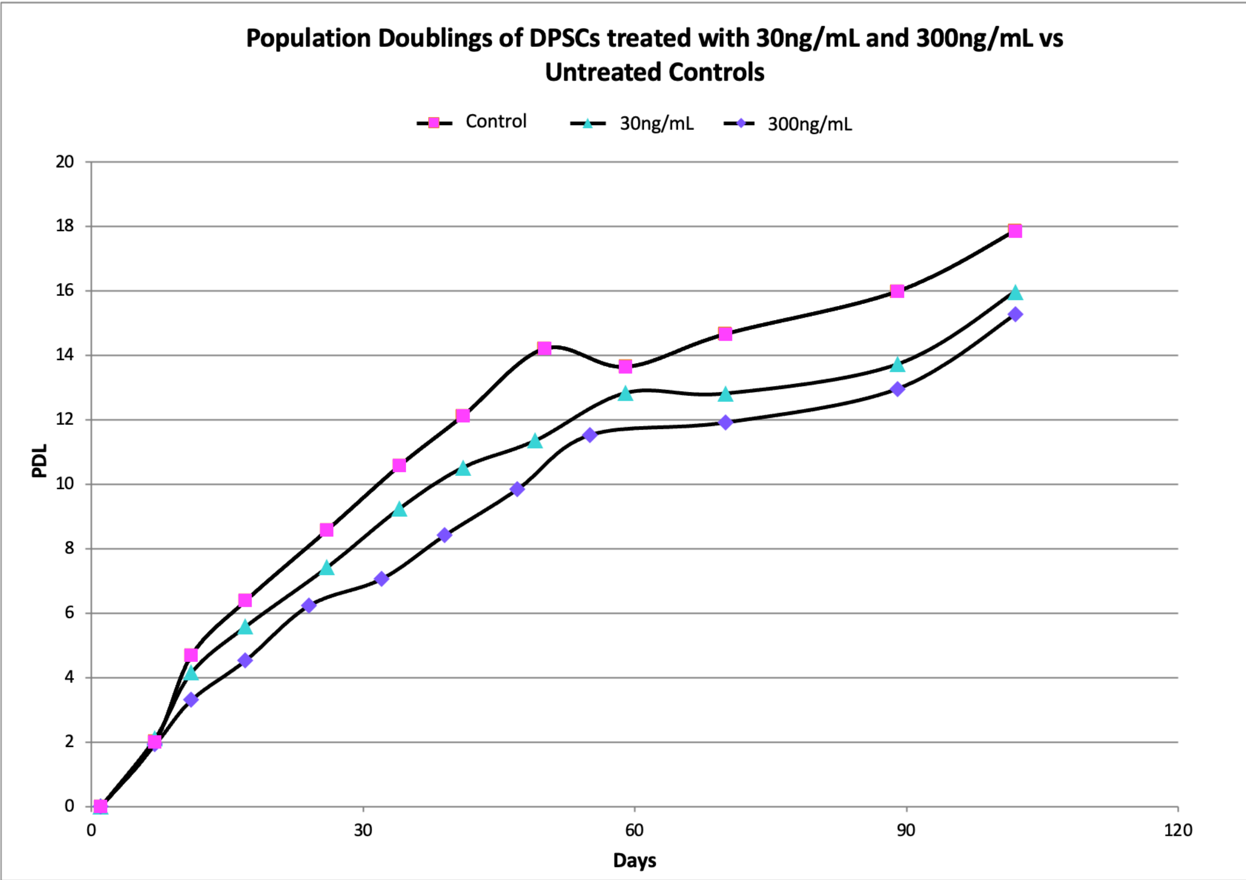

Figure 2. PDL of DPCSs treated with 30ng/mL and 300ng/mL vs Untreated Controls.

Flow Cytometry Characterization

Flow cytometry was used to characterize DPSC and SHED cells as MSCs after being grown in the untreated control media, 30ng/mL, and 300ng/mL nicotinic conditions. **(Figures 3-4)** below display the plotting of forward scatter (FSC) and side scatter (SSC) measurements to represent and characterize the cells of interest within the heterogenous population. A gated percentage of SHEDs and DPSCs, 26% and 7.5% respectively, co-expressed CD73, CD90, CD105, and CD44 (quadruplicate labeled cells) (Dominici et al., 2006). All cells from both populations were negative for hematopoietic markers CD45, CD34, CD11b, CD19, and HLA-DR. **(Table 1)** below shows the percentage of gated cells presenting specific MSC markers from each cell population cultured in all three nicotinic conditions. The results revealed the SHED cell populations consistently exhibited higher positivity (more than 30%) of MSC markers compared to DPSCs in general, although this did not appear to be impacted by the presence of nicotine within each respective population.

