2024

Effects of acetylsalicylic acid on odontogenesis of human dental pulp cells and TGF-ß1 liberation from dentin

BOSTON UNIVERSITY

HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

DISSERTATION

EFFECTS OF ACETYLSALICYLIC ACID ON ODONTOGENESIS OF HUMAN DENTAL PULP CELLS AND TGF-ß1 LIBERATION FROM DENTIN

by

VISSUTA KHAMPATEE

DDS, Chiang Mai University, 2016
CAGS, Boston University School of Dental Medicine, 2023

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Science in Dentistry
In the Department of Restorative Sciences and Biomaterials

2024

Approved by:

First Reader

_____

Laisheng Chou, DMD, CAGS (Oral Pathology), CAGS (Oral Medicine), PhD
Professor of Restorative Sciences and Biomaterials
Professor and Director of Oral Medicine
Professor of General Dentistry
Henry M. Goldman School of Dental Medicine
Professor of Materials Science and Engineering,
College of Engineering
Boston University

Second Reader

_____

Russell Giordano, DMD, CAGS (Prosthodontics), DMSc
Associate Professor of Restorative Sciences and Biomaterials
Director of Biomaterials
Assistant Dean of Biomaterials and Biomaterials Research
Henry M. Goldman School of Dental Medicine
Associate Professor in Materials Science and Engineering
College of Engineering
Boston University

Third Reader

_____

John Ictech-Cassis, DDS, DMD, CAGS (Prosthodontics)
Clinical Professor of Restorative Sciences and Biomaterials
Clinical Director of the Certificate of Advanced Graduate Study in Operative Dentistry Program
Clinical Director of the Certificate of Advanced Graduate Study in Digital Dentistry Program
Director of the BU Dental Health Center
Henry M. Goldman School of Dental Medicine
Boston University

DEDICATION

It is with genuine gratitude and warm regard that I dedicate this thesis to my parents and my nana, the people who have raised me to become such a strong and independent woman. If it weren't for you, I wouldn't have the confidence and courage to accomplish so many achievements in my life. Also, I would like to give special thanks to all my friends for being a great supporting system for me throughout everything, especially when I hit rock bottom. From time to time, it could be difficult being in a place ten thousand miles from home for so long, but I am truly grateful for your constant support and encouragement despite all the challenges during the pandemic. Having all of you in my life is the most precious thing that has ever happened to me.

ACKNOWLEDGMENTS

I wish to acknowledge and express my warmest gratitude to my advisor Prof. Dr. Laisheng Chou, for his contribution to the conception and critically revising of this Doctor of Science in Dentistry thesis. The completion of this study could not have been possible without his expertise and support.

A debt of gratitude is also owed to Dr. Yuwei Fan, Mr. Minglei Zhao, and Mrs. Claire Zhang for taking the time to guide me through the technical training and provide answers to all my curiosities.

I would like to also give a special appreciation to my dearest friend Dr. Sunporn Namano, for being there for me during the most difficult time and constantly inspiring me with her profound thought and knowledge. It is such a privilege to have known her as one of my best friends.

Last but not least, it is almost impossible to extend enough regard to my family. Because of them, I feel like nothing is beyond our reach if we keep the determination to pursue the goal and never be afraid to go for it.

EFFECTS OF ACETYLSALICYLIC ACID ON ODONTOGENESIS OF HUMAN DENTAL PULP CELLS AND TGF-ß1 LIBERATION FROM DENTIN

VISSUTA KHAMPATEE

Boston University, Henry M. Goldman School of Dental Medicine, 2024

Major Professor: Dr. Laisheng Chou, Professor of Restorative Sciences and Biomaterials

ABSTRACT

Acetylsalicylic acid (ASA), aspirin, is a renowned NSAID that its role in the process of bone metabolism has recently come to light. However, the influence of ASA on the odontogenesis of human dental pulp cells (HDPCs) remains elusive. In search of materials that would synergize the healing potential of the dental pulp, this study aimed to investigate the role of ASA on the odontogenesis of HDPCs *in vitro* and the influence of ASA on TGF-ß1 liberation from dentin.

HDPCs were cultured in a culture medium with different concentrations of ASA: 25, 50, 75, 100, 200 μg/mL and 0 μg/mL as a control. The mitochondria activity of HDPCs was assessed using an MTT assay. Crystal violet staining and triton were used to evaluate cell proliferation rates. ALP activity was measured with the fluorometric assay. Expressions of DSP and RUNX2 were determined with ELISA. DSP and RUNX2 mRNA levels were measured with RT-qPCR. Alizarin red staining was conducted to evaluate the mineralized nodule formation. Dentin slices were submerged in PBS (negative control), 17% EDTA (positive control), and ASA before collecting the solution for TGF-ß1

quantification by ELISA. The data were analyzed by *t* tests and ANOVA followed by the Tukey post hoc tests. P values < 0.05 were considered statistically significant.

The results showed that 25-50 μg/mL ASA promoted mitochondria activity of HDPCs at 72h ($P<0.05$) and yielded significantly higher proliferation rates of HDPCs than the control at 14d and 21d ($P<0.001$). All concentrations of ASA promoted odontogenic differentiation of HDPCs by enhancing the mineralization and the levels of DSP, RUNX2, and their mRNA expression in a dose-dependent manner ($P<0.05$). Also, ASA yielded significantly higher TGF-ß1 liberation after conditioning dentin for 5min ($P<0.001$) and 10min ($P<0.05$).

In conclusion, the data suggest that ASA promotes the odontogenic potential of HDPCs and TGF-ß1 liberation from dentin *in vitro* and might be incorporated into the novel pulp capping materials for dental tissue regeneration.

Keywords: acetylsalicylic acid, regenerative medicine, biomaterials, odontoblast, cell differentiation, dental pulp capping, demineralized dentin matrix

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALP .....Alkaline Phosphatase

ANOVA .....Analysis of Variance

ARS……..Alizarin Red S Solution

ASA……..…….....Acetylsalicylic Acid

ASC……..……Adult Stem Cell

BM……...…Basement Membrane

BME……...……Eagle's Basal Medium

BMSC……...……. Bone Marrow Stem Cell

BMMSC……...…..…….Bone Marrow Mesenchymal Stem Cell

BMP .....Bone Morphogenetic Protein

CFR……..… Code of Federal Regulations

Ca^{2+}……...…….Calcium Ion

$Ca(OH)_2$.....Calcium Hydroxide

CITI.....Collaborative Institutional Training Initiative

CNS……..…….…….Central Nervous System

C=O……..……...…….……Carbonyl Group

CO_2……...…….……...Carbon Dioxide

-COOH……..….....……Carboxyl Group

COX…….Cyclooxygenase

CURE……...…..Clopidogrel in Unstable angina to prevent Recurrent Events

CVD……. Cardiovascular Disease

DI…….……. Distilled water

DPP .....Dentin Phosphoprotein

DPSC.....Dental Pulp Stem Cell

DSP .....Dentin Sialoprotein

DSPP .....Dentin Sialophosphoprotein

ECM.....Extracellular Matrix

EDTA…….…Ethylenediaminetetraacetic Acid

ELISA…….Enzyme-Linked Immunosorbent Assay

FBS .....Fetal Bovine Serum

FDA..... Food and Drug Administration

GEO…… Gene Expression Omnibus

GF…….…..Growth Factor

GI .....Gastrointestinal

H_0.....Null Hypothesis

HCl……..Hydrochloric Acid

HDPC….....Human Dental Pulp Cell

hDPSC……...….... Human Dental Pulp Stem Cell

HRP.....Horseradish Peroxidase

IBC.....Institutional Biosafety Committee

IDE……...…….Inner Enamel/Dental Epithelium

IFN-γ……...……..……..Interferon-γ

IRB.....Institutional Review Board

LAP……Latency-Associated Protein

MMP.....Matrix Metalloproteinases

MSC .....Mesenchymal Stem Cell

MTA.....Mineral Trioxide Aggregate

MTT……..……..Methylthiazolyldiphenyl-Tetrazolium Bromide

MUP…….…..…4-Methylumbelliferyl Phosphate Disodium Salt

NaOH……..…….Sodium Hydroxide

NCP……...Non-Collagenous Protein

NH_4OH…….Ammonium Hydroxide

NSAID……..……Nonsteroidal Anti-inflammatory Drug

OCN .....Osteocalcin

OD.....Optical Density

-OH…….....Hydroxyl Group

OPN .....Osteopontin

PBS .....Phosphate Buffered Saline Solution

PDGF.....Platelet-Derived Growth Factor

PDL.....Periodontal Ligament

PDLSC .....Periodontal Ligament Stem Cell

PG ..... Prostaglandin

PGH-synthase .....Prostaglandin-Endoperoxide Synthase

rASC…….……..Rat Adipose-Derived Stromal Cell

RCT…….. Root Canal Treatment

RMGIC .....Resin Modified Glass Ionomer Cement

RPM……..…..Revolutions per Minute

RT .....Room Temperature

RT-qPCR……..…Quantitative Reverse Transcription Polymerase Chain Reaction

RUNX2.....Runt-Related Transcription Factor 2

SABC.....HRP-Streptavidin Conjugate

SC……..……Stem Cell

SCAP .....Stem Cell from the Apical Papilla

SHED .....Stem Cell from Human Exfoliated Deciduous Teeth

ssCMap……..…. Statistical Significant Connectivity Map

TERT…….……. Telomerase Reverse Transcriptase

TGF-β.....Transforming Growth Factor-β

TMB.....Tetramethyl-Benzidine

TNF-α …….……..Tumor Necrosis Factor-α

TSC.....Tricalcium Silicate Cement

TXA_2.....Thromboxane A_2

CHAPTER ONE: INTRODUCTION

Chapter One: INTRODUCTION

1.1 Literature Review

1.1.1 Acetylsalicylic Acid

1.1.1.1 General Information

Acetylsalicylic acid (ASA), aspirin, is a nonsteroidal anti-inflammatory drug (NSAID) that have been used to treat cardiovascular disease (CVD), pain, and inflammation for over centuries (1). In 1534 BC, Egyptian physicians discovered the medicinal value of willow (*tjeret or salix*) and utilized its bark as an anti-inflammatory/pain reliever for nonspecific aches and pains. Later on, the willow bark had become a common remedy globally across the civilized world by 216 AD (2). In 1828, the willow was refined into yellow crystals by Joseph Buchner, a professor of pharmacy at Munich University. Then it was labeled as salicin after the original Latin name of willow, *salix*, before being renamed salicylic acid (3, 4).

As for the molecular structure, the salicylic acid contained a central 6-carbon benzene ring with two attached hydroxyl (-OH) and carboxyl group (-COOH) components, then the phenol group was acetylated and gave rise to the purest form of a renowned acetylsalicylic acid. In 1899, the compound was registered as “aspirin” before acquiring approval by the Food and Drug Administration (FDA) later in 1980 and has been available worldwide ever since (5). Nowadays, 10 to 20 billion aspirin tablets are regularly

administered to millions of adults in the United States as a long-term preventive treatment for CVD (6).

1.1.1.2 Mechanisms of Action

As an NSAID, the ASA preserves both anti-inflammatory and anti-thrombotic properties by working in a dose-dependent manner to inhibit prostaglandin (PG) synthesis (7). The main target of ASA is a membrane-bound hemoprotein and glycoprotein. It is known as cyclooxygenase (COX) or prostaglandin-endoperoxide synthase (PGH-synthase), which presents in 3 isoforms, namely COX-1, -2, and -3 (8). Although both COX-1 and -2 are capable of producing PGs, they involve in different processes. COX-1 predominantly functions in physiological processes and homeostasis to induce physiologically needed platelet aggregation or gastric mucosa protection, while COX-2 mainly works in pathophysiological processes by producing PGE_2 in inflammatory cells leading to symptoms of inflammatory reaction or disease such as hyperalgesia, rheumatoid, and osteoarthritis (9)(Fig. 1). Also, the potencies of individual NSAIDs against COX-1 and COX-2 vary since higher doses of ASA are required to achieve COX-2 inhibition compared to targeting COX-1–dependent platelet function (10, 11).

The mechanism of ASA occurs when the arachidonic acid is inhibited from entering the active binding site of COX to start the process of PG production. The inhibition is caused by selective acetylation of the hydroxyl group (-OH) of a serine residue (Ser 530) of the enzyme by ASA, leading to generating a bulky group on the Ser 530 oxygen that affects the arachidonic acid access to the binding site, which results in irreversible COX

dysfunction (Fig. 2). Without the PG, thromboxane A_2 (TXA_2) production is halted, which explains an antithrombotic property of ASA since TXA_2 directly relates to platelet aggregation by acting as a potent vasoconstrictor and stimulator (12-14)(Fig. 1). For this reason, ASA shows several therapeutic benefits concerning the inflammatory and immune responses, which contribute to wound healing.

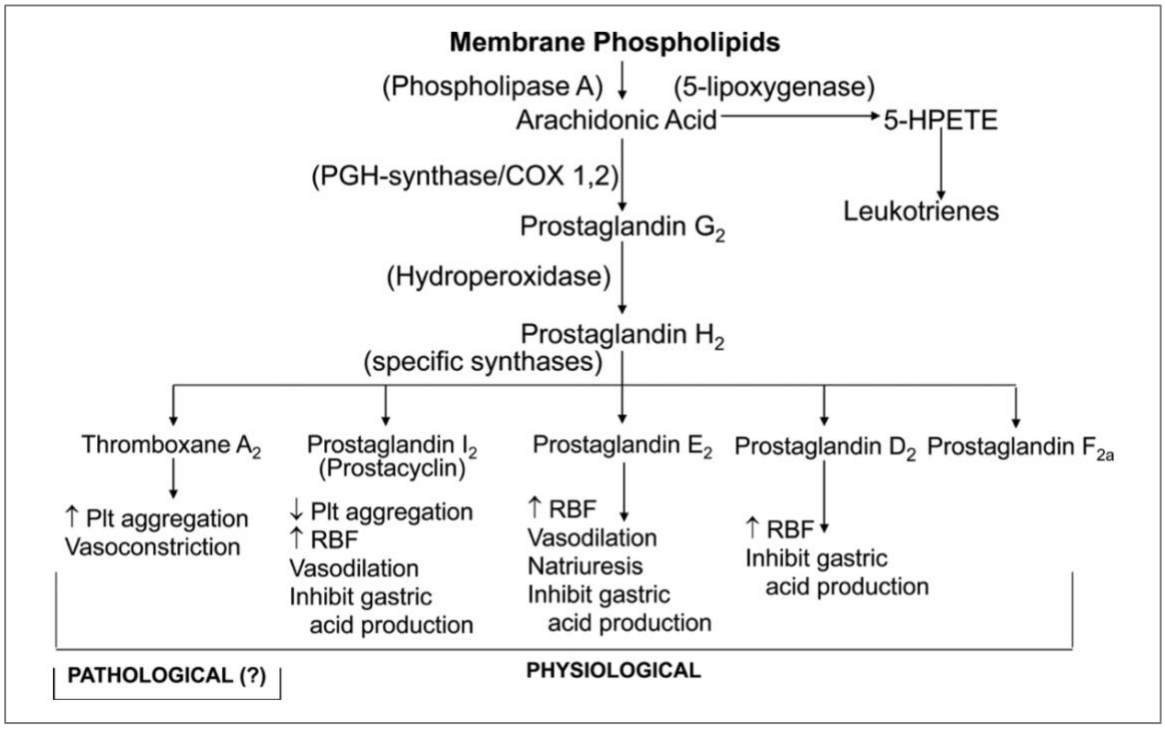


Figure 1. Cyclooxygenase is associated with the production of prostaglandins.

Cyclooxygenase (COX) or prostaglandin-endoperoxide synthase (PGH-synthase) is associated with the production of prostaglandins (PG) from arachidonic acid, leading to several physiological effects. HPETE indicates hydroperoxyeicosatetraenoic acid; COX, cyclooxygenase; PG, prostaglandin; Plt, platelet; and RBF, renal blood flow (1, 15).

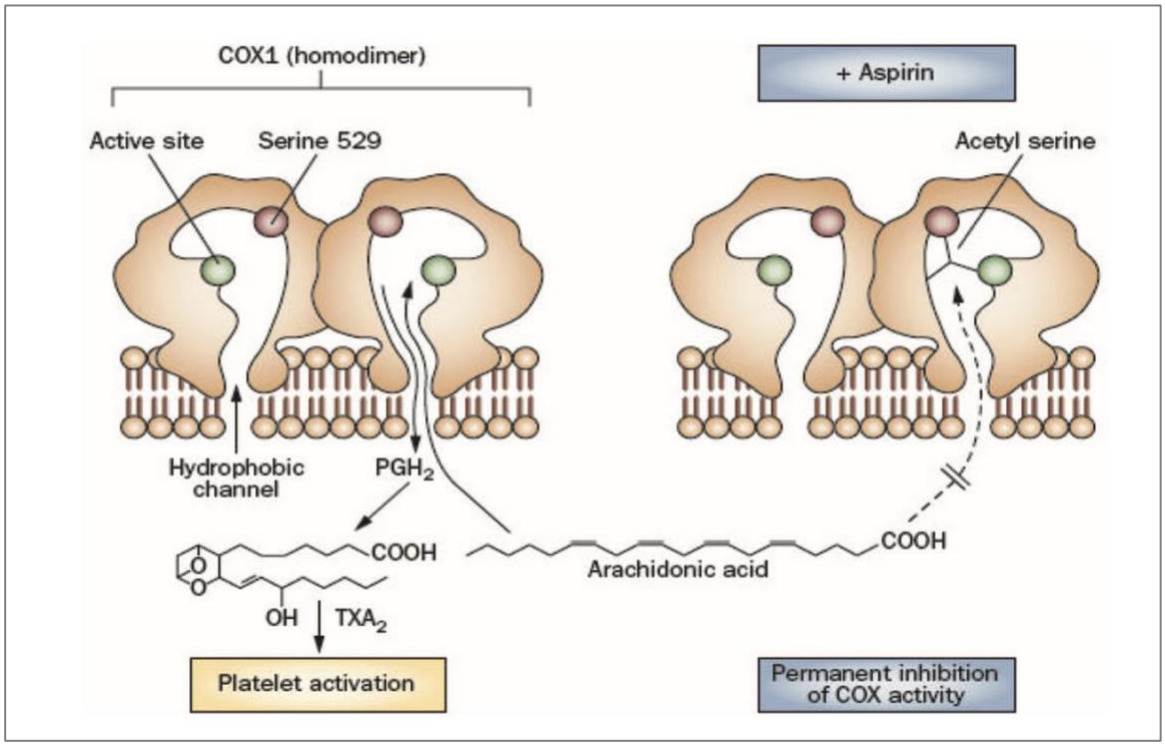


Figure 2. The cyclooxygenase inhibition by acetylsalicylic acid.