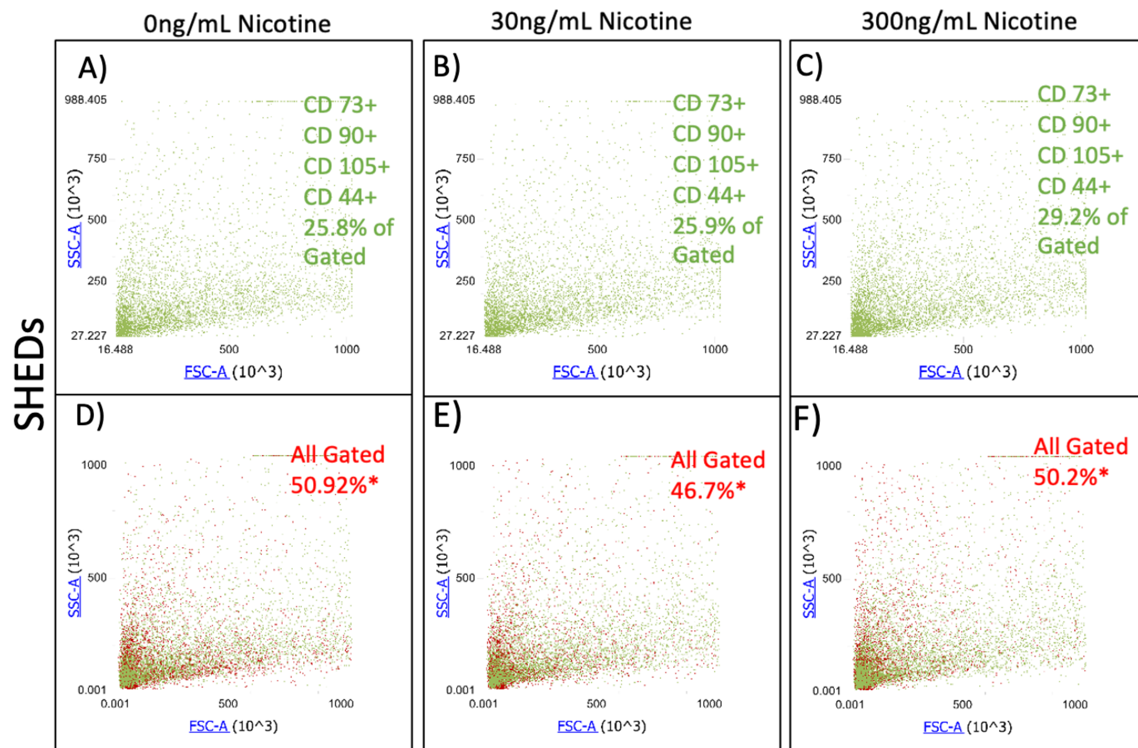

Figure 3. A-F. Flow Cytometry analysis of expanded SHEDs. **A-C.** FSC/SSC plots of SHEDs displaying positive co-expression for all the MSC markers CD 73, CD90, CD 105, and CD 44 at varying concentrations of nicotine (green). The proportions of quadruplicate labelled cells are given within each figured. **D-F.** FSC/SSC plots of SHEDS co-expressing all four MSC markers (green) super imposed over all cells gated to exclude debris and doublet events (red). The proportion of gated cells is given in each figure. . 20,000 events were collected for each experiment.

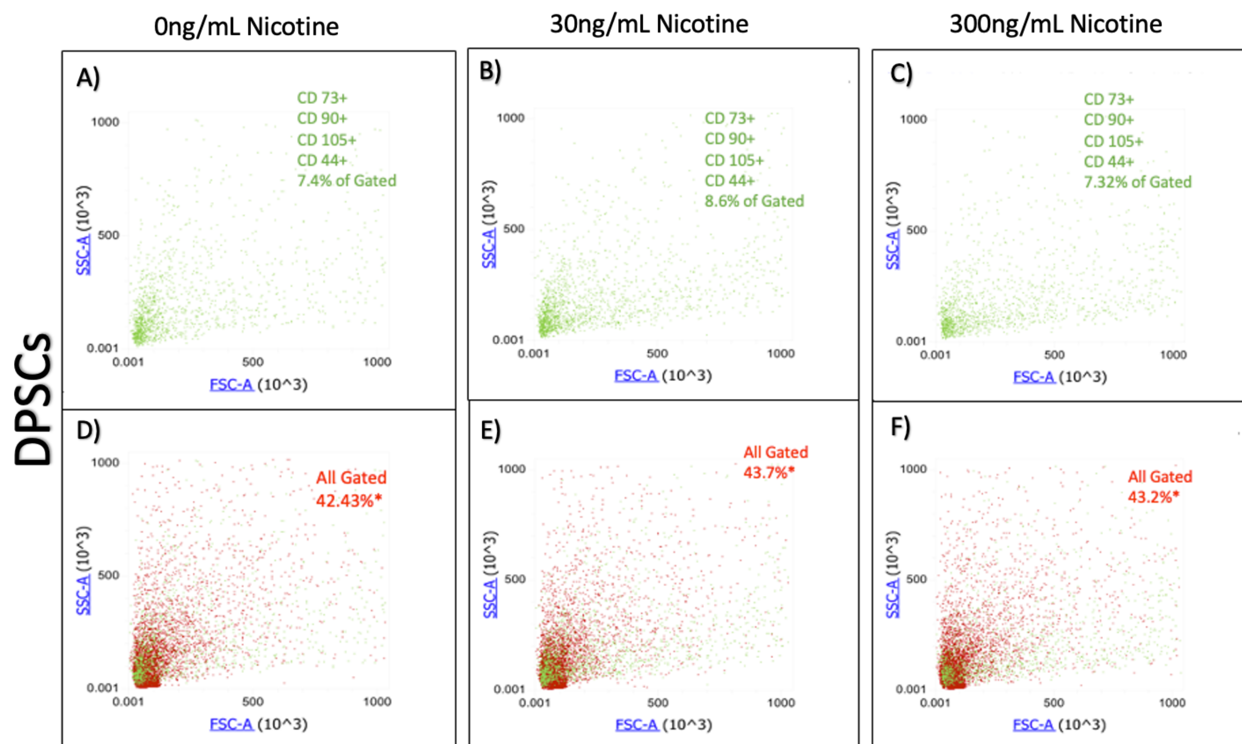

Figure 4. A-F. Flow Cytometry analysis of expanded DPSCs. **A-C.** FSC/SSC plots of DPSCs displaying positive co-expression for all the MSC markers CD 73, CD90, CD 105, and CD 44 at varying concentrations of nicotine (green). The proportions of quadruplicate labelled cells are given within each figured. **D-F.** FSC/SSC plots of DPSCs co-expressing all four MSC markers (green) super imposed over all cells gated to exclude debris and doublet events (red). The proportion of gated cells is given in each figure. 20,000 events were collected for each experiment

Table 1. MSC Individual Surface Marker Comparison of SHEDs and DPSCs utilizing Flow Cytometry.

Nicotinic Condition	SHEDs		Nicotnic Condition	DPSCs	
	Surface Marker	**%Gated**		**Surface Marker**	**%Gated**
SHEDs 0 ng/µL	CD73	67.92	**DPSCs 0 ng/µL**	CD73	25.595
	CD90	76.011		CD90	28.188
	CD105	62.903		CD105	18.972
	CD44	70.336		CD44	36.036
SHEDs 30 ng/µL	CD73	71.875	**DPSCs 30 ng/µL**	CD73	29.73
	CD90	75.71		CD90	32.876
	CD105	63.147		CD105	21.654
	CD44	74.414		CD44	40.643
SHEDs 300 ng/µL	CD90	74.069	**DPSCs 300 ng/µL**	CD90	26.797
	CD44	74.856		CD44	31.196
	CD105	66.218		CD105	19.366
	CD73	71.688		CD73	37.898

Quantitative Polymerase Chain Reaction (qPCR)

Reverse transcription of the mRNA isolates of the SHEDs and DPSCs after growing in osteogenic culture medium was performed to generate cDNA to evaluate *BGLAP*, *SPP1*, and *RUNX2* gene expression using RT-PCR and q-PCR. Cycle threshold or Ct, was measured for each gene of interest and is defined as the number of cycles required before the target of interest accumulates fluorescent signal and exceeds background levels. Three replicate outlier Ct values of DPSCs from the 300ng/mL nicotinic condition grown in both control and osteogenic treated media were omitted (one each from *RUNX2*, *SPP1*, and *BGLAP*). All replicates throughout the triplicate experiments for each nicotine treated and non-treated cell lines were utilized to evaluate gene expression of *BGLAP*, *SPP1*, and *RUNX2* after growth in osteogenic differentiation medium. The relative gene expression was standardized against the expression of the housekeeping, or reference gene, RPLP0. Calculating the mean Ct gene of interest – mean of Ct gene of reference of the cDNA sample was defined as ∆Ct of that gene of interest and controls for variance among samples. The ∆Ct of the gene of interest was used to then calculate the ∆∆Ct which is the ∆Ct — the calibrator (average ∆Ct of the control group). The ∆∆Ct value compares the experimental condition to the control condition and quantifies the experimental effects being observed. The ∆∆Ct is used to calculate the relative quantification (RQ), in order to analyze changes in gene expression, or fold change, in the samples with the gene of interest.

The fold change of *RUNX2*, *SPP1*, and *BGLAP* expression in each cell population sample after growth in osteogenic differentiation medium versus the control, were measured and is shown in **(Figures 5-7)** below. The columns of each graph represent the mean values of triplicate samples and the error bars indicate the standard error of mean (SEM). A fold change above a

value of 1 indicates upregulation of the gene of interest relative to the control. A fold change below 1 indicates a down regulation of the gene of interest relative to the control.