Acetylsalicylic acid (ASA) or aspirin inhibits the arachidonic acid active binding site of cyclooxygenase (COX). COX indicates cyclooxygenase; PG, prostaglandin; TX, thromboxane (1, 14).

1.1.1.3 Optimal Doses and Blood Concentration Levels

Although ASA gives numerous benefits in terms of inflammatory disease and CVD treatments, the interference of PG synthesis from ASA administration could alter the homeostatic or normal protective function of the body and potentially cause serious adverse effects. Therefore, it is crucial to monitor optimal doses of ASA to maintain and maximize the therapeutic advantages.

Effective daily doses of ASA as an antithrombotic agent have been reported to be as low as 30-100 mg/day in randomized clinical trials (16). In terms of long-term cardiovascular prevention, the recommended doses that may optimize efficacy and safety of patients are 75-81 mg/day in current clinical data, and the bleeding tendency amplifies in higher doses (1, 17-19). The observation from the Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) stated that the optimal daily dose of aspirin might be between 75 and 100 mg. Doses of over 100 mg were associated with no clear benefit and might have possibly entailed adverse side effects. (13, 19).

According to the FDA, ASA will be completely absorbed from the gastrointestinal (GI) tract and hydrolyzed to salicylic acid, with peak plasma levels of salicylic acid occurring within 1-2 hours of administration before being distributed to tissues and fluids in the body, including the central nervous system (CNS), with the highest concentrations presents in the plasma, liver, renal cortex, heart, and lungs. A mild level of salicylism or early signs of salicylic overdose manifest as tinnitus or ringing in the ears, which occurs

when the plasma concentration levels of ASA reach 200 µg/mL, and the severe toxic effects potentially start at the plasma concentration levels of 400 µg/mL (20).

The therapeutic, toxic, and lethal blood levels of ASA for adults have been published in Drug and Chemical Blood-Level Data 2001 (Table 1). Although the values in the table are not absolute and cannot be applied to infants and children, they are considered practical guidelines to evaluate ASA doses in each case (21).

Drug	**Therapeutic**		**Toxic**		**Lethal**	
	(mg%)	(µg/mL)	(mg%)	(µg/mL)	(mg%)	(µg/mL)
Acetylsalicylic acid (As met: salicylate) For analgesic use	2-10	20-100	15-30	150-300	50	500
Acetylsalicylic acid (As met: salicylate) For rheumatoid arthritis	2-25	20-250	-	-	-	-

Table 1. Drug and Chemical Blood-Level Data 2001

1.1.1.4 Adverse Effects

The adverse effects of ASA include gastric ulcers, renal failure, and impaired platelet function that can result in hemorrhagic complications such as stroke, which all are

known to be consequences of the alteration of PG functions (15). GI toxicity is a frequent outcome of the deprivation of cytoprotective effects of PGE_2 on the gastric mucosa from ASA-induced COX inhibition. The deactivation of COX also leads to decreased TXA_2 levels and impacts the hemostasis, which aggravates the risk of hemorrhagic stroke. Moreover, an association between renal vasodilatory PG inhibition and the use of NSAIDs has been reported, which results in the risk of renal insufficiency and worsening of hypertension control (22-25).

1.1.1.5 Aspirin and Regenerative Medicine

Another crucial role of PGs is its association with the bone remodeling cycle by playing a part in the regulation of osteoblast and osteoclast functions to promote bone formation or bone loss, specifically PGE_1 and PGE_2, which are produced by bone and reported to be the most potent stimulators of bone loss. PGs exhibit a long-term effect in bone organ culture by increasing the replication and differentiation of new osteoclasts and stimulating osteoclastic bone resorption, leading to osteoporosis (26-28). The level of COX enzyme induction also influences the PGs since the production is reportedly mediated through the COX-2 pathway. Therefore, inhibiting COX-2 by ASA can directly affect PG production and result in bone mass alteration (29-31).

Since the knowledge of the connection between NSAIDs and the process of bone metabolism came to light, researchers have studied the roles of ASA to provide a prospective fundamental for bone regeneration therapy. An association between increased bone mineral density and the use of ASA in the senior population was reported in an

epidemiological study (32, 33). Also, the low dose of ASA improved the osteogenesis of bone marrow mesenchymal stem cells (BMMSCs) in ovariectomized mice by targeting telomerase activity and blocking osteoclastogenesis (34). Co-administration of ASA and allogeneic rat adipose-derived stromal cells (rASCs) helped attenuate bone loss and decrease levels of pro-inflammatory cytokines in the serum (35).

In dentistry, ASA-treated stem cells from exfoliated deciduous teeth (SHED) significantly promoted *in vitro* and *in vivo* osteogenic differentiation and immunomodulation by the up-regulation of the telomerase reverse transcriptase (TERT)/Wnt/β-catenin cascade (36). Another study demonstrated the positive effects of ASA on the expression of growth factor–associated genes and the osteogenic capacity of periodontal ligament stem cells (PDLSCs), suggesting the roles of ASA in periodontal health (37). Furthermore, the odontogenic differentiation of stem cells from the apical papilla (SCAPs) was also enhanced by ASA activating the phosphorylation of AKT in SCAPs (38). The Gene Expression Omnibus (GEO) database combined with Statistical Significant Connectivity Map (ssCMap) method was utilized to identify currently licensed compounds or drugs for humans that potentially correlated with induced osteogenic/ odontogenic differentiation of dental pulp cells and predicted that ASA could be a prospective candidate (39). The ssCMap was developed from an innovative web-based technique to establish connections via gene expression profiles/signatures and has been applied to match the phenotypic target and predicted effective drugs for several diseases (40, 41).

1.1.2 Human Dental Pulp

The dental pulp is a unique un-mineralized oral tissue confined within a dental pulp cavity composed of dentin, enamel, and cementum, which provide rigid mechanical support and protection for the pulp tissue. The roof of the pulp cavity resides in the pulp chamber within the coronal part of the tooth, and the pulp chamber connects to each root canal at the cementoenamel junction level to extend the pulp cavity to the apex of the root, where the apical foramen locates as an entry of vascular, lymphatic, and nervous supplies for the tooth (42, 43).

Human dental pulp cells (HDPCs) consist of a heterogeneous population, including endothelial cells, neurons, fibroblast, osteoblast/osteoclast, odontoblast, and human postnatal mesenchymal stem cells (MSCs). The MSCs have been identified in various tissues throughout the body, including the oral cavity and dental pulp. The angiogenic properties and multipotentiality of MSCs allow them to regenerate dentin and dental pulp tissue, which are considered beneficial for vital pulp therapy and tissue regeneration (44, 45).

1.1.2.1 Multipotent Mesenchymal Stem Cells

Stem cells (SCs) are unspecialized clonogenic cells with the ability to divide or proliferate indefinitely. The SCs also obtain the self-renewing property and can ultimately differentiate into specialized cells under specific conditions to provide growth and development as well as homeostasis and tissue reparation. According to the differentiation

potential, SCs have been categorized into three main groups: 1. totipotent stem cells, 2. pluripotent stem cells, 3. multipotent stem cells or post-natal/adult stem cells (ASCs)(46, 47).

The totipotent stem cells are the first embryonic cells that arise from the division of the zygote, which are considered ultimate stem cells because of the potential to differentiate into any specialized cells needed to enable the growth and development of the organism. The second type of stem cell, pluripotent stem cells, is obtained from the inner cell mass of an embryo in the blastocyst stage. For this reason, pluripotent stem cells are commonly called embryo cells. This category of SCs has the potential to differentiate into any type of human tissue but is unable to support the full development of an organism. The final category of SCs is multipotent stem cells, known as somatic stem cells or ASCs, which acquire the potential to differentiate into different types of cells within a given cell lineage or a small number of lineages (43, 48).

The ASCs can be further divided into two subcategories, hematopoietic stem cells and MSCs. The hematopoietic stem cells develop into blood cells, including red blood cells, white blood cells, and platelet cells, whereas MSCs give rise to several other cells, such as bone, cartilage, fat tendon, and pulp cell. Both genuine multipotent stem cells and committed progenitor cells with restricted differentiation potentials are populated in MSCs, suggesting their heterogeneity (49-51). The characteristics of MSCs depend on the location they are isolated from and the method of isolation and growth (43, 52, 53).

The MSCs can be acquired from various places in the body, such as adipose tissues, umbilical cord blood, and bone marrow (54, 55). Despite the relative abundance of the acquired progenitors and the potential to remedy tissue damage, the procedures to collect adipose tissue and bone marrow require frequent usage of general anesthesia and are quite invasive, which can cause morbidity at the harvest site (56-58). Therefore, oral MSCs were proposed as an alternative source for the collection as they manifest tissue regeneration potential both *in vitro* and *in vivo* (59-61). The oral MSCs are available in pulp and peri-oral tissues, including periodontal ligament (PDL), apical papilla, and gingival tissues.

Human dental pulp stem cells (hDPSCs) have become an attractive cell source for regenerative therapy after the first report of MSCs isolation and characterization from the pulp tissue of the impacted third molar by Gronthos et al. in 2000 (62). The hDPSCs are ectodermal-derived SCs originated from migrating neural crest cells and possess MSCs properties. Some surface markers regarding mineralized nodule formation exhibited by hDPSCs show similarities to BMMSCs, such as alkaline phosphatase (ALP), osteocalcin (OCN), and osteopontin (OPN). The collecting procedures of hDPSCs are non-invasive since they can be easily obtained from the teeth that are freshly extracted and discarded. Possessing the MSCs properties, the hDPSCs exhibit a spindle-shaped fibroblast-like morphology and are capable of self-renewal and multilineage differentiation into several cell populations, such as odontoblasts, osteoblasts, neural cells, chondrocytes adipocytes, myoblasts, fibroblasts, and endothelial cells. In other words, the oral MSCs, including hDPSCs, are considered multipotent MSCs (60, 63).

1.1.2.2 Dentin-Pulp Complex

The ultimate goal of the dentin-pulp complex is to maintain the vitality of the dental pulp by regulating homeostatic and self-protective mechanisms when the pulp encounters a multitude of stimuli, such as caries. Many studies have reported the strong parallels between the induction of odontoblast differentiation in the later developments of the tooth and the dental tissue repair processes, as the pulp is capable of reinitiating dentinogenesis once it sustains external injuries. Both tissue formation and repair mainly involve growth factor (GF) signals to start the event (64, 65). The approaches toward dental pulp tissue regeneration and repair nowadays tend to follow the common theme of mimicking the normal tissue formation since the methods might enable the re-establishment of normal structure-function relationships of the primary dentin (66). Therefore, understanding the molecular and cellular processes involved in dentinogenesis and repair should provide insights and pave the way for the novel approach to dental tissue repair in vital pulp therapy.

1.1.2.2.1 Dentinogenesis

The transformation of a tooth primordia into complex mineralized structures involves interactions between dental epithelial and mesenchymal cells. These sequential and reciprocal epithelial-mesenchymal interactions are critical events in tooth development that enable GFs and cell-signaling molecules to temporospatial diffuse through each compartment of the tooth germ, which leads to histo-morphogenesis and cytodifferentiation of epithelial and mesenchymal cells into ameloblasts and odontoblasts, respectively. The formation of dentin, dentinogenesis, is a complex process that begins after the cusp patterns have been formed in the bell stage of tooth development. The process

involves tissue, cellular and extracellular elements as well as multiple signaling pathways that are controlled by several GFs and transcription factors (67).

Dental papilla, ectomesenchymal SCs residing in each cusp, is progressively withdrawn from the cell cycle at a specific timing to give rise to competent post-mitotic odontoblasts. During the late bell stage of tooth development, mitotic division takes place to expand the numbers of the dental papilla, and the last division occurs right underneath the basement membrane (BM) to form two daughter cells of pre-odontoblast. The first group of pre-odontoblast resides next to the BM at the cusp tip and receives the inductive signal of epithelial-derived transforming growth factor-ß superfamily (TGF-ßs) secreted by the inner enamel/dental epithelium (IDE) then sequestrated and immobilized on the dental BM for temporospatial presentation and signaling pre-odontoblast cells to undergo the cytodifferentiation, which the cells elongate and develop more organelles suitable to later function as odontoblast cells. Once cytodifferentiation is complete, the odontoblasts begin to express TGF-ßs to stimulate themselves by an autocrine pathway and functional differentiate into mature odontoblasts (65, 68). Another group of pre-odontoblast remains in the Höhl layer, which is considered a reservoir of incompletely differentiated cells. This group will play a part in reparative dentinogenesis when the odontoblast layer is damaged (69).

The mature odontoblasts are considered fully functioned and ready to secrete pre-dentin, a scaffolding collagen web of the dentin organic matrix composed of collagen and non-collagenous proteins (NCPs), including GFs. Mineralization is also carried out by

calcium ion (Ca^{2+}) transportation of mature odontoblasts via a transcellular route. This transmembrane transport mechanism helps the cells to maintain Ca^{2+} balance and results in an extracellular accumulation of Ca^{2+} in pre-dentin (70, 71).

The mineralization of pre-dentin leads to the sequestration of cytokines and GFs within the dentin, especially the most identified TGF-ß family. Once these bioactive components become fossilized, their biological activities are protected by interacting with the proteoglycan that resided in dentin extracellular matrix (ECM) components such as decorin and biglycan (65, 72). Many studies, both *in vitro* and *in vivo*, have shown that the fossilized bioactive dentin matrix components, including TGF-ßs, could be extracted from dentin and play crucial roles in dentin repair since they induce dentinogenesis and dental tissue regeneration. When dentin exposes to a specific condition or injury, the dentin will release the fossilized bioactive components, which signal the pre-odontoblast residing in the pulp to start the repair process (73-75).

1.1.2.2.2 Reparative Dentinogenesis

After the completion of the primary dentin secretion, the odontoblasts are also responsible for physiologically producing the secondary dentin throughout the lifetime, starting after the tooth has erupted into the oral cavity or following the apical closure of the tooth. During this stage of tooth development, the primary odontoblasts return to a quiescent state and reduce the rate of secretion. This change includes a shortening of the cell body and a decrease in the number of cellular organelles responsible for the synthetic and secretory behavior of the cells, but it will still function through the life of the tooth

although the rate is very minimal, unless the tooth undergoes injurious processes which disturb the quiescent state of odontoblasts and re-activate them to transform back to their full function to secrete tertiary dentin in response to external factors, such as decay or abrasion, in order to protect and maintain the integrity of the underlying pulp (69, 72).

The complex interplay and the relative balance among injury, defense, and regenerative processes in response to tooth injury are the prime elements to determine tooth survival. When the hard tissues of the tooth are broken down, the barrier against bacteria is invaded, which leads to the formation of two types of tertiary dentin categorized by the severity of the damage (Table 2). The demineralization of the dental tissue is accompanied by the release of the fossilized bioactive components from the dentin. Once they are liberated, these GFs traverse the tubules to the pulp and induce various cellular responses, including activation of the pre-odontoblasts and formerly quiescent odontoblasts lying underneath to re-enter an active state (65, 76).

In a mild tooth injury, such as moderate progression caries, the quiescent primary odontoblasts survive the stimuli and are re-activated to generate tertiary reactionary dentin. The structure of the reactionary dentin will be mostly similar to the physiologic dentin because they are both created by primary odontoblasts. On the other hand, in severe tooth injury, odontoblasts in adult pulp are differentiated post-mitotic cells and unable to divide to produce new secretory cells when these cells are lost. Therefore, another form of cellular replacement occurs involving SCs or progenitor cells in the pulp if the injury to the pulp is intense and results in local cell death in the odontoblast layer. The liberated bioactive

components from dentin, such as bone morphogenetic proteins (BMPs) and TGF-ßs, will induce recruitment and differentiation of the pre-odontoblasts with SC properties in the sub-odontoblast layer, giving rise to odontoblast-like cells to replace the lost odontoblasts and secrete dentin bridge. The dentin bridge is also known as tertiary reparative dentin, which has a variety of dentinal tubule characters ranging from fewer dentinal tubule numbers to an irregular pattern of dentinal tubules (66, 77, 78) (Table 2).

Tertiary Dentin	**Reactionary Dentin**	**Reparative Dentin**
Stimulus	Mild	Aggressive
Formative Cells	Surviving post mitotic odontoblasts	New odontoblast-like cells from pre-odontoblasts/ progenitor cells
Structure	▪ Physiologic dentin ▪ Alternations in the direction of new dentinal tubules	▪ Heterogenous ▪ Dentinal tubules are less in number and irregular in the arrangement

Table 2. Categories of tertiary dentin

1.1.2.3 Growth Factors and Biomarkers in Dentin-Pulp Complex

1.1.2.3.1 Growth Factors

GFs are peptide signaling molecules expressed by pulp cells and involved in major events of tooth morphogenesis and differentiation to keep homeostasis of the healthy pulp as well as healing the pulp after injury. Even at low concentrations, the potency of the GFs can control cell behavior and activity by signaling a stimulation or inhibition of cell functions. Some examples of commonly known GFs identified in dental tissues are in the family of TGF-ßs, BMPs, Platelet-derived growth factors (PDGFs), etc. Both injury events and clinical restorative procedures are the mechanisms that cause the release of GFs from the dentin matrix. Demineralization of dentin from bacterial acids in caries, cavity preparation, or application of dental materials may solubilize and expose matrix-bound GFs at certain degrees, leading to a subsequent diffusion of GFs along the dentinal tubules to interact with the odontoblasts and pulp cells (78).

Specific receptors of GFs present on either the producing cells themselves or other cells in which the GFs interact with their receptors in several manners, including endocrine, autocrine, paracrine, juxtacrine, and intracrine modes. A chain of intracellular signals starts once the GFs bind to the receptor. The signal transduction to the nucleus influences gene transcription and regulates both intracellular and extracellular events relating to cell activities, leading to a variety of cellular responses, including proliferation, differentiation, and biomarker secretion (65).

Among GFs found within the dentin, TGF-ßs are the most studied, and their activity is well-established. The key benefits of these TGF-ßs are their abilities to induce MSCs migration and cytodifferentiation into odontoblast-like cells to repair the injury site of the dentin, which appears to be parallel to the event of physiological odontoblast differentiation during tooth development except for the origin of the TGF-ßs production (65). The TGF-ßs exist in three isoforms, TGF-ß1, TGF-ß2, and TGF-ß3; however, only TGF-ß1 is identified at the highest level within the human dentin matrix, whereas odontoblasts and other cells of the pulp express all three isoforms. Increased levels of TGF-ß1 are also detected in odontoblasts and pulpal cells of carious human teeth, explaining an enhanced TGF-ß1 in tertiary dentin compared to the primary physiological dentin (79, 80). Each isoform of TGF-ßs is secreted by odontoblasts into pre-dentin in an inactive latent complex form bound with a latency-associated protein (LAP), which requires proteolytic cleavage for an activation. It is suspected that the matrix metalloproteinases (MMPs) involved in matrix remodeling may cleave the LAP from TGF-ß1 before the mineralization process and then leave TGF-ß1 within the mineralized dentin in an active form protected by isoform-specific interactions with ECM components, such as decorin and biglycan (79, 81).

Possible strategies to utilize TGF-ßs as an element of regenerative therapy have been introduced since many studies have demonstrated the stimulatory effects of isolated dentin matrix components on reactionary and reparative dentinogenesis both *in vitro* and *in vivo* (74, 82, 83). The ability of recombinant TGF-ß1 is reported to enhance odontoblast reactionary response to the stimuli and odontoblast-like differentiation, which pave the way

toward the innovation of possible biomaterials incorporating exogenous signaling molecules (82, 84). Moreover, dentin, a natural endogenous reservoir of GFs, can be another resource to deliver the TGF-ßs once the GF-ECM interaction is altered. The solubilizing effects of current cavity conditioning agents and pulp capping materials on matrix-bound bio-active molecules are mentioned, but further investigation is still required to improve the material properties for a better clinical outcome (85, 86).

1.1.2.3.2 Biomarkers

Biological markers, or biomarkers, are functional elements at the cellular or molecular level defined by the National Institutes of Health Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (87). Several biomarkers present at different stages of tooth development can be measured accurately to distinguish health and disease since distinct biomarkers are produced in response to pathological and physiological processes. The reversible and irreversible pulpitis can also be determined via the biomarker assessment in fluid from pulp, dentinal tubule, or gingiva (88). Furthermore, the expression of varied related markers, such as ALP, dentin sialophosphoprotein (DSPP), and runt-related transcription factor 2 (RUNX2), is commonly used to evaluate the odontogenic and osteogenic differentiation ability of hDPSCs (89).

ALP is known as a membrane-bound glycoprotein used in the hydrolysis of phosphate monoesters (90). It is believed that the ALP functions to reduce the

concentration of extracellular pyrophosphate, an inhibitor of mineral formation, and supply inorganic phosphate to facilitate mineralization (91). Since the ALP is mainly involved in mineral deposition and calcification, ALP activity is highly present in dental pulp cells and found in different tissues of mammals, such as bone, kidney, and liver (92). Although the physiological function of ALP is still unclear, an association between ALP and bone metabolism in normal skeletal mineralization makes ALP an indicator of osteoblast differentiation and osteogenic properties (93). By providing inorganic phosphate for hydroxyapatite formation, ALP plays a part in the early stages of odontoblast differentiation and also frequently serves as a marker for odontoblast-like differentiation, which involves in dental tissue repair after pulpal injury (89).

Among dentin ECM, the main component of NCPs secreted by odontoblast is DSPP, which is a phosphorylated protein with an essential role in tooth development, specifically odontoblast differentiation and mineralization (94). The DSPP precursor protein is translated from DSPP mRNA consisting of dentin sialoprotein (DSP) sequences at the NH_2 terminus and the dentin phosphoprotein (DPP) domain at the COOH region. After the translation, DSPP is secreted into the matrix and cleaved into two dentin NCPs, DSP and DPP, by a zinc-dependent matrix protease (95). High levels of carbohydrate and sialic acid, along with aspartic acid and phosphoserine, contained in both DSP and DPP suggest their relation to dentin mineralization (96). The expression of DSPP is tooth-specific and restricted to the differentiation process of dental cells as it is predominantly expressed in odontoblasts, with a transient level in pre-ameloblasts and rarely found

elsewhere (97). For this reason, mutations concerning the DSPP gene will possibly result in dentinogenesis imperfecta types II and III (98, 99).

Another essential biomarker involved in bone and dental mineralization is RUNX2, which belongs to the Drosophila runt family (100). The primary role of RUNX2 is related to osteoblast and odontoblast differentiation, as well as the determination of osteoblast and odontoblast lineages from MSCs. The RUNX2 has been reported to play a part in regulating other bone- and tooth-related gene expressions, including DSPP. An *in vivo* study showed an upregulated DSPP gene expression in mouse pre-odontoblasts and downregulated expression in mouse odontoblasts when RUNX2 was overexpressed (94). RUNX2 expression pattern during bone and tooth formation has been reported along with its differential biological functions on DSPP expression during odontoblast cytodifferentiation (101, 102). At the beginning of bone development, RUNX2 modulates osteoblast differentiation and increases the number of immature osteoblasts to generate immature bone. On the other hand, RUNX2 is downregulated at the late stage of osteoblast differentiation since it inhibits osteoblast maturation (103). These events suggest the importance of RUNX2 in the homeostasis of mineralized tissues. Hence, RUNX2 mutation or deficit can lead to impaired functions of bone and tooth, such as cleidocranial dysplasia or tooth hypoplasia (104, 105).

1.1.3 Vital Pulp Therapy

Preservation of tooth vitality has been the ultimate goal of dental treatment since a protective resistance to mastication forces provided by a vital tooth is more favorable than the tooth that undergoes a root canal treatment (RCT)(106). Vital pulp therapy aims to maintain the integrity of the dental-pulp complex after sustaining injuries and facilitate the tissue repair process to establish the tertiary dentin as the physical barrier against the invasion. High success rates of vital pulp therapy depend on the ability to eliminate infected tissue, relevant hemorrhagic control, and the placement of seal biocompatible materials to create a proper environment that stimulates healing, which is the most crucial factor since restorative leakage can contribute to treatment failure in the long run (107-109). Hence, the vital pulp therapy in class I restoration showed a significantly better prognosis than class II, III, IV, and V restorations that are more prone to leakage due to proximal wall involvement (110, 111). Studies showed that some symptomatic permanent teeth with deep caries could be managed successfully by direct pulp capping, partial pulpotomy, and full pulpotomy (112-114). The event was explained by the evidence showing that most inflamed or necrotic pulp tissue stayed within the coronal pulp of vital teeth (115). Therefore, vital pulp therapy is a suitable option that provides less costly and less invasive treatment for reversible and irreversible pulpitis with a proper case selection.

1.1.3.1 Vital Pulp Therapy Categories

Vital pulp therapy can be generally classified into three main categories: 1. Indirect pulp capping, 2. Direct pulp capping, and 3. Pulpotomy.

1.1.3.1.1 Indirect Pulp Capping

The concept of indirect pulp capping is based on the ecologic and metabolic balance regained after eliminating the source of infection and sealing the cavity properly. The reduction of bacteria number over time prompts the remineralization process and eventually arrests caries (116). Indirect pulp capping is a treatment option in asymptomatic or reversible pulpitis teeth without pulp exposure or apical lesion present (107). The procedures can be completed in one or two visits, depending on the amount of infected dentin removed. The single-visit technique only requires partial caries removal, leaving the carious dentin closest to the pulp to avoid exposure. The peripheral seal needs to be maintained tightly with bioactive lining material and permanent restoration to support the pulp healing. Another technique is known as step-wise excavation, which requires complete caries removal done in two visits. Carious dentin in proximity to the pulp remains in the cavity after the first visit and will be completely managed with definitive restoration in the re-entry visit when the carious dentin becomes harder. A recent systematic review showed a reduced risk of pulpal exposure and postoperative symptoms in a partial caries removal technique compared with step-wise excavation (117). In addition, a retrospective study showed a 93% 3-year survival rate in the permanent teeth after treatment with the indirect pulp capping technique (118).

1.1.3.1.2 Direct Pulp Capping

Direct pulp capping is the treatment of vital pulp exposure involving the placement of a capping biomaterial directly over the exposed pulp tissue to facilitate dentin bridge

formation and pulpal healing (109). The indications for the teeth suitable to receive direct pulp capping treatment include reversible pulpitis or asymptomatic teeth with non-carious mechanical pulp exposure while excavating the last portions of deep caries. The diameter of an exposure site must be lesser than 1.5 mm with well hemorrhagic control to assess the level of pulp inflammation (107). Contamination of caries or saliva at the exposure site, constant bleeding, or dark color of the blood may indicate more severe pulpal inflammation, which requires more invasive tissue removal as pulpotomy until only healthy pulp remains (119). Studies reported that the success rate of direct pulp capping ranged from 87.5% to 94%, depending on the ability to follow up with the patients regardless of age or gender (106, 120). Remaining bacteria or marginal leakage of the restoration leading to microorganism invasion are other factors contributing to the failure of direct pulp capping (106, 110). Thus, rubber dam usage and tightly sealed restoration are required to achieve a favorable treatment outcome.

1.1.3.1.3 Pulpotomy

According to the American Association of Endodontists glossary, two approaches to pulpotomy can be employed. The partial pulpotomy or Cvek pulpotomy is defined as the amputation of a small portion of the vital coronal pulp tissue to preserve the remaining coronal and radicular pulp, whereas the full pulpotomy involves complete removal of the coronal pulp to the orifice level aiming to conserve the vitality of the radicular pulp tissue as an alternative to the RCT (121, 122). Success rates of pulpotomy range from 93% to 96%, specifically in children and young adults with traumatic pulp exposure (123-125). In

irreversible pulpitis teeth, full pulpotomy exhibits 78% to 100% success rates, but the survival chance may decrease if the periapical lesion is present concurrently (109). The pulpotomy has merits over RCT in terms of lesser treatment duration and technical difficulties, while comparable pain relief has been reported without a significant difference in the 5-years treatment outcomes regardless of age and gender of the patients (126-128). For this reason, a pulpotomy can be performed as an appropriate alternative treatment for RCT in symptomatic pulpitis.

1.1.3.2 Current Materials for Vital Pulp Therapy

Many pulp capping materials have been commonly used in the clinical setting nowadays and preserve the purpose of promoting the dentinogenic potential of the pulp cells to generate the dentin bridge and wall off the injury. Previous studies showed that, due to the acidic or alkaline pH, some restorative and pulp capping materials were capable of releasing GF from the dentin matrix to induce odontoblast and odontoblast-like cells to initiate dentin regeneration (66, 85, 86, 129). Determination of the treatment success is done by evaluating the biocompatibility of each material along with the morphology, quality, and thickness of the dentin bridge produced by induced odontoblast cells (130, 131). Current materials include calcium hydroxide ($Ca(OH)_2$) and tricalcium silicate cement (TSC).

Historically, $Ca(OH)_2$ was considered the goal standard to perform vital pulp therapy and was also proposed as a lining material (132). However, some concerns were raised regarding long-term solubility and the poor ability to seal or adhere to dentin leading

to microleakage. For this reason, the application of resin-modified glass ionomer cement (RMGIC) as a protective liner layer over the $Ca(OH)_2$ is necessary due to the better capacity to bond with dentin and the mechanical strength. However, direct contact of RMGIC with the pulp tissue needs to be avoided since an intense inflammatory response might occur and hinder dentin bridge formation (133). The dentin bridge produced following the $Ca(OH)_2$ application presents some porosities and tunnel defects caused by dissolving $Ca(OH)_2$ beneath the restoration that allows the recolonization of bacteria (130, 131). The mechanism of $Ca(OH)_2$ mainly comes from the extreme alkaline pH that provides an antimicrobial effect and pulp tissue irritation, which initiate the pulpal defense and repair (131, 134). Despite the benefits of the alkaline property, it is also cytotoxic, which causes a layer of necrosis of the pulp tissue and weakens the mechanical property of dentin in long-term observation (135-137). Thereby, taken together with the aforementioned factors may contribute to the failure of the pulp capping procedure.

Recently, TSCs such as mineral trioxide aggregate (MTA) and Biodentin have drawn much attention due to their superiority in biological and mechanical properties with consistently good outcomes, leading to long-term success rates (138-140). Studies showed that MTA exhibited histologically superior dentin bridge formation to $Ca(OH)_2$ following a direct pulp capping treatment, and the MTA-induced dentin bridge preserved a better quality with lesser porous (140-142). The pulp tissue treated with MTA also demonstrated lower inflammation levels or inflammation free, which was explained by studies showing the ability of MTA to down-regulate inflammatory cytokines and suppress the proliferation of some microorganisms (143, 144). Direct contact of MTA with HDPCs induced

recruitment, proliferation, and differentiation of MSCs into odontoblast-like cells to form a dentin bridge without a detected necrotic layer. (138, 145). Nevertheless, tooth discoloration due to the oxidation of MTA has been reported along with other drawbacks, including high cost and long setting time, which results in the manipulation difficulty (146, 147). Although similar efficacy in pulp capping treatment is reported in Biodentin with a slightly better handling technique, some limitations still exist and require further investigation to improve the properties and clinical outcomes (148).

1.2 Rationale and Clinical Significance for this Research

ASA is a renowned orally administered NSAID and antithrombotic agent used as a treatment of inflammatory and cardiovascular conditions (15). Besides the general medicinal property, many published studies have been focusing on other compelling activities of ASA, such as the roles in the process of bone metabolism with inductive effects on proliferation and osteogenic differentiation of BMMSCs and SHED (36, 149). Some epidemiologic studies revealed that current ASA users had significantly higher bone mineral density compared with non-users (32). Moreover, there was a study that showed the positive effect of ASA in the treatment of ovariectomy-induced osteoporosis through osteoblast activation (150). The ASA has been studied with different types of cells concerning the teeth and oral cavity and manifested positive effects on osteogenesis (37, 38). However, the influence of ASA on the odontogenesis of HDPCs and dentin remains elusive.

HDPCs are a heterogeneous cell population, including postnatal multipotent MSCs and progenitor cells that exhibit the potential for tissue regeneration (42). Surrounded by dentin, HDPCs reside in the dental pulp and play a crucial part in dental tissue repair when the teeth are subject to injury (64). Various conditions such as caries, erosion, and dental restorative procedures can cause dentin demineralization leading to the liberation of growth factors sequestrated within the dentin matrix during tooth development (85, 86). Among the bioactive molecule reservoir, the TGF-ß family is believed to obtain a variety of cell signaling properties that invoke dentinogenic responses of HDPCs, including cell

proliferation, differentiation, and mineralization to initiate the production of reparative dentin (151).

Despite the natural regenerative capacity of the dentin-pulp complex, an amount of tertiary dentin gained from self-healing is hardly sufficient to maintain the pulp vitality without the need for restorations. Hence, constantly searching for the materials that help synergize the healing potential of the pulp is critical to improving clinical outcomes. $Ca(OH)_2$ and TSC have long been considered options to opt for in pulp capping procedures. Regardless of their popularity and ability to release TGF-ß1 from dentin via collagen degradation (85), only 60-80 % success rates were reported after a 3-year follow-up in randomized clinical trials (152, 153), and long-term exposure to highly alkaline conditions created by $Ca(OH)_2$ and TSC could cause local necrosis of the pulp tissue (136) and hinder the mechanical properties of dentin (135, 137).

On the other hand, ASA, with the reported bone regenerative capacity and acidity, might be an additional ingredient that helps neutralize the pH of current pulp capping materials and supports dental tissue regeneration. This project hypothesizes that the ASA at an optimal concentration has an inductive effect on the HDPCs odontogenesis as well as TGF-ß1 liberation from dentin which could benefit the development of pulp capping materials.

CHAPTER TWO: HYPOTHESIS

Chapter Two: HYPOTHESIS

It is hypothesized that the acetylsalicylic acid (ASA) at an optimal concentration has an inductive effect on human dental pulp cell (HDPCs) odontogenesis *in vitro* as well as an influence on transforming growth factor-β1 (TGF-ß1) liberation from dentin.

The first null hypothesis (H_0) is no difference in the *in vitro* proliferation, differentiation, and mineralization between the experimental sample groups of ASA-treated HDPCs and the untreated control group.

The second H_0 is no difference in the released TGF-ß1 level between the dentin conditioned with the negative control and the ASA-treated group.

CHAPTER THREE: OBJECTIVES OF THE STUDY

Chapter Three: OBJECTIVES OF THE STUDY

Objective 1:

To determine the effect of different concentrations of acetylsalicylic acid (ASA) (25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 200 μg/mL) on the **attachment efficiency** of human dental pulp cells (HDPCs) at 16 hours compared to the untreated control group.

Objective 2:

To evaluate the effect of different concentrations of ASA (25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 200 μg/mL) on the **proliferation rate** of HDPCs on day 7, day 14, and day 21 compared to the untreated control group.

Objective 3:

To examine the effect of different concentrations of ASA (25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 200 μg/mL) on HDPCs **differentiation at the transcriptional level** on day 4, day 7, and day 14 compared to the untreated control group.

Objective 4:

To examine the effect of different concentrations of ASA (25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 200 μg/mL) on HDPCs **differentiation at the post-transcriptional level** on day 7, day 14, and day 21 compared to the untreated control group.

Objective 5:

To evaluate the effect of different concentrations of ASA (25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 200 μg/mL) on the **mineralization level** of HDPCs on day 7, day 14, and day 21 compared to the untreated control group.

Objective 6:

To determine the influence of ASA on **transforming growth factor-β1** (**TGF-ß1) liberation from dentin** conditioned with ASA (25 μg/mL and 200 μg/mL) at 5 minutes and 10 minutes compared to the negative control group.