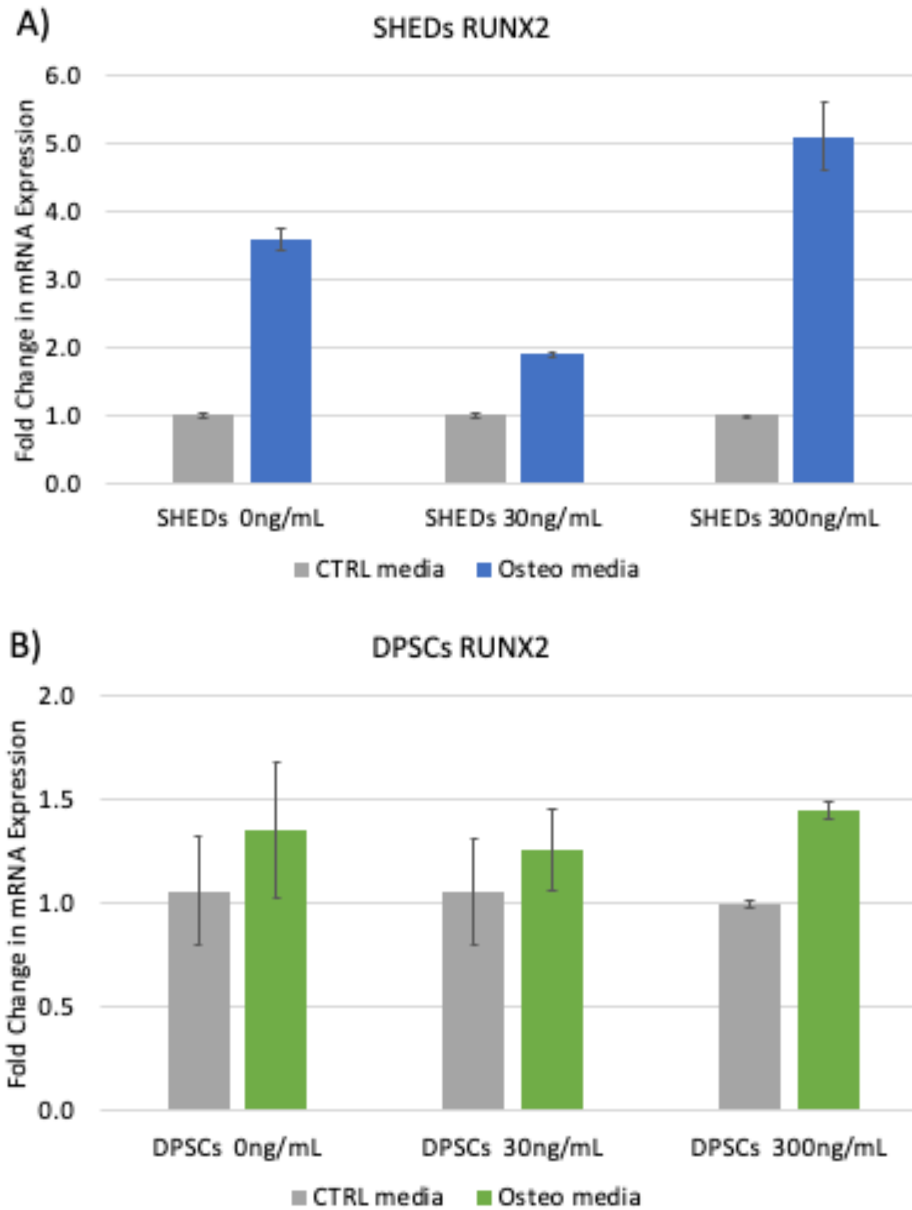

Figure 5. A-B. The fold change in RUNX2 expression in SHEDs and DPSCs. (A) RUNX2 expression in SHEDs under 0ng/mL and 30ng/mL nicotinic conditions after 7 days of osteogenic induction showed a significant increase (P<0.05). SHEDs under the 300ng/mL sample showed an increased expression of RUNX2 but is unreliable due to the housekeeping gene not normalized between the sample and control in all three replicates. (B) DPSCs showed a comparable increase in RUNX2, but was not considered significant (P>0.05).

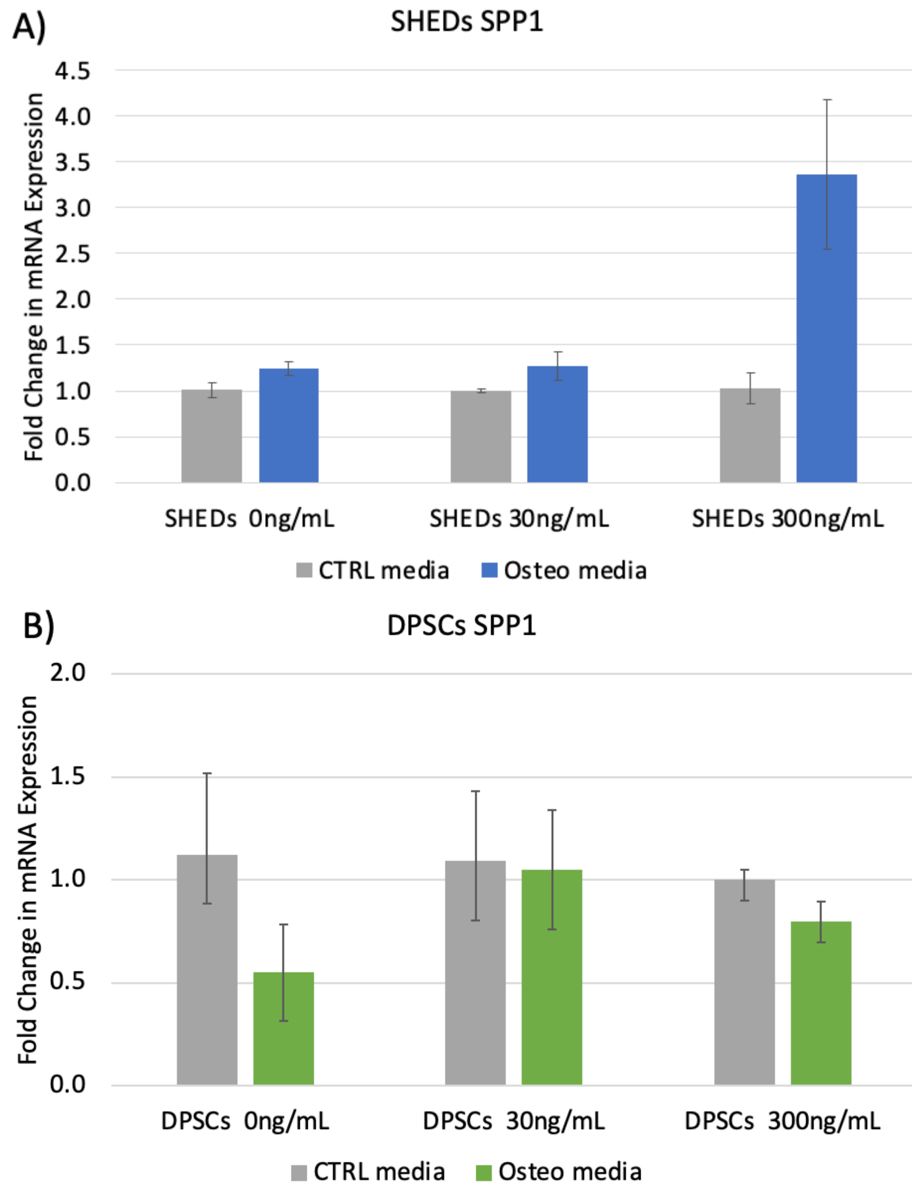

Figure 6. A-B. The fold change in SPP1/OPN expression in SHEDs and DPSCs. (A) SHEDs under the 0ng/mL control and 30ng/mL nicotinic condition showed a slight increase in expression of SPP1/OPN after 7 days of osteogenic induction. SHEDs under the 300ng/mL nicotinic condition showed an increased expression of SPP1 but is unreliable due to the housekeeping gene not normalized between the sample and control in all three replicates. (B) DPSCs showed little to no change. The expression profiles for SPP1/OPN across all samples were not considered significant ($p>0.05$).

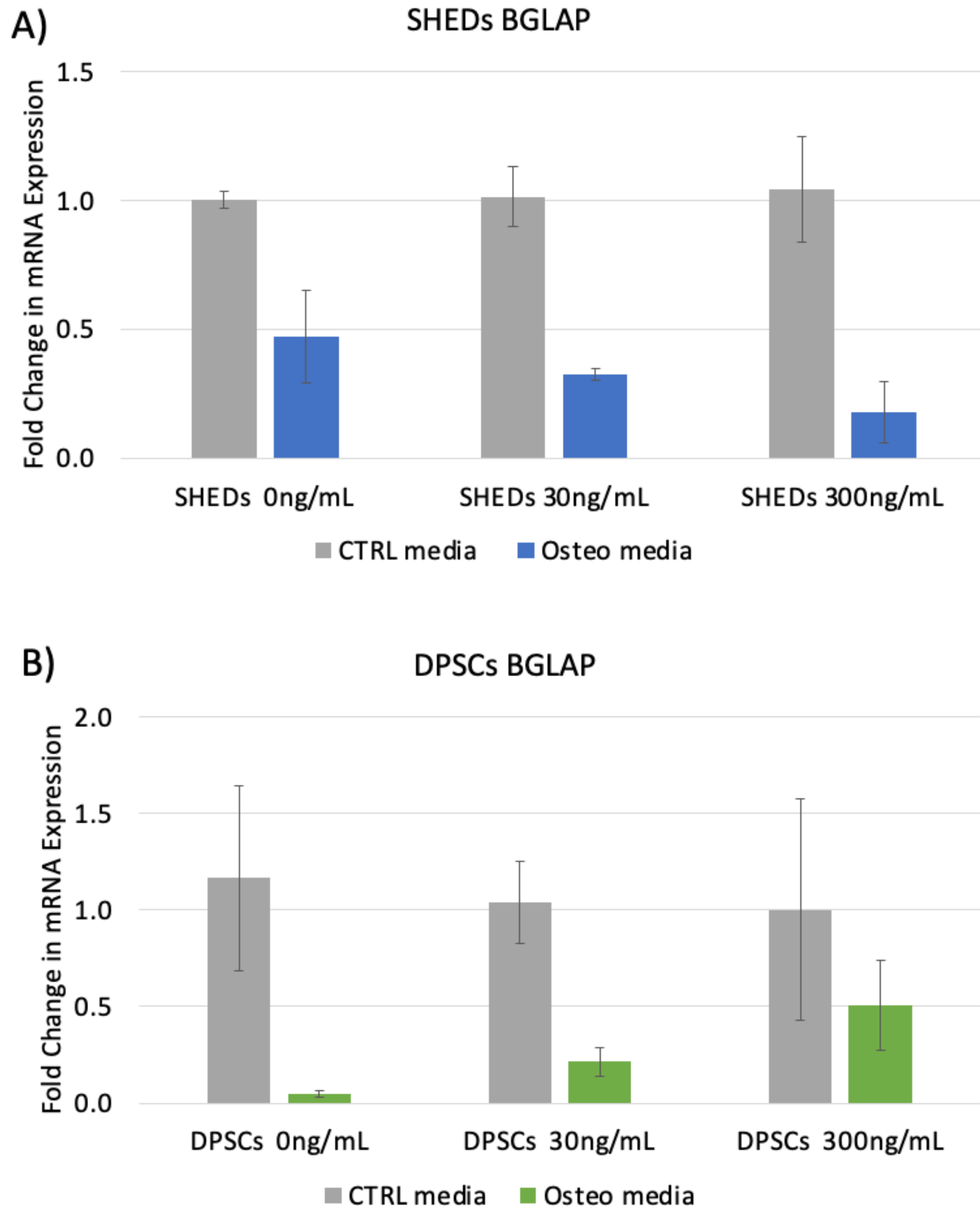

Figure 7. A-B. The fold change in BGLAP/OCN expression in SHEDs and DPSCs. (A) Fold change in the mRNA expression of BGLAP/OCN in SHEDs under the 0ng/mL and 30ng/mL nicotinic conditions after osteogenic induction was significant ($p<0.05$). (B). Fold change in mRNA expression of BGLAP/OCN in all DPSC cell populations after induction showed decreased gene expression. Expression of BGLAP in DPSCs under the 30ng/mL nicotinic condition showed less expression of BGLAP after osteogenic induction and was considered significant compared to those grown in control medium. ($p<0.05$).

Chapter 4: Discussion

Smoking and the increasingly popular vaping, maintains high nicotine concentrations in the human body especially in the oral cavity where it is first exposed. Bone remodeling, periodontal health, and the dentin-pulp regeneration complex have all been negatively impacted by the use of cigarettes and ENDs. The aim of this present study was to determine if protonated nicotine significantly affects the growth, surface marker expression, and osteogenic differentiation capacity of SHEDs and DPSCs. A typical blood concentration of nicotine representative of smokers and vapers (30ng/mL) was utilized and the results suggested that these habits may affect proliferation and gene expression of dental derived stem cells, leading to impaired oral tissue and bone healing. Substantially higher concentrations of 300ng/mL appeared to exaggerate these effects.