CHAPTER FOUR: MATERIALS AND METHODS

Chapter Four: MATERIALS AND METHODS

4.1 Materials

4.1.1 List of General Supplies

Table 3. List of General Supplies

Material	Manufacturer	Catalog number
Aspirating pipettes	Fisher Scientific	13-675-16
15 mL Centrifuge tubes	Fisher Scientific	14-959-70C
50 mL Centrifuge tubes	Fisher Scientific	14-432-22
Corning™ 12.5 cm^2 tissue culture flasks (T12.5)	Fisher Scientific	08-772-1F
Corning™ 225 cm^2 tissue culture flasks (T225)	Fisher Scientific	1482680
Disposable masks	Kimberly-Clark	12978
EMD MilliporeSigma™ Stericup™ sterile vacuum filter units, 0.22 μM	Millipore Sigma	SCGVU02RE

330 High speed bur	Midwest	386261
Kimwipes™	Fisher Scientific	06-666
1.5 mL Microcentrifuge tubes	Fisher Scientific	05-408-129
2 mL Pipettes	Fisher Scientific	13-675-3C
10 mL Pipettes	Fisher Scientific	13-678-11E
25 mL Pipettes	Fisher Scientific	13-678-11
Pipette tips (blue) 200-1000 μL	Fisher Scientific	02-707-509
Pipette tips (yellow) 1-200 μL	Fisher Scientific	02-707-503
Surgical gloves	Fisher Scientific	19-130-1597
Weighing dish (small)	Fisher Scientific	02-202-100
3x3 Weighing paper	Fisher Scientific	NC9691607
6-Well plate flat clear bottom	Fisher Scientific	07-200-83

24-Well plate flat clear bottom	Fisher Scientific	07-200-83
96-Well plate flat clear bottom	Fisher Scientific	08-772-2C
Multichannel pipette reagent reservoirs	Millipore Sigma	CLS4870
Nunc™ Petri Dishes	Thermo Scientific	08-757-099

4.1.2 List of Chemical Supplies and Kits

Table 4. List of Chemical Supplies and Kits

Material	Manufacturer	Catalog number
10% Acetic acid	Fisher Scientific	135-16
Acetylsalicylic acid	Sigma-Aldrich	A5376
Alizarin red S	Sigma-Aldrich	A5533
Alkaline phosphatase assay kit (Fluorometric)	Abcam	ab83371

Ammonium hydroxide	Fisher Scientific	AC423305000
Amphotericin B	Thermo Fisher Scientific	15290018
L-Ascorbic acid	Sigma-Aldrich	A4544
Basal medium eagle	Fisher Scientific	21-010-046
Cell culture grade water	Fisher Scientific	MT25055CV
Crystal violet, powder	Fisher Scientific	21-010-046
1X DPBS, no calcium, no magnesium	Fisher scientific	14-190-250
1α,25-Dihydroxyvitamin D3	Sigma-Aldrich	D1530
17% Ethylenediaminetetraacetic acid solution (EDTA)	Vista Apex	317011-2
70 % Ethanol	Fisher Scientific	S25306A

100% Ethanol molecular biology grade	Fisher Scientific	BP2818500
Fast optical 96-well reaction plate with barcode, 0.1 mL	Fisher Scientific	43-469-06
Fetal bovine serum	Sigma-Aldrich	229288-100G
Fetal bovine serum, charcoal stripped	Fisher Scientific	A3382101
10 % Formalin	Fisher Scientific	15-290-018
L-Glutamine	Fisher Scientific	SH3003401
β-Glycerophosphate disodium salt hydrate	Sigma-Aldrich	G9422
Human DSP ELISA kit	Xpress Bio	XPEH4864
Human RUNX2 ELISA kit	Xpress Bio	XPEH2428
Hydrochloric acid solution 1.0 N	Sigma-Aldrich	H9892

Menadione	Sigma-Aldrich	M5625
MTT assay kit	Abcam	ab211091
Optical adhesive film	Fisher Scientific	43-609-54
Penicillin/Streptomycin antibiotic	Fisher Scientific	15-140-122
Phasemaker™ tubes	Thermo Fisher Scientific	A33248
PureLink™ DNase set	Fisher Scientific	12-185-010
PureLink™ RNA mini kit	Fisher Scientific	12-183-018A
Sodium hydroxide solution 1.0 N	Sigma-Aldrich	S2770
SuperScript™ III Platinum™ one-step qRT-PCR kit	Thermo Fisher Scientific	11732020
TAQMAN GENE EX ASSAYS: Hs00171962_m1	Thermo Fisher Scientific	4448892

TAQMAN GENE EX ASSAYS: Hs00231692_m1	Thermo Fisher Scientific	4453320
TAQMAN GENE EX ASSAYS: Hs02786624_g1	Thermo Fisher Scientific	4453320
TAQMAN GENE EX ASSAYS: Hs03023943_g1	Thermo Fisher Scientific	4453320
TGF-ß1 Human ELISA kit	Thermo Fisher Scientific	BMS249-4
Triton™ X-100	Sigma-Aldrich	T8787
TRIzol™	Fisher Scientific	15-596-026
0.05% Trypsin-EDTA	Fisher Scientific	HT501128-4L
UltraPure™ DNase/RNase-free distilled water	Fisher Scientific	10-977-023
XTT assay kit	Abcam	ab232856

4.1.3 List of Equipment

Table 5. List of Equipment

Equipment	Manufacturer	Catalog number
Biological safety cabinet	LABCONCO	3430809
#7 Chandler bi-bevel chisel	Hu-friedy	CC
Hemocytometer	Reichert	26715
High speed hand-piece	Midwest	HP200
Incubator	Thermo ELECTRON	3110
Light microscope	Leica	LEITZ DM IL
Manual pipette P20	Gilson, Pipetman	FA10003P
Manual pipette P100	Gilson, Pipetman	FA10004P
Manual pipette P200	Gilson, Pipetman	FA10005P
Manual pipette P1000	Gilson, Pipetman	FA10006P

Microcentrifuge	Fisher Scientific	0497716KM
Microplate reader (spectrophotometer)	Tecan	Infinite 1000 Pro
Multichannel pipette 20-100 µL	Sigma-Aldrich	BR705908
Multichannel pipette 50-200 µL	Sigma-Aldrich	BR705910
NanoDrop™ 1000 Spectrophotometer	Thermo Fisher Scientific	ND1000
pH meter with electrode	Corning	320
Pro-pette pipette aid (pipette controller)	MTC Bio	P6080
Real Time PCR 7900HT	Thermo Fisher Scientific	4329001
Real Time PCR StepOnePlus™	Thermo Fisher Scientific	4376600
Roto Mix shaker	Thermolyne	M51335
Surgical dental mallet ML.01	Osung	ML25
Table top centrifuge	Beckman	TJ-6

Timer	Fisher scientific	02-401-7
Ultrasonic dismembrator	Fisher scientific	50 Sonic F50
Weight scale	OHAUS	TS400S
Weight scale	Denver Instruments	P-314

4.2 Approvals and Training

This study was approved by the Institutional Biosafety Committee at Boston University (IBC #19-661). All necessary human subject training was completed through the Collaborative Institutional Training Initiative (CITI). Online lab training was provided through the Office of Research at Boston University. Exempt review from the human subjects was granted by the Institutional Review Board (IRB; H-33173, H-42227) to use biological waste material from human teeth. Patient identification for human waste extracted teeth is not possible.

4.3 Methods

4.3.1 Agents

4.3.1.1 Acetylsalicylic Acid Preparation

Material Basic Physical and Chemical Properties

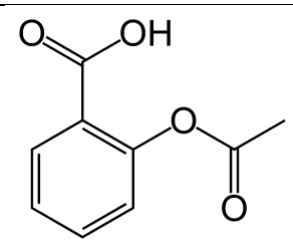
Product name	Acetylsalicylic acid (Sigma-Aldrich)
Synonyms	▪ ASA ▪ Aspirin ▪ O- Acetylsalicylic acid ▪ 2-Acetoxybenzoic acid
Appearance	White powder
Odor	Odorless
Purity	≥99%
Formula	$C_9H_8O_4$
Structure	
Molecular weight	180.16 g/ mol
pH	3.5 at 2.5 g/ L at 20° C (68° F)
Melting point	134-136 ° C (273-277° F)
Water solubility	4.6 g/ L at 25° C (77° F)

Table 6. Physical and chemical properties of acetylsalicylic acid (ASA).

Experimental Groups

1. Control (0 μg/mL ASA)
2. 25 μg/mL ASA in culture medium
3. 50 μg/mL ASA in culture medium
4. 75 μg/mL ASA in culture medium
5. 100 μg/mL ASA in culture medium
6. 200 μg/mL ASA in culture medium

Concentration Selection

The preparation of ASA followed the protocol from a previous study (38). The ASA at 25, 50, 75, and 100 μg/mL were selected as optimum concentrations according to preceding publications that exhibited promising results of ASA on other cell types, including stem cells from the apical papilla (SCAPs) (38), periodontal ligament stem cells (PDLSCs)(37), stem cells from exfoliated deciduous teeth (SHED) (36), and bone marrow mesenchymal stem cells (BMMSCs) (149).

In terms of ASA pharmacokinetics, the United States Food and Drug Administration (FDA) disclosed in the Code of Federal Regulations (CFR) Title 21 CFR 343 (20) that the early signs of salicylic overdose (salicylism), including tinnitus or ringing in the ears, occur at plasma concentrations approximating 200 μg/mL, whereas severe toxic effects are associated with levels above 400 μg/mL ASA. For this reason, an extreme

concentration of ASA at 200 μg/mL was incorporated into the experiment to evaluate the effect of ASA on human dental pulp cells (HDPCs) when the optimal limit was exceeded.

Preparation Protocols

1. Prepare 200 mL of 2000 μg/mL ASA stock solution by diluting 0.404 g of 99% ASA powder in 1X sterilized phosphate-buffered saline solution (PBS) and stir for 1 hour 30 minutes or until all powder completely dissolves.
2. Filter the 2000 μg/mL ASA stock solution using MilliporeSigma™ Stericup™ sterile vacuum filter units.
3. Dilute the 2000 μg/mL ASA stock solution down to the designated concentrations of 1000 μg/mL, 750 μg/mL, 500 μg/mL, and 250 μg/mL using 1X sterilized PBS. These are considered 10X stock solutions, which will reach the working concentration of 1X once combined with the designated amount of culture medium in the well plate.
4. Calculate the needed amount of 10X ASA stock solution (2000 μg/mL, 1000 μg/mL, 750 μg/mL, 500 μg/mL, 250 μg/mL) that will acquire 1X end-concentrations (200 μg/mL, 100 μg/mL, 75 μg/mL, 50 μg/mL, 25 μg/mL) when incorporated into the culture medium in the well plate.
5. Add the 10X ASA stock solution into each experimental well accordingly.

4.3.1.2 Growth Medium Preparation

1X Eagle's Basal Medium (BME) was utilized to prepare the complete growth medium for HDPCs culture. In a total volume of 500 mL, the growth medium contained 50 mL supplement of 100% fetal bovine serum (FBS), 5 mL of 100X penicillin/streptomycin antibiotic (10,000 U/mL), and 0.5 mL of 1000X amphotericin B anti-fungal (250 μg/mL) in 444.5 mL of BME to prevent microbial infection as well as facilitate the cell growth. The end concentration of each component is shown in Table 7.

Component	Volume	End concentration
100% FBS	50 mL	10% (v/v)
100X Pen/ Strep (10,000 U/mL)	5 mL	100 U/mL (1X)
1000X Amphotericin B (250 μg/mL)	0.5 mL	0.25 μg/mL (1X)
1X BME	444.5 mL	-
Total volume	500 mL	

Table 7. Growth medium components and the end concentrations in the complete medium.

4.3.1.3 Differentiation Medium Preparation

An odontogenic medium with 1α,25-dihydroxyvitamin D3 (Vit D_3) was used to induce the odontogenic differentiation of HDPCs. In a total volume of 30 mL, the

odontogenic medium contained 3 mL of charcoal-stripped FBS, 3 ml of 100 mM β-glycerophosphate, 1 mL of 8.5 mM L-ascorbic acid, 0.3 mL of 100X penicillin/streptomycin antibiotic (10,000 U/mL), 0.3 mL of 1 µM menadione, and 0.3 mL of 200 mM L-glutamine in 22.1 mL of BME. The end concentration of each component of the odontogenic medium and the supplement Vit D_3 are displayed in Table 8 and Table 9 subsequently.

Component	Volume	End concentration
Charcoal-stripped FBS	3 mL	10% (v/v)
β-Glycerophosphate (100 mM)	3 mL	10 mM
L-Ascorbic acid (8.5 mM)	1 mL	0.3 mM
100X Pen/ Strep (10,000 U/mL)	0.3 mL	100 U/mL (1X)
Menadione (1 µM)	0.3 mL	10^{-8} M
L-Glutamine (200 mM)	0.3 mL	2 mM
1X BME	22.1 mL	-
Total volume	30 mL	

Table 8. Odontogenic medium components and the end concentrations in the complete medium.

Component	Volume	End concentration
Vit D_3 (1 µM)	0.3 mL	10^{-8} M
Odontogenic medium	30 mL	-
Total volume	30.3 mL	

Table 9. The end concentration of Vit D_3 in the odontogenic medium with supplement Vit D_3

4.3.1.4 Crystal Violet Dye Preparation

0.2 % (w/v) Crystal violet dye solution was prepared by dissolving 0.2 g of crystal violet powder in 100 mL distilled water (DI) and mixing until the powder was fully dissolved. The solution needs to be kept at room temperature (RT) and away from the light.

4.3.1.5 Triton X Preparation

1% (v/v) Triton X-100 solution was prepared by dissolving 1 mL of 100% triton X in 99 mL DI and mixing until completely blended. Let the solution sit for at least 1 hour to eliminate bubbles before use. The solution needs to be kept at RT and away from the light.

4.3.1.6 Alizarin Red S Preparation

2 % (w/v) Alizarin red S solution (ARS) was prepared by dissolving 2 g of alizarin red S powder in 100 mL DI. After mixing for at least 2 hours, the pH was carefully adjusted to reach pH 4.1-4.3 with 0.1% ammonium hydroxide (NH_4OH). The pH was monitored for 30 minutes to ensure the pH stability of the ARS and then filtered using EMD

MilliporeSigma™ Stericup™ sterile vacuum filter units before storing away from the light at RT.

4.3.2 Cell Culture Preparation

4.3.2.1 Isolation of Progenitor Cells from the Human Dental Pulp

Human permanent molars were extracted by oral surgeons from the Oral Surgery Department of Boston University School of Dental Medicine. The procedures conformed to approved guidelines by the Boston University Medical Campus Institutional Review Board (IRB; H-33173, H-42227). Before the procedure, healthy donors with an age range of 18-25 years old went through the screening process to rule out any metabolic, systemic diseases, acute infections, or a history of steroid intake within the six months preceding the surgery. The patients who met the inclusion criteria would provide written informed consent to the department. The teeth presented with caries, restorations, trauma, non-vital signs, endodontic lesions, or teeth associated with pathologic conditions affecting the pulp were considered to be exclusion criteria. All methods of HDPCs isolation in the study were performed following previously published protocols (62, 154, 155) with some modifications.

Freshly extracted teeth were stored in a cold 1X PBS-containing tube within an ice container immediately after the operation. The teeth were then transferred to the lab in a secure biohazardous labeled container to prevent infection and contamination. Before the pulp isolation, the teeth were rinsed and cleaned out any remaining soft tissue residue. A

sagittal indentation was made on each tooth using a high-speed handpiece and a 330 bur in an intermittent manner with water coolant to avoid heat generation and pulp exposure. Once the groove was created, the tooth was secured to the pliers and later sectioned in half using #7 chandler bi-bevel bone chisel and hammer. The pulp tissue was located and meticulously extracted from the pulp chamber using sterile surgical forceps and Gracey curette, then placed into a petri dish containing sterile PBS in the ice container.

The extracted pulp tissue samples were transported to a class II biological lab safety hood to undergo the following isolating procedures within sterile conditions. Petri dishes containing cold sterilized PBS were prepared under the biological hood to further rinse the samples before dividing them into smaller fragments using sterilized micro-dissecting scissors. Each pulp fragment was transferred into a T12.5 flask containing 8 mL of warm growth medium and labeled with sample number and date. The flasks were maintained in the incubator at 37°C with 5% carbon dioxide and saturated humidity. The culture medium was changed twice a week, and passage 3 (P3) cells were used for further experiments. The human dental pulp tissue extraction and HDPCs isolating procedures were displayed in Figures 3 and 4.

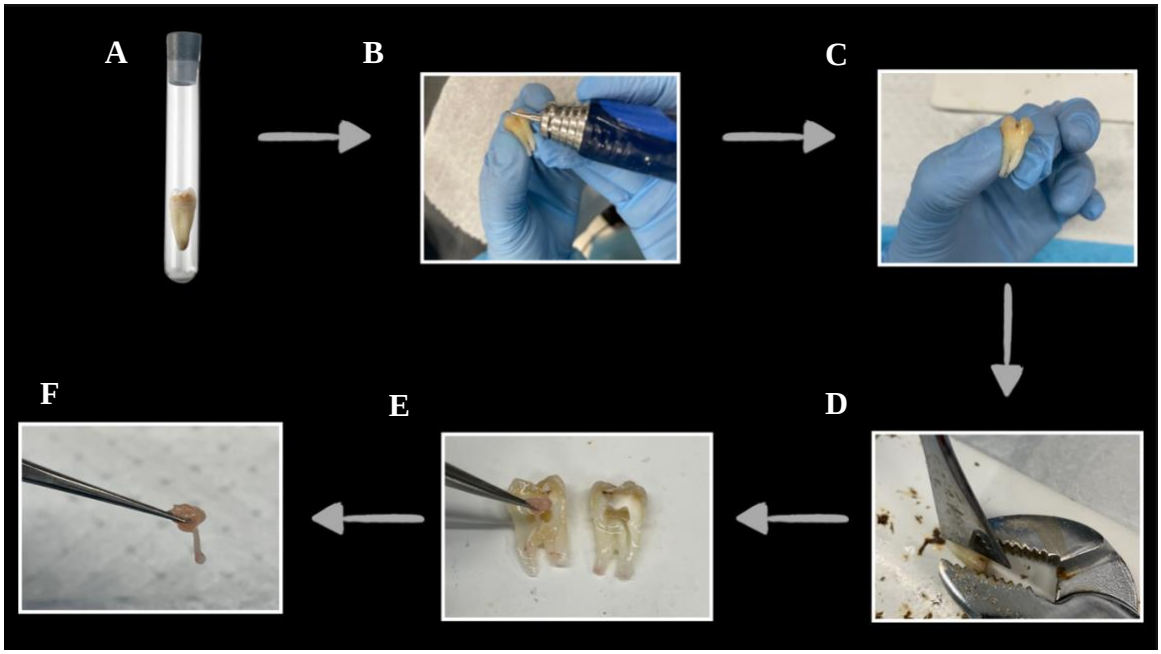


Figure 3. Human dental pulp tissue was extracted from the tooth.

Human dental pulp tissue was extracted from the tooth. **(A)** A freshly extracted tooth was stored in a cold 1X PBS-containing tube and transported to the lab in a secured biohazard labeled container. **(B, C)** A sagittal indentation was made on each tooth using a high-speed handpiece and a 330 bur in an intermittent manner with water coolant. **(D, E)** The tooth was secured to the pliers and later sectioned in half using #7 chandler bi-bevel bone chisel and hammer. **(F)** The pulp tissue was located and meticulously extracted from the pulp chamber.