Previous studies reported that nicotine does not affect the percentage of apoptosis and viability of SHEDs grown in media dosed with nicotine over the course of 10 days (Wittchow 2022). However, population doubling of these cells grown in nicotinic media over the course of several weeks were not investigated. The results of this study provide evidence that the population doubling capacities of SHED and DPSC cells were affected by increasing concentrations of nicotine. SHED cells grown in both 30ng/mL and 300ng/mL nicotinic concentrations showing a significant decrease in PDL growth compared to cells grown in untreated media suggest the sensitivity of the proliferation capacity of SHEDs when cultured in a nicotinic environment. The measured PDL of the DPSC cell populations showed nicotine's negative influence on DPSC growth as soon as 15 days of exposure. The cell populations grown in 30ng/mL and 300ng/mL nicotinic

concentrations also presented with lower PDL rates indicating that a nicotine concentration as low as 30ng/mL can adversely affect DPSC cell proliferation.

These findings are supported by the research of Zhou et al. concluding that nicotine suppresses the proliferation of periodontal ligament stem cells (PDLSCs), another type of dental derived MSC, in a dose dependent manner (Zhou et al., 2013). When 10^{-3} through 10^{-6} mol/L of nicotine was added to cultured cells, the proliferation rate significantly decreased when compared to untreated controls, whereas 10^{-7} mol/L did not (Zhou et al., 2013). The findings were further substantiated by the observation of vacuolar degeneration and increased granular substance found in the cell cytoplasm of PDLSCs treated with nicotine as little as 10^{-4} mol/L (Zhou et al., 2013). Additionally, Kamel et. al. found that DPSCs cultured from non-smokers were more numerous after three passages when compared to DPSCs from smokers (Kamel et al., 2020). Additionally, in their study, the use of a MTT proliferation assay, a technique used to measure cellular metabolic activity as an indicator of cell viability and proliferation, confirmed a higher mean absorbance rate of the non-smoker group indicating smoking and its contents negatively contribute to the proliferation capacity DPSCs (Kamel et al., 2020).

It has been well studied that these uncommitted dental derived stem cells are vital in the homeostatic response of oral hard and soft tissues, migrating to the area of inflammation and differentiating to a new generation of odontoblasts or cementoblasts in order to secrete tertiary dentin or contribute to pulpal or periodontal regeneration (Zhou et al., 2013) (Sloan & Smith 2007). The relationship of smoking and delayed healing of the oral tissues may be attributed to the poor proliferation rates of SHEDs and DPSCs observed in this study.

The minimal standard criteria to define multipotent human MSCs has been proposed by the International Society of Cell Therapy or ISCT: 1) adherence to plastic, 2) specific surface antigen expression, and 3) multipotent differentiation potential (Dominici et al., 2006). Flow cytometric analysis was conducted in order to characterize the SHED and DPSC populations grown in each nicotinic condition by evaluating the expression of MSC cell markers CD73, CD90, CD105, and CD44 and discriminating against contaminating hematopoietic cells from their heterogenous cell populations. Our analysis revealed nicotine did not appear to affect the proportional expression of mesenchymal progenitor cell surface markers in SHEDs or DPSCs. Previous studies have characterized SHEDs and DPSCs as having shared stem cell properties, although SHEDs differ from DPSCs in their quality of expression (Dominici et al., 2006) (Wang et al. 1231-1240). Consistent with this present study, Wang et. al. also found MSC cell markers to be higher in SHEDs compared to DPSCs and suggested this correlates with greater differentiation potential (Wang et al., 2012). Further investigation of this unique finding can contribute to a greater understanding of the useful applications of dental tissue derived stem cells.

The commitment of MSCs and dental derived stem cells to the osteoblastic lineage can be demonstrated by evaluating the expression of key genes characteristic of osteogenesis. The master transcription factor, *RUNX2*, is crucial for the development and maintenance of bone through the activation and regulation of major bone matrix protein genes in addition to its role in early differentiation and proliferation of immature osteoblasts (Komori 2019) (Chan et al., 2021). In the early stages of osteogenesis, osteoblast specific genes such as COL1A1 (collagen type I), *SPP1* (osteopontin/OPN), *BGLAP* (osteocalcin/OCN) and IBSP (bone sialoprotein) are regulated by *RUNX2,* which is highly expressed in immature osteoblasts and declines in mature

osteoblasts emphasizing its vital role in early differentiation (Chan et al., 2021). OPN and bone sialoprotein are non-collagenous bone matrix proteins involved in forming mineralized tissues such as bone, dentin, and cementum (Wang et al., 2012). OPN also serves an important role in cell adhesion and osteoclast function in the bone remodeling process (Marinucci et al., 2014). OCN is another non-collagenous bone matrix protein excreted both by osteoblasts and odontoblasts in the formation and turnover of hard tissues and has been considered to be a late bone marker for mature osteoblasts (Komori 2022). This study specifically evaluated the expression of *RUNX2*, *SPP1/*OPN and *BGLAP/*OCN genes demonstrating the effect of nicotine on the differentiation ability of SHEDs and DPSCs toward the osteogenic lineage.

The *RUNX2* transcription factor is essential in determining the osteogenic lineage from MSCs and is first detected in pre-osteoblasts then upregulated in immature osteoblasts (Komori 2010). Although there are several other influences, such as *Sp7*/Osterix transcription factor and WnT signaling that direct osteoblast differentiation, *RUNX2* is the first upstream regulator of osteoblast differentiation and is regularly characterized as an early marker for osteogenesis (Komori 2010).