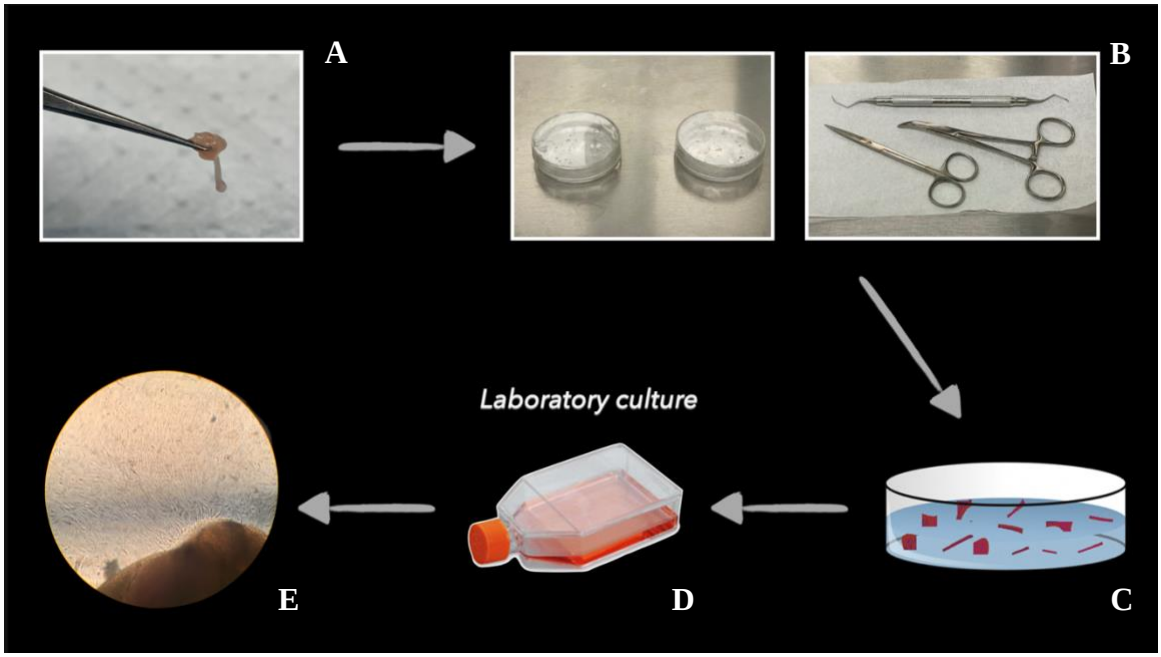


Figure 4. Human dental pulp cells isolating procedures.

Human dental pulp cells (HDPCs) isolating procedures were conducted in class II biological lab safety hood. **(A)** The pulp tissue was extracted from the pulp chamber. **(B, C)** Petri dishes containing cold sterilized PBS were prepared to rinse the pulp tissue samples before dividing them into smaller fragments using sterilized micro-dissecting scissors. **(D)** The pulp fragment was transferred into a T12.5 flask containing a warm growth medium and maintained in the incubator at 37°C. **(E)** The picture displayed HDPCs migration from the pulp tissue onto the surface of the flask.

4.3.2.2 Primary Tissue Culture Maintenance and Expansion

The primary cell culture in T12.5 flasks was allowed until the number of explanted cells reached 70%-80% confluence, which could take up to 3-4 weeks after cell culture initiation. The pulp fragments were kept in the flasks for no longer than 14 days to allow cell propagation (Fig. 4E). As soon as efficient cells were present and attached to the flask, the pulps were removed to prevent microbial contamination. The growth medium was changed every 3-4 days by aspirating the old solution and then using 10 mL of warm 1X PBS to rinse the flask thoroughly to ensure that no remaining old medium was on the internal wall. After 1-2 minutes, the PBS was aspirated and replaced by 8 mL of warm growth medium immediately to avoid cell dehydration. The process was repeated until the cells proliferated to 70%-80% confluence and were ready for the second passage (Fig. 5).

Once the flask reached the designated confluency, the cells would be passaged from a T12.5 flask to a T225 flask. The cell transferring procedure began with aspirating growth medium then the cells were rinsed with 10 mL of warm 1X PBS. After aspirating the PBS, trypsinization was carried out by adding 1 mL of 0.05% trypsin-EDTA into the flask and placing the flask in the incubator at 37°C for 3-4 minutes. During the incubating period, the flask was retrieved to observe the cell condition under the light microscope. The trypsin accelerated the detachment of the cells from the flask surface, which aided in cell transferring. When all cells were completely detached, 10 mL of warm growth medium was added to deactivate the trypsinization. The cell suspension was collected in a sterile 50 mL tube for five-minute centrifugation at 1000 rpm at RT.

After the centrifugation, the pellet of cells coagulated at the bottom of the 50 mL tube. The 50 mL tube was then taken back to the biological hood, and the suspension on the top was carefully aspirated and replaced with 10 mL of fresh growth medium. Finally, the new growth medium with cells was re-suspended several times in the tube before being transferred to a T225 flask, labeled with sample number and date and pre-filled with 50 mL of the growth medium, then stored in the incubator at 37°C with 5% CO_2 and saturated humidity.

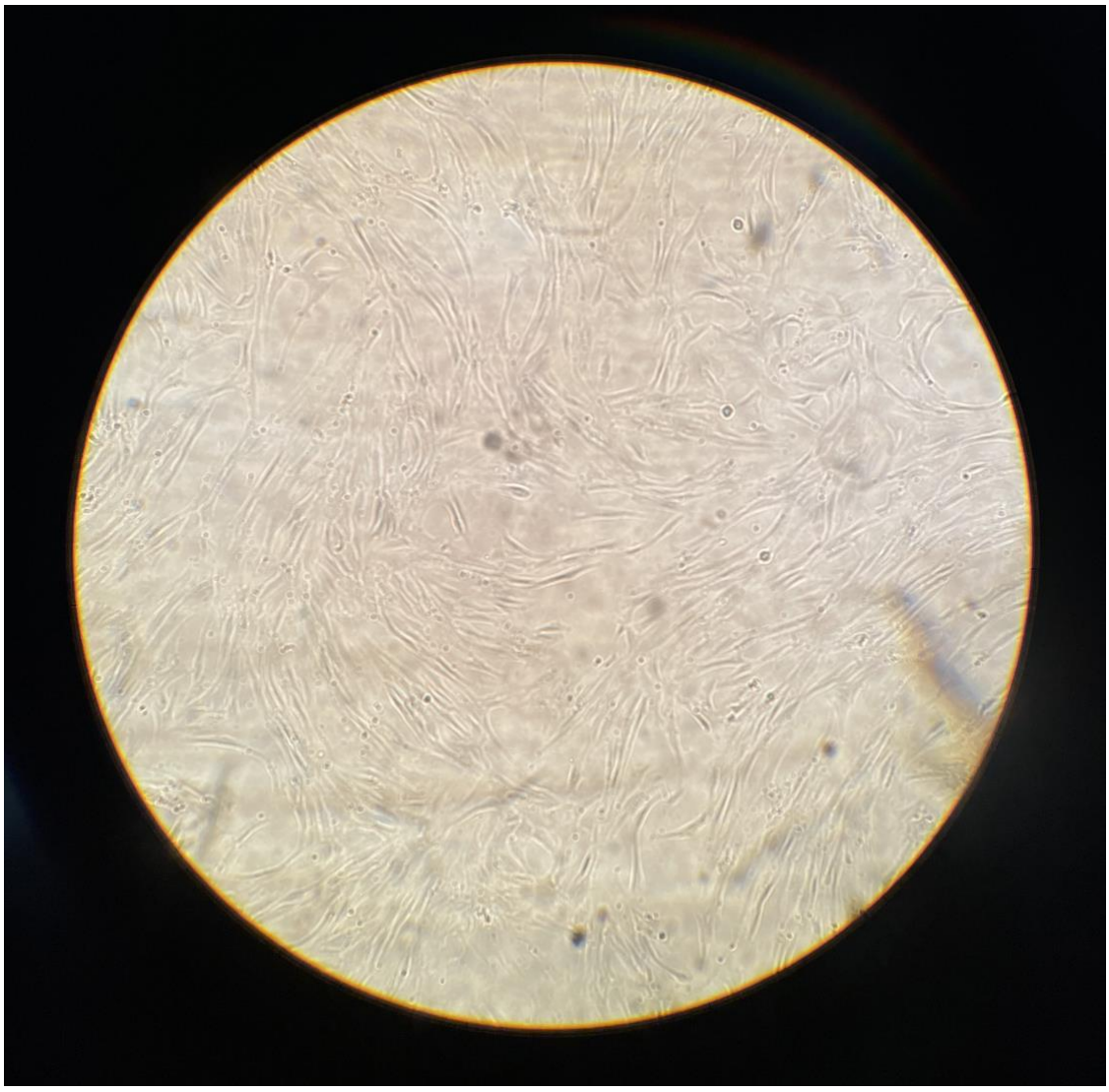


Figure 5. Human dental pulp cells culture at 70%-80% confluency.

The picture exhibited passage 1 of explanted human dental pulp cells (HDPCs) culture at 70%-80% confluency in a T12.5 flask before trypsinization and secondary passage procedures.

4.3.2.3 Secondary Tissue Culture Maintenance and Processing

For the second passage cell culture in the T225 flask, the growth medium was changed every 3-4 days by rinsing the cells with 30 mL of 1X PBS before aspirating and replacing the solution with a total of 60 mL of fresh growth medium. The cell propagation continued for 1-2 weeks until the cells reached proper confluency and were ready for the experiment.

Once the second passage reached 70%-80% confluence, the cells would be processed by the following protocol and used in the experiment. First, the old medium was aspirated, and the cells were rinsed with 30 mL of warm 1X PBS. Then, the trypsinization began by aspirating the PBS and adding 3 mL of 0.05% trypsin-EDTA into the flask, which was then kept inside the incubator at 37°C for 4-5 minutes. The cells fully detached from the flask, which was confirmed using the light microscope. Following that, to stop the trypsinization, 30 mL of warm growth medium was added to the flask, and the cell suspension was transferred in a sterile 50 mL tube for five-minute centrifugation at 1000 rpm at RT.

After the centrifugation, the pellet of cells coagulated at the bottom of the 50 mL tube. The 50 mL tube was then taken back to the biological hood, and the suspension on the top was carefully aspirated and replaced with a fresh growth medium in the designated amount. Subsequently, 20 μL of the cell suspension was added to the hemocytometer to perform cell density count. The process began with using a small pipette tip to fill the suspension into the hemocytometer gently to avoid generating bubbles. Then, the cell

suspension spontaneously flowed into the chamber via capillary action. After some adjustments under 20x magnification of the light microscope, gridlines were explicit and considered proper to begin the cell count. The number of cells in 1 mL of suspension was an average from cell number counted from four separate square areas shown in the hemocytometer then multiplied by 10^4. Each experiment required a different number of seeding cells. Hence, the dilution of the cell suspension would be adjusted with the growth medium accordingly.

4.3.2.4 Determination of the Optimal Cell Seeding Concentration

In this study, the HDPCs would be observed in 6-well plates and 24-well plates until the final time point on day 14 and day 21 subsequently. Therefore, an optimal cell seeding pilot was required in each plate before initiating the experiment to determine the number of seeding cells needed. The pilot helped ensure that the cells would not reach the maximum confluency before the designated time points and could survive throughout the time required for each experiment.

The optimal cell seeding density pilot was carried out by seeding different numbers of cells in a 6-well plate and a 24-well plate then the cell densities were monitored using a light microscope for daily records. For the 6-well plate, the tested concentrations were $2x10^5$, $2.5x10^5$, and $3x10^5$ cells per well. As for the 24-well plate, the tested concentrations were $0.7x10^3$, $3x10^3$, $1x10^4$, $1.5\ x10^4$, $1x10^5$, and $2x10^5$ cells per well. All concentrations were done in triplicates and monitored for 14 days and 21 days in a 6-well plate and a 24-well plate accordingly. In conclusion, the results showed that, for HDPCs, the optimal cell

density of $3x10^3$ cells per well was suitable for a 21 day-experiment in a 24-well plate (Table 10), whereas, for a 6-well plate, $3x10^5$ cells per well were suitable for a 14 day-experiment (Table 11).

Cell number	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Day 14	Day 21
$0.7x10^3$	0%	1%	3%	5%	10%	15%	20%	40%	60%	80%
$3x10^3$	1%	5%	10%	15%	20%	30%	40%	60%	80%	100%
$1x10^4$	5%	10%	20%	40%	60%	70%	80%	100%	-	-
$1.5\ x10^4$	5%	10%	30%	40%	60%	80%	90%	100%	-	-
$1x10^5$	20%	40%	60%	80%	100%	-	-	-	-	-
$2x10^5$	40%	60%	80%	100%	-	-	-	-	-	-

Table 10. Optimal cell seeding density in 24-well plate.

Cell number	Day 3	Day 7	Day 14
$2x10^5$	20%	30%	70%
$2.5x10^5$	20%	40%	80%
$3x10^5$	40%	70%	100%

Table 11. Optimal cell seeding density in 6-well plate.

4.3.3 ASA pH Evaluation

Before proceeding to the experiments relating to cell culture, a 3-day pH evaluation on ASA was conducted to confirm that the pH of each concentration of ASA was within the biological range and safe for the HDPCs.

The growth medium with ASA concentrations of 25, 50, 75, 100, and 200 µg/mL was prepared in 15 mL tubes in triplicate (n = 3) with the 0 µg/mL ASA as a control. The pH evaluation was conducted immediately after the preparation using a pH meter with an electrode tip. The growth medium with all concentrations of ASA was incubated at 37°C in 5% CO_2, and pH evaluation was taken at 20 minutes, then on day 1, day 2, and day 3 to determine the pH stability of ASA over time.

4.3.4 Attachment Efficiency Assay

HDPCs were cultured in 24-well plates at a density of 100,000 cells per well in a culture medium with 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, or 200 µg/mL of ASA with the 0 µg/mL ASA as a control (n = 6). At 4, 8, 12, and 16 hours, the HDPCs were rinsed with 1X PBS and fixed with 10% neutral buffered formalin for 1 hour at RT. The supernatant was harvested to count the number of floating cells under a light microscope using a hemocytometer. The attachment efficiency was considered 100% when no remaining floating cell was seen under the light microscope.

After the HDPCs were fixed, the formalin was removed, and the cells were rinsed twice with DI. A crystal violet staining was conducted by incubating the HDPCs with 0.2%

crystal violet solution for 1 hour at RT before washing with DI twice. The quantification was done by solubilizing the crystal violet staining with 1% triton X-100 solution. The HDPCs were incubated with 0.9 mL of 1% triton X-100 solution for 30 minutes on the shaker at RT then the supernatant was collected. The HDPCs were further incubated with 0.3 mL of 1% triton X-100 solution for another 30 minutes on the shaker at RT then the supernatant was collected and mixed with the first collection. The mixed supernatant of each group was added to a 96-well plate in the amount of 120 µL/well, and the absorbance was measured at 590 nm using a spectrophotometer.

A standard curve with a trend line was generated using the attached cell numbers calculated from floating cells and the optical density (OD) values of the control group, then use the graph equation to find the cell numbers of other groups from the OD values. The percentage of attachment efficiency was calculated using the provided equation.

$$Percentage\ of\ attachment\ efficiency = \frac{Cell\ number}{Initial\ seeding\ number} x\ 100$$

4.3.5 Cell Viability

4.3.5.1 Methylthiazolyldiphenyl-Tetrazolium Bromide (MTT) Assay

MTT assay is a non-radioactive colorimetric assay used for measuring cell proliferation, cell viability, and cell cytotoxicity in an *in vitro* model. The viable cells with active metabolism work to converse water-soluble MTT or (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compounds into purple insoluble formazan crystals (Fig. 6), which serve as a marker for viable cells and a measure of the metabolic activity of mitochondria. The quantification can be carried out by solubilizing the formazan crystal using a proper solvent and measuring the absorbance. The more metabolic activity in the sample, the higher the detected signal.

The mitochondria activity was assessed at 24, 48, and 72 hours using an MTT assay kit (Abcam). HDPCs were cultured in 96-well plates with a culture medium containing 0, 25, 50, 75, 100, or 200 μg/mL ASA at a density of 7,000 cells per well (n = 6). According to the manufacturer's protocols, at each time point, serum-free medium and 50 μL of MTT Reagent were added to each well, then the plates were incubated at 37°C for 3 hours. After the incubation, the old reagent was removed, then 150 μL of MTT Solvent was added to each well. The plate was wrapped with foil and incubated on the shaker at RT for another 15 minutes, then the absorbance was read using a spectrophotometer at the OD of 590 nm. The level of the signal is proportional to the number of viable cells.

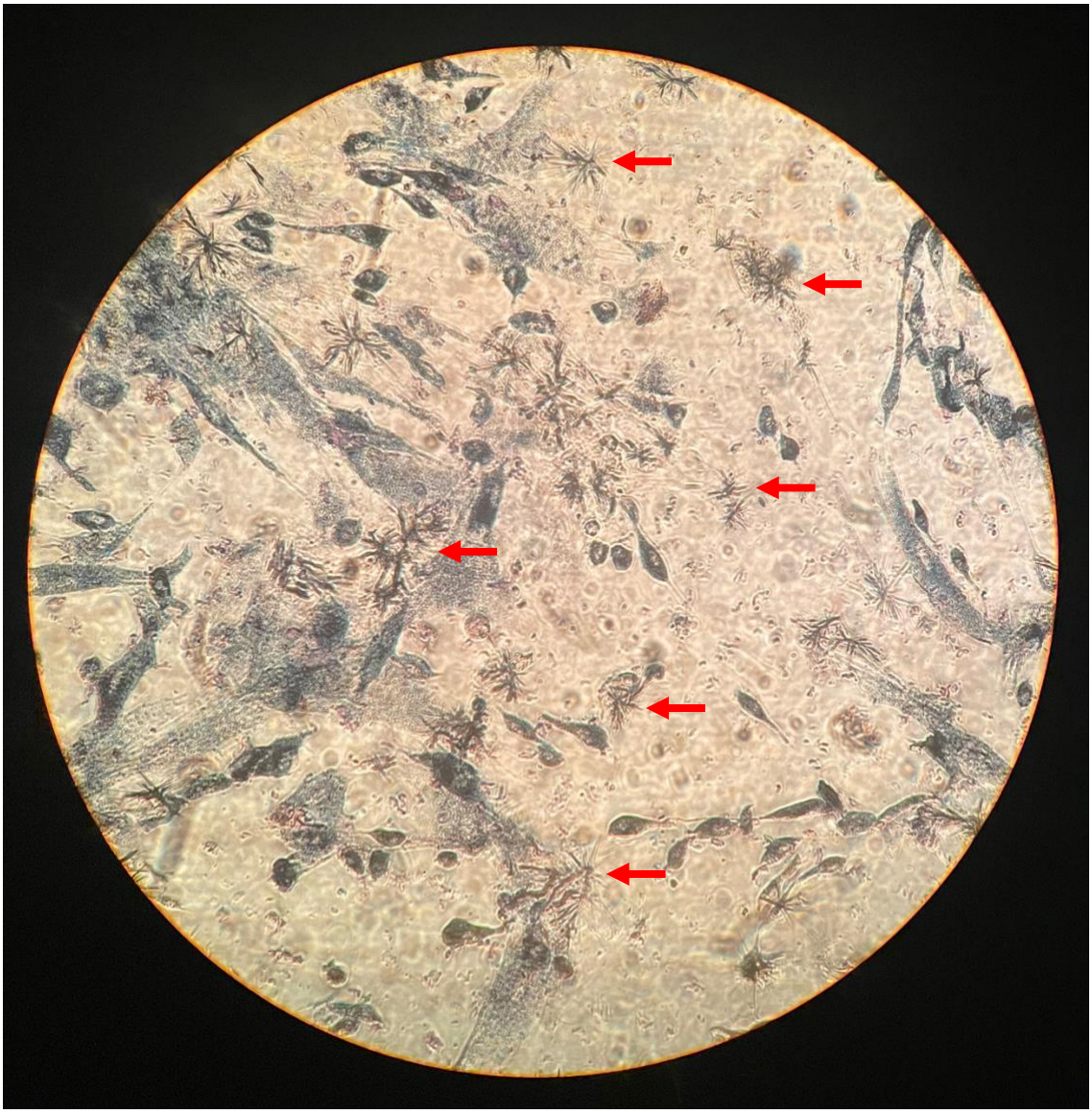


Figure 6. Formazan crystal formation.