The results from qPCR show an increase in the expression of *RUNX2* in both SHEDs and DPSCs of each nicotinic condition when cultured in osteogenic media. While this study confirmed the increased expression of *RUNX2* in osteogenic differentiating media, it did not show that nicotine negatively affected the osteogenic properties of either of these cell populations. The SHED cell population in all conditions and the control showed about a two-fold or more increase in *RUNX2* compared to the DPSCs, which showed somewhat less of an increase. It can also be appreciated that SHEDs may inherently have a stronger capacity for osteogenic differentiation as concluded by an *in vivo* experiment where both SHEDs and DPSCs were transplanted into mice

but found more bone-like structures and hard tissue mineralization in mice with the SHED transplantation (Wang et al., 2012). Our results show how *RUNX2* may not be immediately affected by nicotine in the early stages of osteoblast differentiation. This is substantiated by the experiment conducted by Marinucci et. al. who did not find a down-regulation of *RUNX2* in cultured osteoblasts after 13 days of chronic nicotine exposure, showing little negative influence of nicotine in acute exposure of less than 13 days (Marinucci et al., 2014). Similarly, our cell population samples were taken from the osteogenic differentiation media at day 7, but may or may not have shown a greater response after longer in culture.

RUNX2 induces the expression for major bone matrix proteins encoded by the genes *SPP1* and *BGLAP* for OPN and OCN proteins respectively (Komori 2022). The progression of osteoblast differentiation is a dynamic process and is reflected by the changing expressions of *RUNX2*, *SPP1*, and *BGLAP*. OPN has been characterized as an early protein product and marker for immature osteoblasts during immature bone matrix formation while OCN is found to be prominent in mature osteoblasts (Komori 2010). A study in mice showed that while *RUNX2* is prominently detected in the early stages of osteogenic differentiation, its expression has been shown to be downregulated in mature osteoblasts and not required for the maintenance of major bone matrix proteins but can actually inhibit osteoblast maturation and development into osteocytes. (Komori 2022).

Our results from qPCR showed no significant difference in the expression of *SPP1*/OPN in either SHED and DPSC cell populations cultured in osteogenic media from any treatment group. Gene expression of *BGLAP*/OCN showed a decrease in fold change between both cell populations

in all nicotinic conditions and the control when cultured in osteogenic differentiating media, although this may not be related to the concentrations of nicotine.

Our findings show how protonated nicotine does not have a significant difference in altering the osteogenic differentiation potential of SHEDs and DPSCs. Results from qPCR presented in **(figures 5-7)** highlighted the slight changes in mRNA expression of *RUNX2*, *SSP1*/OCN, and *BGLAP*/OCN among treatment groups and the control. The results differ from studies that showed suppressed levels of expression of *RUNX2* and OCN in human alveolar bone marrow derived MSCs concluding that nicotine has a negative effect on the differentiation into osteoblasts (Kim et al., 2012). Additionally, our conclusion also differs from studies looking at the effects of nicotine on dental derived MSCs and suggested nicotine deteriorates osteoblast differentiation and mineralization of DPCSs and stem cells of the periodontal ligament after 14 days of culture with osteogenic inducing media (Zhou et al., 2013) (Yanagita et al., 2018). The results in **(figures 6-7)** showed a slight difference in OPN and OCN expression from all treatment groups after culture in osteogenic media, suggesting that nicotine does not alter the osteogenic differentiation capacity of our SHEDs and DPSCs. This is comparable to our results for *RUNX2* expression **(figure 5)** suggesting that the increase in *RUNX2* expression in all treatment groups and the control during differentiation is not adversely affected by concentrations of nicotine. As stated previously, *RUNX2*, *SPP1*, and *BGLAP* are temporally expressed throughout the beginning stages of osteogenesis. The upregulated expression of *RUNX2* in our SHED and DPSC cell populations may indicate the early transition into pre-osteoblasts. However, it can be observed that gene expression of OPN and OCN, markers for immature and mature osteoblast respectively, may not

be upregulated during the event the samples were taken at 7 days of culture in osteogenic media, perhaps suggesting a need to culture for a longer time period.

One interesting finding that certainly interfered with the presented qPCR results from SHEDs and DPSCs replicate samples is that exposure to 300ng/mL nicotine showed that the housekeeping gene, RPLP0, gave inconsistent Ct values compared to our control. A suitable housekeeping gene is utilized to normalize mRNA levels between the experiments and the control by constantly being transcribed among all samples and is critical for reporting accurate quantitative and meaningful data (Dheda et al., 2004). It is plausible that RPLP0 may be biologically affected by the excess amounts of nicotine in our 300ng/mL experimental treatment groups. This may warrant a separate investigation on use of an alternative housekeeping gene and repeating these experiments to decrease variation in our results.

Our study investigated nicotine's effects on cell population growth, surface MSC marker expression, and osteogenic differentiation capacity of SHEDs and DPSCs. There are various dental derived stem cells that can further be evaluated such as periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP), and alveolar bone derived mesenchymal stem cells (ABMSCs) that can aid in the investigation of the effects of nicotine (Nguyen et al., 2021). Many have studied other effects of nicotine on MSCs and osteoblast differentiation but few on specifically odontoblast differentiation from dental derived stem cells. Gronthos et al revealed that odontoblasts and osteoblast expressed similar matrix proteins such as COLL I, ALP, OCN and OPN but specific genes for odontoblasts include dentin sialoprotein and dentin phosphoprotein, which are expressed after dentinogenesis and secretion of the pre-dentin matrix (Gronthos et al., 2000). The study also demonstrated the odontogenic potential of DPSCs by transplantation into

mice and observing sparse and dense calcified nodules, whereas osteoblasts derived from bone marrow stem cells developed extensive sheets of calcified deposits and lipid-laden adipocytes (Gronthos et al., 2000). A study by Yanagita et. al. evaluated the expression of dentin matrix acidic phosphoprotein 1 (DMP-1), an extracellular protein critical for the proper mineralization of dentin, in nicotine exposed DPSCs and found a decrease in expression (Yanagita et al., 2008). Evaluation of odontoblast specific genes in our DPSCs and SHEDs could be beneficial to assess possible clinical significance. Additional assessment in osteogenic differentiation through fixation and staining after growth in osteogenic media may reveal mineral matrix deposition and confirm osteogenic induction. Further *in vitro* studies of these nicotine treated and untreated SHEDs and DPSCs after osteogenic induction may help elucidate development of the correct tissue morphology and function in their respective microenvironments.

Chapter 5: Conclusion

It is understood that smoking and now recently, vaping, is a risk factor for many preventable systemic diseases and the progression of osteoporosis, oral cancer, periodontitis, and compromised bone remodeling. The concentration of nicotine in saliva can reach high levels, especially after habitual and deep inhales of ENDS, making the oral tissues the first to be exposed (Cheng 2014). Of particular interest is youth exposure to higher levels of nicotine from the popular use of vaping products, not only leading to a cycle of product dependence, but also chronic sub-toxic levels of nicotine in the saliva and blood negatively impacting oral and systemic health on a cellular level.

Our study explored the effects of protonated nicotine on the growth, surface marker expression, and osteogenic differentiation capacity of the dental derived human stem cells, SHEDs and DPSCs. The results demonstrated decreased cell proliferation and growth at 30ng/mL and 300ng/mL nicotinic concentrations through the measurement of population doublings over the course of more than 30 days. Flow cytometry analysis revealed nicotine did not have an effect on the inherent expression pattern of these dental derived stem cells, displaying the typical markers for MSCs: CD73, CD90, CD105 and CD44. Analysis with PCR demonstrated that nicotine did not reduce the expression of osteoblastic gene markers *RUNX2, SPP1/*OPN, and *BGLAP*/OCN after 7 days of osteogenic induction. The results reveal that nicotine does not appear to affect osteogenic differentiation capacity after induction.

Although we have concluded that protonated nicotine does not appear to alter SHED or DPSC cell surface marker expression, nor their ability to differentiate towards the osteogenic lineage, nicotine does have a profound influence on their ability to proliferate and therefore may

have several clinical implications. Populations of dental derived stem cells reside within the dentin-pulp complex and surrounding oral tissues, contributing to its vitality through the proliferation, recruitment, differentiation, and migration to sites needing repair and regeneration (Gronthos et al., 2000) (Sloan & Smith 2007). Nicotine and the toxins from smoking and vaping have been shown to contribute to carious lesions and deteriorate periodontal tissues and surrounding bone. Root resorption and bone breakdown during orthodontic treatment underscores nicotine's impact on osseous and periodontal ligament tissues (Jyothish et al., 2021). Goals of orthodontic tooth movement require a balance of mechanical forces, cellular inflammation, and bone remodeling in order to prevent compromised periodontal status (Michelogiannakis et al., 2018) (Jyothish et al., 2021). The preadolescent and adolescent demographic make up a large portion of the population undergoing orthodontic treatment and are prone to experiment with attractive ENDS products, exposing them to unprecedented amounts of protonated nicotine and other toxins. It is imperative for both orthodontists and dental professionals to be aware of nicotine's deleterious effects on oral health and comprehensively address and educate the specific needs of each patient.

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Curriculum Vitae

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Education:

University of Nevada, Las Vegas School of Dental Medicine — April 2024
Master of Science in Oral Biology
Certificate: Orthodontics and Dentofacial Orthopedics

University of California, San Francisco School of Dentistry — June 2017
Doctor of Dental Surgery

University of California, San Diego — June 2010
Bachelor in Science in Human Biology

Professional Work Experience:

Part Time Clinic Instructor, UNLV School of Dental Medicine — April 2023-Present
Las Vegas, NV

Dental Officer, Naval Air Station North Island — June 2020-July 2021
Coronado, CA

Dental Department Head, USS ESSEX (LHD 2) — July 2018-June 2020
United States Navy

Dental Officer, Marine Corps Recruiting Depot — July 2017-June 2018
San Diego, CA

Leadership and Volunteer Work:

AAO Resident Champion, UNLV Orthodontic Residency — April 2023-December 2023
Las Vegas, NV

Voluntary Clinical Instructor, UC San Diego Health Sciences — November 2019-June 2021
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Volunteer General Dentist, Veteran's Village of San Diego — November 2019-June 2021
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Research and Publications:

Masters Research Thesis
Dr. John Colombo, Associate Professor of Biomedical Sciences
"The effects of nicotine on growth, surface marker expression, and osteogenic capacity of stem cells from human exfoliated deciduous teeth (SHED) and dental pulp stem cells (DPSC)"

Published review in *Pacific Coast Society of Orthodontist Bulletin*, Winter 2023
"Differences in Facial Types and Jaw Loading" by Dr. Laura Iwasaki

Scholarship and Awards:

Navy Commendation Medal, United States Navy	June 2020
Surface Warfare Medical Designation Officer, United States Navy	February 2019
Inherent Resolve Campaign Medal, United States Navy	January 2019
Global War on Terrorism Expeditionary Medal, United States Navy	November 2018
Health Professions Scholarship Program, United States Navy	July 2013
Martin Scholarship, UCSF School of Dentistry	September 2013

Professional Affiliations:

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