The picture showed formazan crystal formation (red arrows) under the light microscope after incubation with Methylthiazolyldiphenyl-tetrazolium bromide (MTT) Reagent.

4.3.5.2 XTT Assay

XTT assay is another non-radioactive colorimetric assay used for measuring cell proliferation, cell viability, and cell cytotoxicity in an *in vitro* model. The assay principle is based on the extracellular reduction of XTT, or yellow tetrazolium salt (sodium3´-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy6-nitro), by NADH (nicotinamide adenine dinucleotide (NAD) + hydrogen (H)) produced in the mitochondria via trans-plasma membrane electron transport and an electron mediator. The reduction of XTT by metabolically active cells generates an orange water-soluble formazan dye used to measure cellular metabolic activity. The greater the number of viable cells, the higher the overall activity of mitochondrial dehydrogenases. The formazan dye, an indicator of cell viability, is directly quantified by measuring the absorbance using a spectrophotometer. No additional solubilization step is required.

The mitochondria activity was assessed at 24, 48, and 72 hours using an XTT assay kit (Abcam). HDPCs were cultured in 96-well plates with a culture medium containing 0, 25, 50, 75, 100, or 200 μg/mL ASA at a density of 7,000 cells per well (n = 6). According to the manufacturer's protocols, at each time point, 10 μL of the XTT Mixture, consisting of XTT Developer Reagent and Electron Mediator Solution, was added to each well and mixed for 1 minute on a shaker at RT. The HDPCs were incubated for 2 hours at 37^{o}C in a CO_2 incubator, then another 1 minute on a shaker to ensure a homogenous distribution of color before measuring the absorbance at 450 nm using a spectrophotometer. The level of the signal is proportional to the number of viable cells.

4.3.6 Proliferation Rates

HDPCs were cultured in 24-well plates at a density of 3,000 cells per well in a culture medium with 25, 50, 75, 100, or 200 µg/mL of ASA with the 0 µg/mL ASA as a control (n = 6). From the attachment efficiency result, HDPCs showed the highest attachment at 16 hours which would be considered a baseline for this experiment. At 16 hours, 7, 14, and 21 days, the HDPCs were rinsed with 1X PBS and fixed with 10% neutral buffered formalin for 1 hour at RT. The HDPCs were then observed under the light microscope to ensure a proper fixation before removing the formalin and rinsing the cells twice with DI. The HDPCs were then incubated with 0.2% crystal violet solution for 1 hour at RT and washed twice with DI. The quantification was done by solubilizing the crystal violet staining with 1% triton X-100 solution. The HDPCs were incubated with 0.9 mL of 1% triton X-100 solution for 30 minutes on the shaker at RT then the supernatant was collected. The HDPCs were further incubated with 0.3 mL of 1% triton X-100 solution for another 30 minutes on the shaker at RT then the supernatant was collected and mixed with the first collection. The mixed supernatant of each group was added to a 96-well plate in the amount of 120 µL/well, and the absorbance was measured at 590 nm using a spectrophotometer. The proliferation rate was calculated using the provided equation.

$$Proliferation\ rate = \frac{OD\ of\ test\ condition}{OD\ at\ 16h\ in\ the\ same\ test\ condition}$$

4.3.7 Odontogenic Differentiation

HDPCs were cultured in 24-well plates at a density of 3,000 cells per well in a culture medium with 25, 50, 75, 100, or 200 µg/mL of ASA with the 0 µg/mL ASA as a control (n = 4). At 16 hours, the HDPCs were fixed with 10% neutral buffered formalin for 1 hour at RT to be used as a baseline to calculate cell numbers. Live cell morphology image was taken on day 7, day 14, and day 21 using Nikon Deconvolution Wide-Field Epifluorescence System, then the supernatant was harvested after adding the differentiation medium to HDPCs according to the indicated timeline below.

1) For day 7 time point
    - Day 4: Differentiation medium without Vit D_3 was added to the HDPCs
    - Day 5: Differentiation medium with Vit D_3 was added to the HDPCs
    - Day 7: The supernatant was collected

2) For day 14 time point
    - Day 11: Differentiation medium without Vit D_3 was added to the HDPCs
    - Day 12: Differentiation medium with Vit D_3 was added to the HDPCs
    - Day 14: The supernatant was collected

3) For day 21 time point
    - Day 18: Differentiation medium without Vit D_3 was added to the HDPCs
    - Day 19: Differentiation medium with Vit D_3 was added to the HDPCs
    - Day 21: The supernatant was collected

The collected supernatant was immediately stored in a -20°C freezer for further biomarker evaluations. HDPCs from each time point, including 16 hours, would be quantified for cell numbers to be used for normalizing biomarker levels and mineralized nodule production. The HDPCs were rinsed with 1X PBS and fixed with 10% neutral buffered formalin for 1 hour at RT then observed under the light microscope to ensure a proper fixation before removing the formalin and rinsing the cells twice with DI. 0.2% crystal violet solution was applied to incubate the cells for 1 hour at RT before rinsing twice with DI. The quantification was done by solubilizing the crystal violet staining with 1% triton X-100 solution. The HDPCs were incubated with 0.9 mL of 1% triton X-100 solution for 30 minutes on the shaker at RT then the supernatant was collected. The HDPCs were further incubated with 0.3 mL of 1% triton X-100 solution for another 30 minutes on the shaker at RT then the supernatant was collected and mixed with the first collection. The mixed supernatant of each group was added to a 96-well plate in the amount of 120 µL/well, and the absorbance was measured at 590 nm using a spectrophotometer. The cell number was calculated using the provided equation.

$$Cell\ number = \frac{OD\ of\ test\ condition}{OD\ per\ cell}$$

4.3.8 Odontogenic Biomarkers

4.3.8.1 Transcriptional Level

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The levels of dentin sialophosphoprotein (DSPP) and runt-related transcription factor 2 (RUNX2) mRNA productions were measured with RT-qPCR (n = 6). HDPCs were cultured in 6-well plates at a density of 300,000 cells per well in a culture medium with 25, 50, 75, 100, or 200 µg/mL of ASA with the 0 µg/mL ASA as a control. At 4, 7, and 14 days, TRIzol reagent and Phasemaker™ Tubes (Invitrogen; Thermo Fisher Scientific) were applied to lyse the cells for RNA extraction and purification using PureLink® RNA Mini Kit incorporating with PureLink™ DNase Set (Invitrogen) according to the manufacturer's instructions.

RNA yield and RNA quality were determined by the NanoDrop 2000 UV Visible Spectrophotometer (Thermo Fisher Scientific) at the UV absorbance of 260 nm and 260/280 nm subsequently. An $OD_{260/280}$ of over 1.8 indicates that RNA is reasonably clean of proteins and other UV chromophores that could interfere with downstream applications or negatively affect the stability of the RNA. Total extracted RNA was calculated using the provided equation.

$$Total\ RNA\ (\mu g) = OD260\ x\ \frac{40\ \mu g}{1\ OD260\ x\ 1\ mL}\ x\ dilution\ factor\ x\ total\ sample\ volume\ (mL)$$

The extracted RNA of 0.2 μg from each sample was utilized in a one-step RT-qPCR using a SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific). The target TaqMan probes (Thermo Fisher Scientific) are listed in Table 12. The housekeeping gene, β-actin, was used to normalize gene expression levels of each target gene, and the $2^{-\Delta\Delta Ct}$ calculation was conducted to deliver the relative differences in the gene expressions. The PCR was set in standard cycling conditions as described: 50°C for 15 minutes followed by 95°C for 2 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

Names	Gene Assay ID Number
DSPP	Hs00171962_m1
RUNX2	Hs00231692_m1
β-actin	Hs03023943_g1

Table 12. The List of TaqMan Probes. DSPP, dentin sialophosphoprotein; RUNX2, runt-related transcription factor 2.

4.3.8.2 Post-Transcriptional Level

Fluorometric Assay

Alkaline phosphatase (ALP) activity was detected using an Alkaline Phosphatase Fluorometric Assay Kit (Abcam) ($n = 4$). The collected supernatant contains ALP produced by HDPCs. The ALP will cleave the phosphate group of the non-fluorescent 4-

methylumbelliferyl phosphate disodium salt (MUP) substrate creating an intense fluorescent signal.

According to the manufacturer's protocols, the collected supernatant was incubated with non-fluorescent MUP substrate for 30 minutes at RT, protected from light. Stop solution was added to each well, and the fluorescent signal was measured at Ex/Em = 360/440 nm. The standard curve was plotted from the OD of standard samples provided by the kit, and the trend line equation was established. The level of ALP of each test condition was calculated and normalized by cell numbers per million cells.

Enzyme-Linked Immunosorbent Assays (ELISA)

The Human Dentin Sialoprotein (DSP) and the RUNX2 ELISA kits (Express Biotech International) were applied, following the manufacturer's protocols to evaluate the DSP and RUNX2 activities subsequently (n = 4). The kits are based on the sandwich ELISA concept and provide 96-well plates with the surface precoated with capture antibody to capture target biomarkers in the supernatant. Once the biotin-conjugated antibody (detection antibody) is added to the well, the target biomarkers are re-captured in a sandwich manner. HRP-streptavidin conjugate (SABC) is used to bind with the detection antibody and visualized by tetramethyl-benzidine (TMB) substrate. The TMB is catalyzed by horseradish peroxidase (HRP) enzymatic reaction and produces a blue-colored product that changes into yellow after adding an acidic stop solution. The density of the color is proportional to the level of target biomarkers captured in the plate.

According to the manufacturer's protocols, the collected supernatant was added to the pre-coated wells and incubated for 90 minutes at 37°C. The plate was washed twice and incubated with a biotin-labeled antibody for 60 minutes at 37°C. After washing the plate 3 times, SABC was added to each well and incubated for 30 minutes at 37°C. The plate was washed 5 times and incubated with TMB substrate for 20 minutes at 37°C before adding the stop solution to end the reaction. The absorbance was measured at 450 nm using a spectrophotometer. The standard curve was plotted from the OD of standard samples provided by the kit, and the trend line equation was established. The levels of DSP and RUNX2 of each test condition were calculated and normalized by cell numbers per million cells.

4.3.9 Mineralization

During the mineralization, odontoblasts produce calcium deposition indicating the formation of dentin. This mineralized nodule formation can be detected using an alizarin red staining method.

HDPCs were cultured in 24-well plates at a density of 3,000 cells per well in a culture medium containing 25, 50, 75, 100, or 200 µg/mL of ASA with the 0 µg/mL ASA as a control (n = 6). The differentiation medium was added to HDPCs for day 7, day 14, and day 21-time points as indicated in 4.3.7. After collecting the supernatant, the HDPCs were rinsed with 1X PBS and fixed with 10% neutral buffered formalin for 1 hour at RT, then observed under the light microscope to ensure a proper fixation before removing the formalin and rinsing the cells twice with DI. The HDPCs were incubated with ARS in the

dark for 45 minutes on the shaker at RT. The ARS was carefully aspirated, and the cells were rinsed 4 times with DI. Mineralized nodules are visualized in a bright orange-red color (Fig. 7).

The alizarin red staining was solubilized using 10% acetic acid to quantify the mineralized nodule formation. The HDPCs were incubated with 0.9 mL of 10% acetic acid solution for 1 hour on the shaker at RT then the supernatant was collected. The HDPCs were further incubated with 0.3 mL of 10% acetic acid solution for another 1 hour on the shaker at RT then the supernatant was collected and mixed with the first collection. The mixed supernatant of each group was added to a 96-well plate in the amount of 120 µL/well, and the absorbance was measured at 405 nm using a spectrophotometer. The alizarin red level was calculated from the OD of each test condition normalized by cell numbers per million cells.

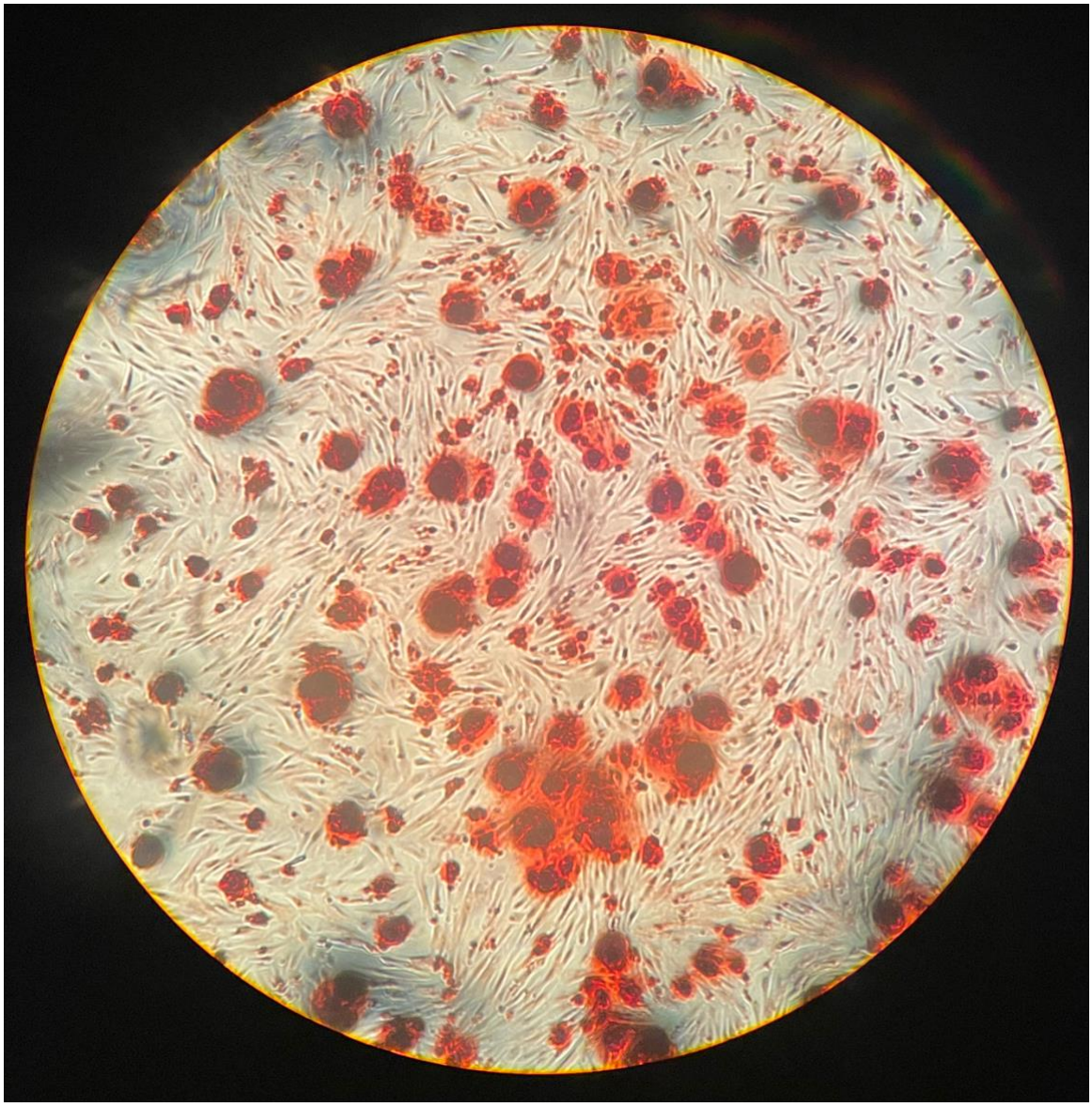


Figure 7. Mineralized nodule formation.

The picture illustrated mineralized nodules in bright orange-red color after the cells were incubated with alizarin red S solution (ARS).

4.3.10 Evaluation of Liberated Transforming Growth Factor-ß1 (TGF-ß1) from Dentin Slices

4.3.10.1 Dentin Slice Preparation

Dentin slices were prepared according to a previously reported protocol (86). Human permanent molars were sectioned into the thickness of 0.6-0.8 mm using a linear precision saw (IsoMet™ 5000; Buehler), then pulp tissues, cementum, and enamel were removed. The dentin was further cut into slices with an estimated surface area of 1.5 cm^2 before being used for the experiment.

4.3.10.2 Evaluation of Liberated Growth Factor from Dentin Slices

Dentin slices were submerged in 500 μL of PBS (negative control), 17% ethylenediaminetetraacetic acid solution (EDTA; positive control), or ASA at a concentration of 25 or 200 μg/mL (n = 5). After 5 or 10 minutes, the solution was collected and stored in a -20°C freezer for further TGF-ß1 evaluation using Human TGF-ß1 ELISA kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Before using the kit, 1.0 N hydrochloric acid (HCl) solution was added to the prepared supernatant to activate the TGF-ß1 and then neutralized with 1.0 N sodium hydroxide (NaOH) solution. The supernatant was added to the anti-human TGF-ß1antibody coated microwells and incubated for 2 hours on the shaker at RT. Human TGF-ß1 present in the sample would bind to antibodies adsorbed to the microwells. Then a

biotin-conjugated anti-human TGF-ß1 antibody was added to bind to human TGF-ß1 captured by the first antibody, and the plate was incubated for 1 hour on the shaker at RT. Unbound biotin-conjugated anti-human TGF-ß1 antibodies would be removed during a wash step, then SABC was used to bind to the biotin-conjugated anti-human TGF-ß1 antibody, and the plate was incubated for another 1 hour on the shaker at RT. Following the incubation, unbound SABC was removed during a wash step, and the TMB substrate solution, reactive with HRP, was added to the wells. After 30 minute-incubation at RT, a blue-colored product was formed in proportion to the amount of human TGF-ß1 present in the sample. The reaction was terminated and turned to yellow color by an acidic stop solution. The absorbance was measured at 450 nm using a spectrophotometer. The standard curve was plotted from the OD of standard samples provided by the kit, and the trend line equation was established to calculate the level of TGF-ß1 in each test condition.

CHAPTER FIVE: STATISTICAL ANALYSIS

Chapter Five: STATISTICAL ANALYSIS

Statistical analyses were performed using JMP Pro version 15.2 software (SAS Institute Inc., Cary, NC). All results were presented as mean ± standard deviation of at least three replicates. Comparisons between two groups were analyzed by independent 2-tailed Student's *t* tests, and multiple groups comparisons were acquired by 1-way analysis of variance (ANOVA) followed by the Tukey post hoc tests. P values < 0.05 were considered statistically significant.

CHAPTER SIX: EXPERIMENTAL RESULTS

Chapter Six: EXPERIMENTAL RESULTS

6.1 ASA pH Evaluation

The highest concentration of acetylsalicylic acid (ASA) for the experiments, 200 μg/mL, was determined based on the minimum plasma concentration that early signs of salicylic overdose occurred, according to the United States Food and Drug Administration (FDA), Code of Federal Regulations (CFR) Title 21 CFR 343. After preparing ASA, a 3-day pH evaluation was done to ensure that the pH of all ASA concentrations in the culture medium was within the biological range to conduct the cell cultures.

On day 0, immediately after preparing the ASA concentrations in the culture medium, the pH of all concentrations was within the range of 7.5-7.9, showing the largest interval presented in every observed time point. The control group presented the highest pH at 7.9, and the pH gradually decreased as the ASA concentration increased to the lowest point at pH 7.5 in 200 μg/mL ASA. 50, 75, 100, and 200 μg/mL ASA demonstrated significantly lower pH than the control group ($P < 0.05$ and $P < 0.001$; Fig. 8). After 20 minutes of incubation, the pH of each group was measured again and demonstrated a smaller interval of 7.73-7.91, with the control showing the highest pH and the 200 μg/mL ASA showing the lowest value. However, 50-200 μg/mL ASA still manifested significantly lower pH than the control ($P < 0.001$; Fig. 9).

All test conditions were kept in the incubator overnight for 1-3 days, then the pH evaluation of the culture medium was conducted. On day 1, the overall pH was brought

down to the range of 7.48-7.63 and maintained in these values until day 2, then gradually increased to 7.54-7.75 on day 3 (Fig. 10-12). The trend during the 3 day-evaluating periods was consistent with one another, with the control showing the highest pH value and ASA concentrations over 25 μg/mL demonstrating significantly lower pH than the control ($P < 0.05$ and $P < 0.001$; Fig. 13).

Overall, the ASA 3-day pH evaluation showed that the pH of the ASA-treated culture medium was within the biological range for the human dental pulp cells (HDPCs) culture. ASA at concentrations higher than 25 μg/mL significantly lowered the pH of culture medium in a dose-dependent manner. The pH of all ASA concentrations ranged from a larger interval of 7.5-7.9 to 7.7-7.9 after being incubated for 20 minutes. The incubator condition kept the pH stable in the 7.5-7.7 range throughout the evaluation (Fig. 13).

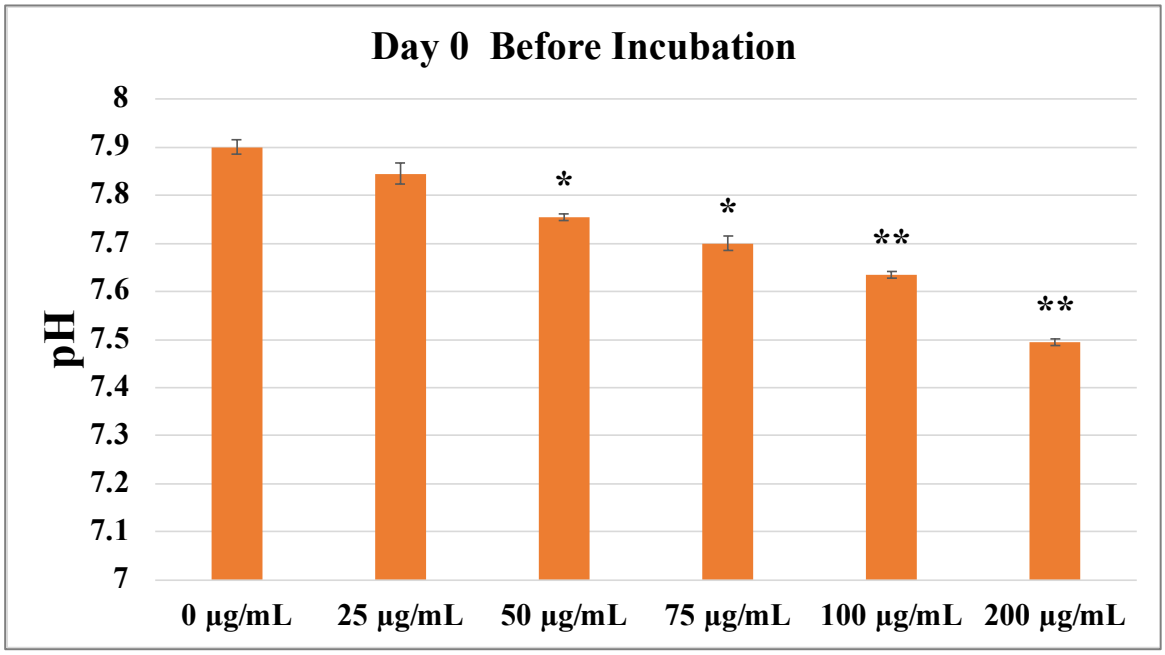


Figure 8. pH evaluation on day 0 before the incubation.

pH evaluation of culture medium in the treatment groups was conducted after the preparation. The acetylsalicylic acid (ASA) affected the pH of the culture medium in a dose-dependent manner, and the pH ranged from 7.5 to 7.9. $n = 3$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

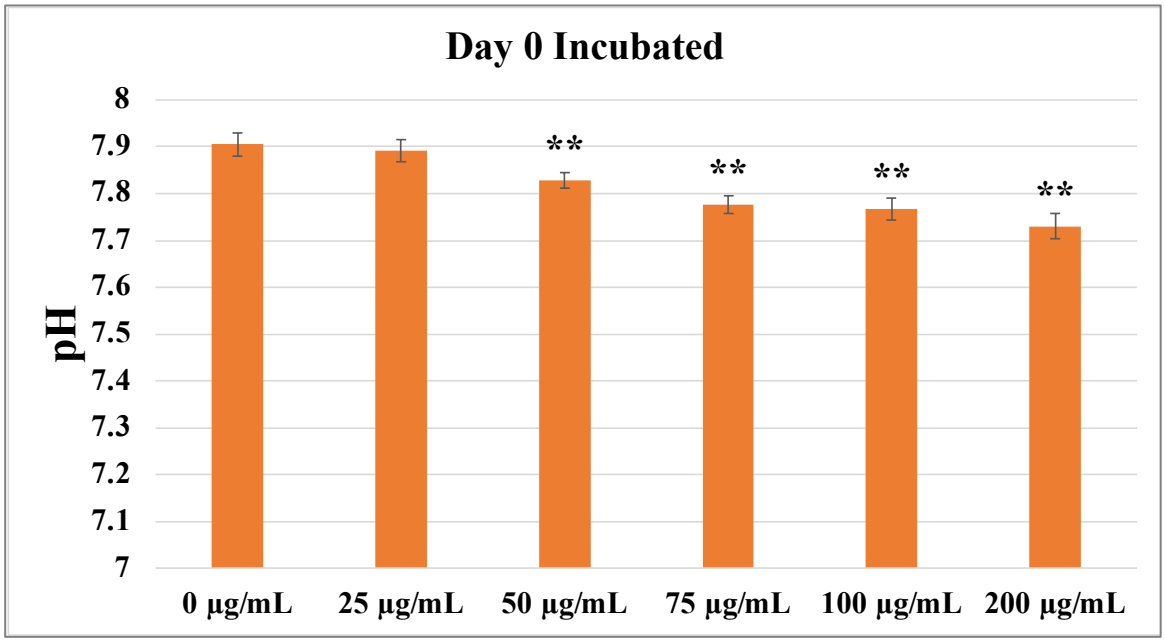


Figure 9. pH evaluation on day 0 after 20 minutes of incubation.

pH evaluation of culture medium in the treatment groups was conducted on day 0 after 20 minutes of incubation. The acetylsalicylic acid (ASA) affected the pH of the culture medium in a dose-dependent manner, and the pH ranged from 7.73 to 7.91. $n = 3$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

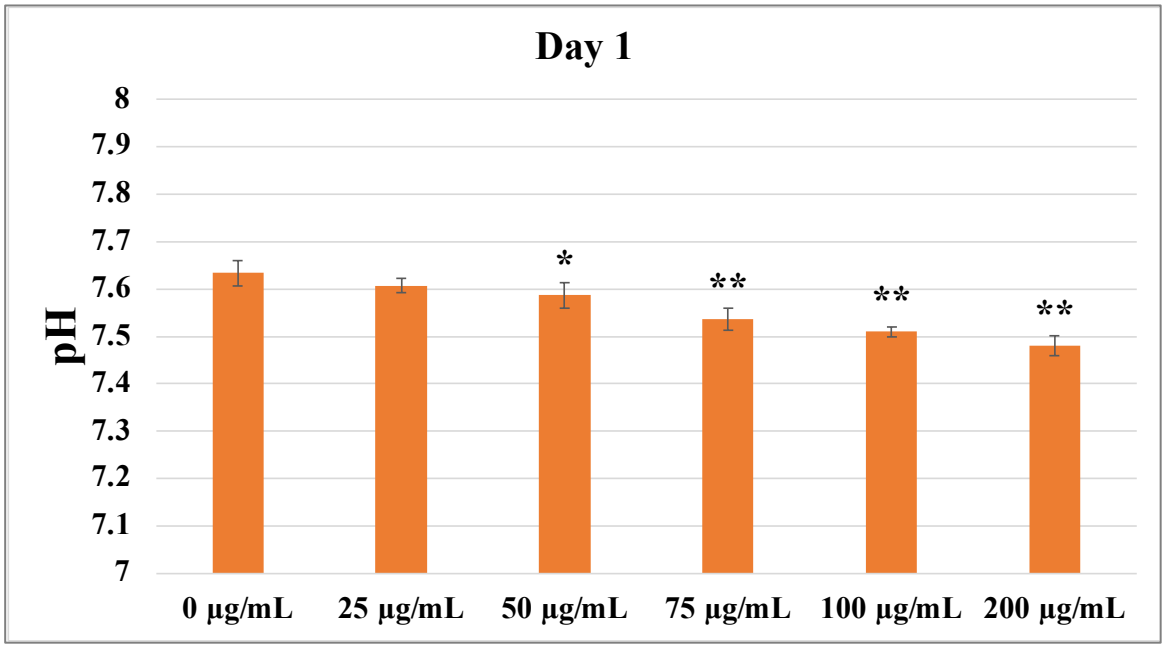


Figure 10. pH evaluation on day 1.

pH evaluation of culture medium in the treatment groups was conducted on day 1. The acetylsalicylic acid (ASA) affected the pH of the culture medium in a dose-dependent manner, and the pH ranged from 7.48 to 7.63. $n = 3$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

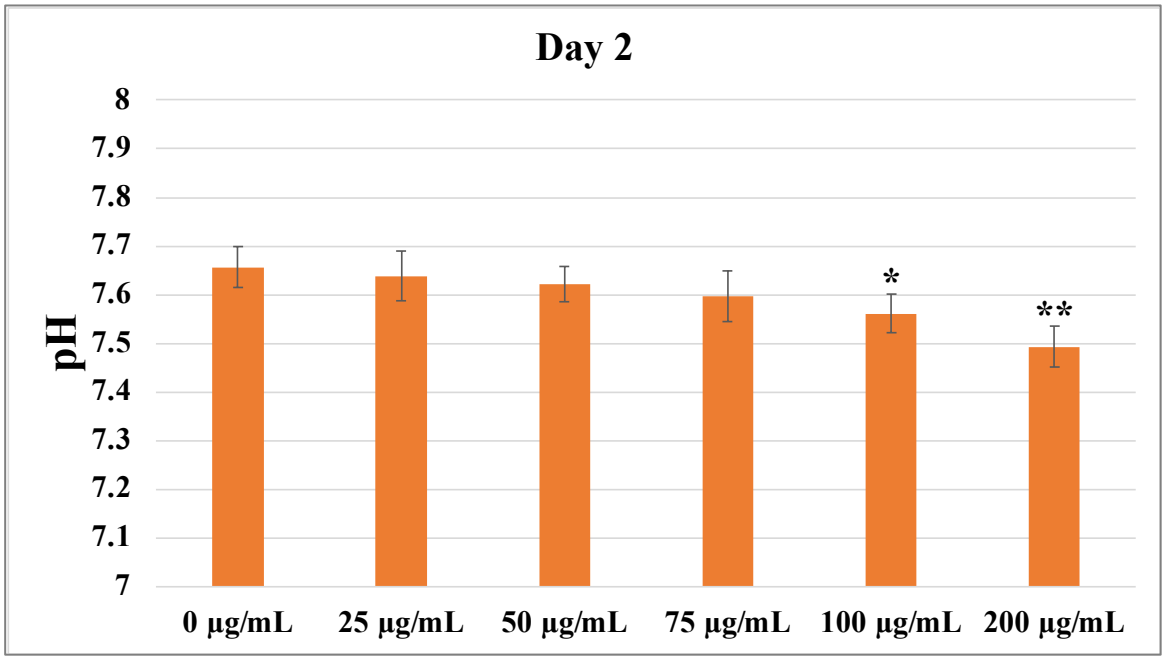


Figure 11. pH evaluation on day 2.

pH evaluation of culture medium in the treatment groups was conducted on day 2. The acetylsalicylic acid (ASA) affected the pH of the culture medium in a dose-dependent manner, and the pH ranged from 7.49 to 7.66. $n = 3$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

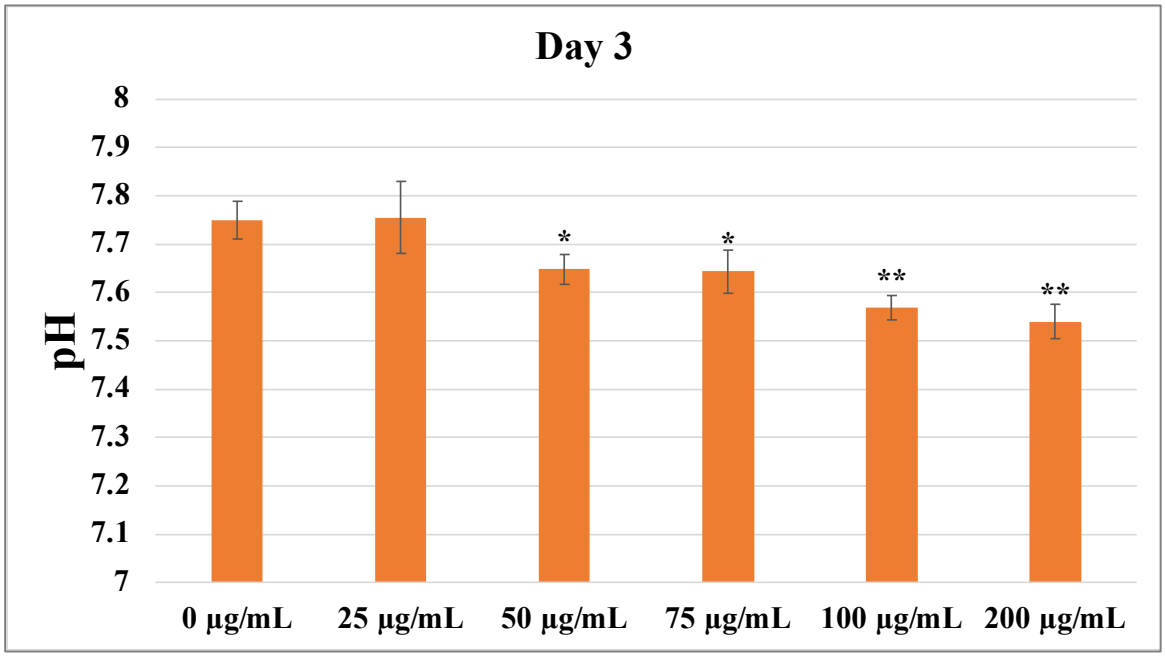


Figure 12. pH evaluation on day 3.

pH evaluation of culture medium in the treatment groups was conducted on day 3. The acetylsalicylic acid (ASA) affected the pH of the culture medium in a dose-dependent manner, and the pH ranged from 7.54 to 7.75. $n = 3$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

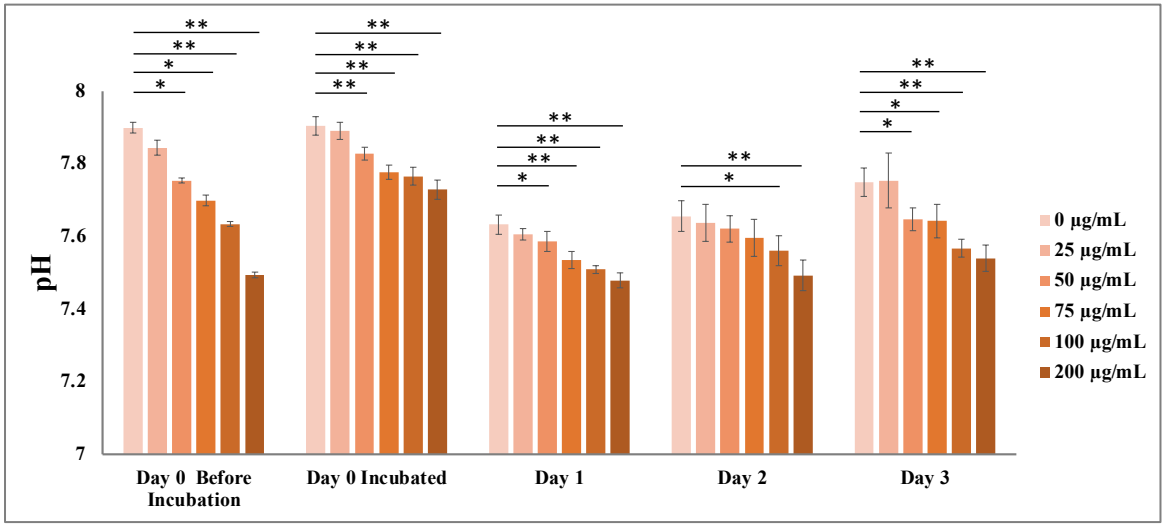


Figure 13. ASA 3-day pH evaluation results.

Acetylsalicylic acid (ASA) 3-day pH evaluation showed that the pH of the ASA-treated culture medium was within the biological range for the human dental pulp cells (HDPCs) culture. The ASA affected the pH of the culture medium in a dose-dependent manner. $n = 3$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

6.2 Attachment Efficiency

According to the reports by preceding residents, normal HDPCs would generally express the highest attachment efficiency at 16 hours, and no cell doubling was detected within this period (156-158). Pilot studies on the attachment efficiency of HDPCs treated with concentrations of ASA were conducted to confirm the phenomenon. The attachment efficiency of HDPCs in the pilot studies was evaluated every 4 hours, with 20 hours as the final time point. Results also showed that the number of attached cells in all test conditions reached the cell seeding number at 16 hours, and the number started to exceed the initial seeding number when observed at 20 hours. The results were repeatable and in line with the previous reports. Therefore, 16 hours were used as the final time point to establish the baseline in the main experiments.

After the time points were confirmed in the pilot studies, the main experiment was conducted by culturing HDPCs with 0, 25, 50, 75, 100, or 200 μg/mL ASA, and the attachment efficiency was assessed at 4, 8, 12, and 16 hours using crystal violet staining solubilized by triton X-100.

The results showed increased attachment efficiency over time, and the HDPCs reached the highest attachment efficiency at 16 hours in all test conditions. Also, the attachment efficiency at 16 hours was significantly enhanced by 50 μg/mL of ASA compared to the control group ($P < 0.05$; Fig. 14).

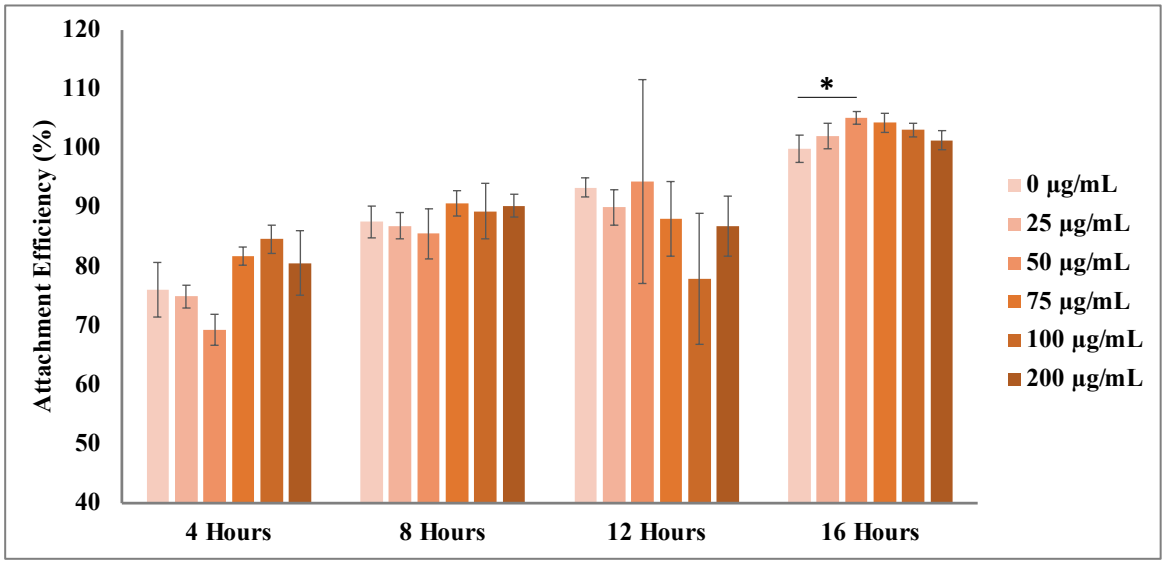


Figure 14. The human dental pulp cells in all test conditions expressed the highest attachment efficiency at 16 hours.

The attachment efficiency of human dental pulp cells (HDPCs) was evaluated every 4 hours using crystal violet staining solubilized by triton X-100. The HDPCs in all test conditions expressed the highest attachment efficiency at 16 hours. HDPCs-treated with 50 µg/mL acetylsalicylic acid (ASA) also showed significantly higher attachment efficiency than the control at 16 hours. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

6.3 Cell Viability

To investigate the effect of concentrations of ASA on the viability of HDPCs, methylthiazolyldiphenyl-tetrazolium bromide (MTT) and XTT assays were conducted to evaluate the mitochondria activity of HDPCs treated with 25, 50, 75, 100, 200, and 0 μg/mL ASA as a control for 24, 48, and 72 hours.

6.3.1 MTT Assay

After 24 hours of ASA treatment, the mitochondria activity observed in all ASA treated groups demonstrated higher levels than in the control group, with statistical significance in the 50-100 μg/mL ASA group ($P < 0.05$; Fig. 15). The results indicated that no cytotoxicity effect of ASA on HDPCs was observed at 24 hours.

An evaluation at 48 hours showed a significantly lower mitochondria activity in the 100-200 μg/mL ASA group to the control indicating a down-regulating effect of high concentrations of ASA at 48 hours ($P < 0.05$ and $P < 0.001$; Fig. 16). However, the levels of mitochondria activity observed at this time were increased from the previous observation at 24 hours, showing signs of proliferation.

The last observation was conducted at 72 hours and showed that overall levels of the mitochondria activity in all test conditions increased from the previous time points. No statistically significant difference in the mitochondria activity of HDPCs was observed in the groups treated with 50-200 μg/mL ASA, but a significantly enhanced mitochondria activity was expressed by the group of HDPCs treated with 25 μg/mL ASA ($P < 0.05$; Fig.

17). The result at this time point was later found to be consistent with the XTT results and the proliferation pilot on day 3.

Over 72 hours, the mitochondria activity observed with the MTT assay showed that all concentrations of ASA were non-toxic to HDPCs as the mitochondria activity of all groups increased within the experimental period (Fig. 18).

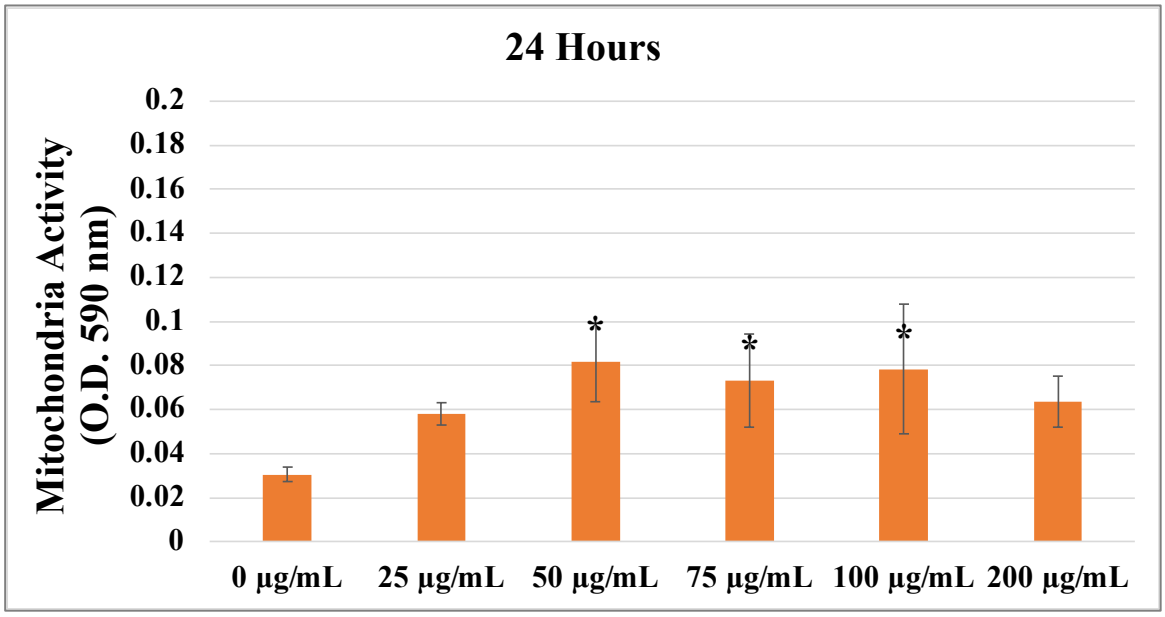


Figure 15. Methylthiazolyldiphenyl-tetrazolium bromide assay at 24 hours.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay at 24 hours showed that all acetylsalicylic acid (ASA) treated groups of human dental pulp cells (HDPCs) demonstrated higher levels of mitochondria activity than the control. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

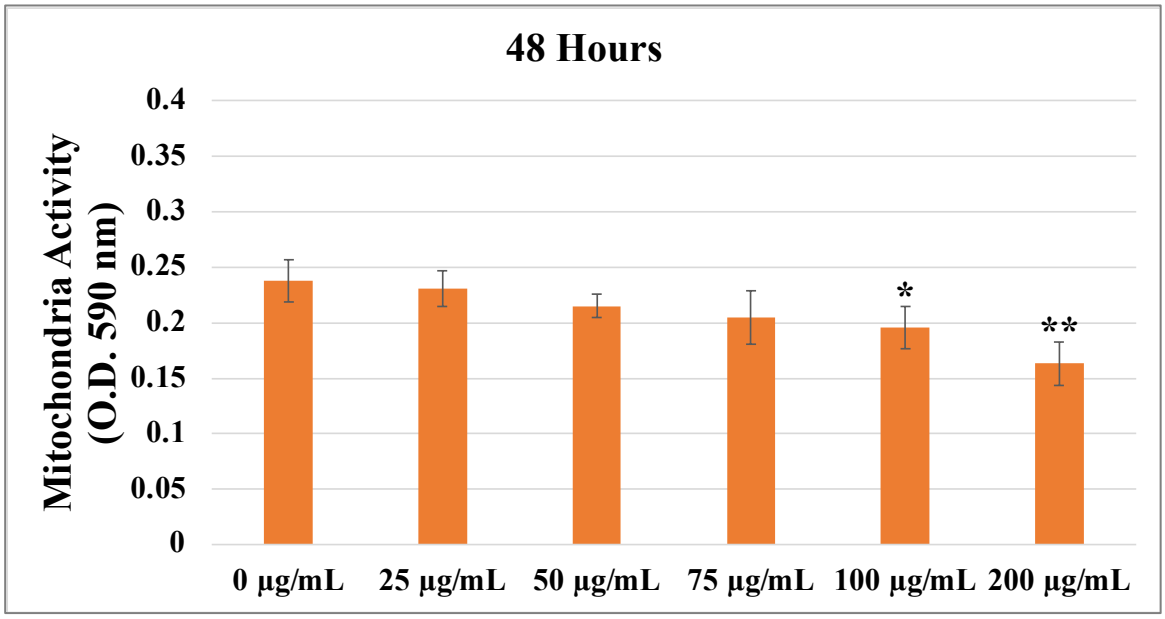


Figure 16. Methylthiazolyldiphenyl-tetrazolium bromide assay at 48 hours.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay at 48 hours showed a significant down-regulating effect of high concentrations of acetylsalicylic acid (ASA) on the mitochondria activity of human dental pulp cells (HDPCs). $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

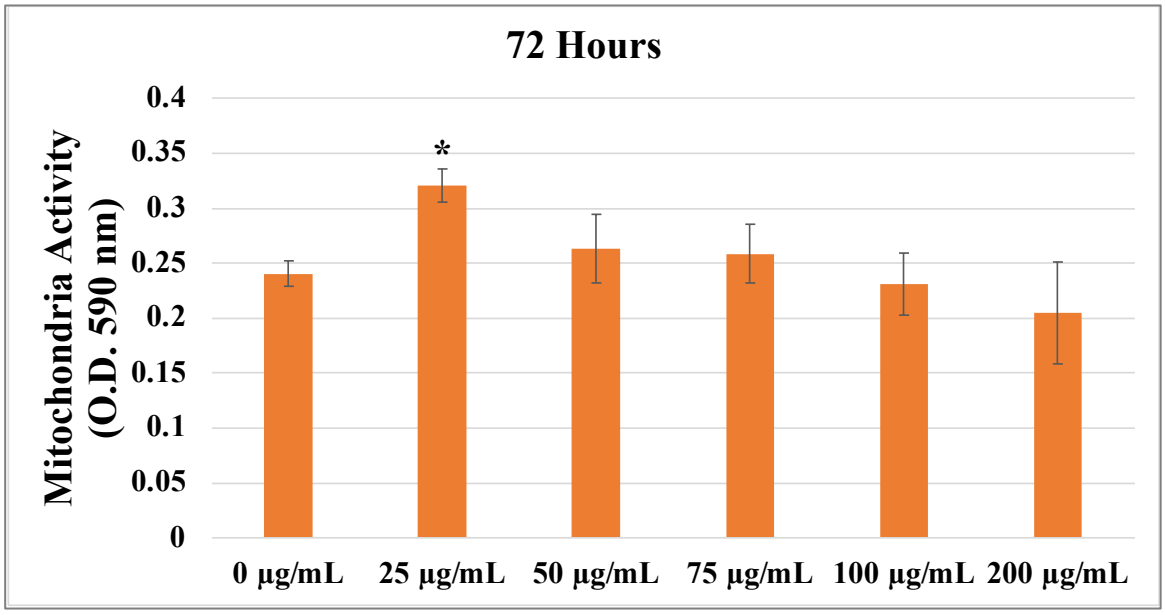


Figure 17. Methylthiazolyldiphenyl-tetrazolium bromide assay at 72 hours.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay at 72 hours showed significantly enhanced mitochondria activity expressed by the group of human dental pulp cells (HDPCs) treated with 25 µg/mL acetylsalicylic acid (ASA). $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

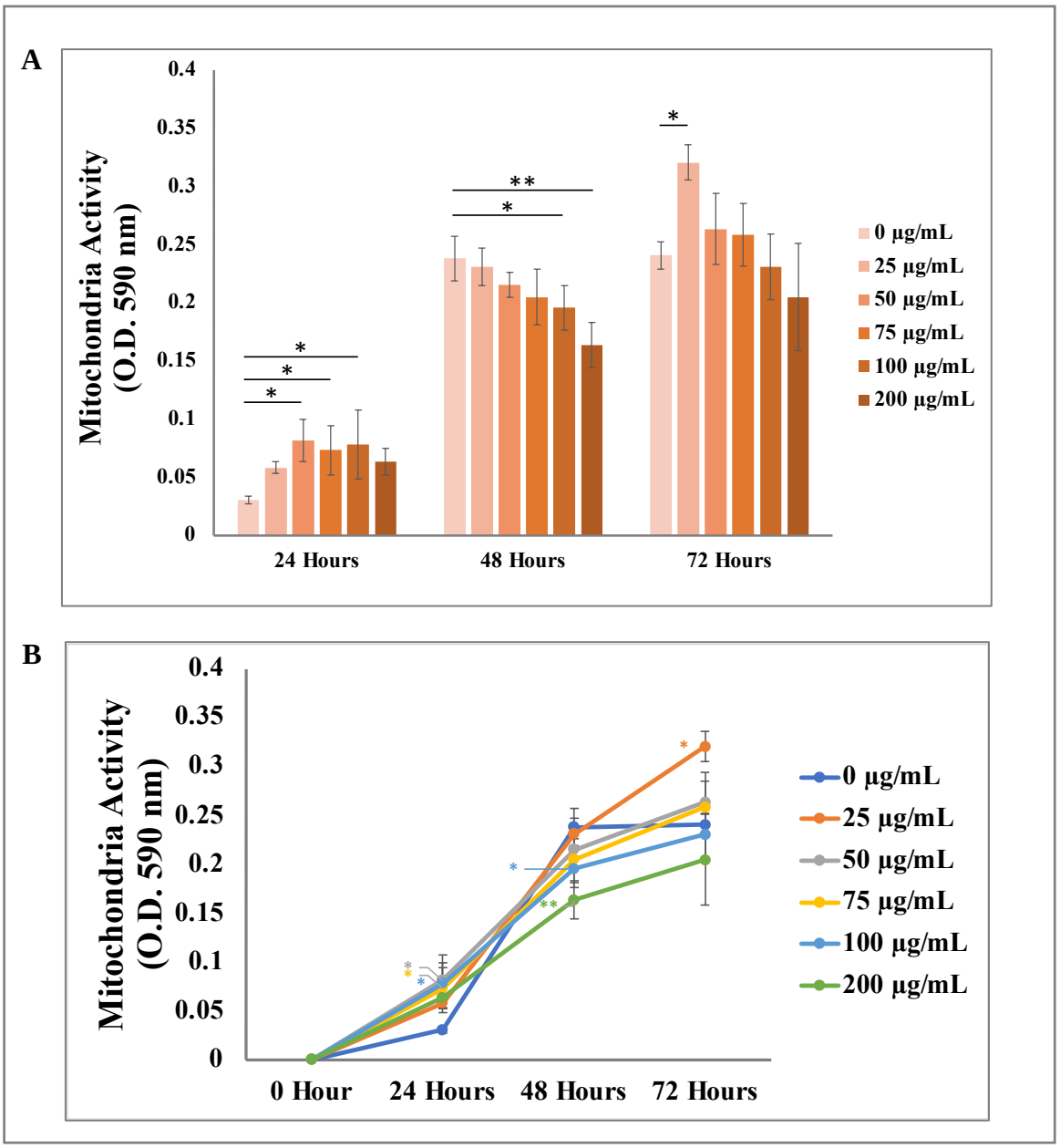


Figure 18. Methylthiazolyldiphenyl-tetrazolium bromide assay overall results.

(A, B) The mitochondria activity observed with the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay showed that all concentrations of acetylsalicylic acid (ASA) were non-toxic to human dental pulp cells (HDPCs) as the mitochondria activity of all groups increased over the experimental period. $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

6.3.2 XTT Assay

After 24 hours of ASA treatment, the XTT assay showed that ASA influenced the mitochondria activity of HDPCs in a dose-dependent manner, with 200 μg/mL ASA demonstrating significantly lower mitochondria activity than the control ($P < 0.001$; Fig. 19). The same trend was observed at 48 hours when 25 μg/mL ASA developed the significantly highest mitochondria activity of HDPCs ($P < 0.05$; Fig. 20). Then the activity gradually decreased as the ASA concentration increased. The HDPCs treated with 200 μg/mL ASA expressed the lowest mitochondria activity compared to other groups and the control ($P < 0.001$). However, the mitochondria activity of each test condition was maintained at the same level or slightly increased from the level observed at 24 hours.

At 72 hours, the levels of the mitochondria activity in all test conditions increased from the previous time points indicating cell proliferation. ASA constantly exerted a dose-dependent effect on the mitochondria activity of HDPCs, with 25 μg/mL ASA significantly enhancing the mitochondria activity ($P < 0.05$), and the activity was slightly down-regulated by ASA over 75 μg/mL to the significantly bottom level at 200 μg/mL ASA ($P < 0.001$; Fig. 21). The trend was consistent with the MTT result and the proliferation pilot on day 3.

Over 72 hours, the mitochondria activity observed with the XTT assay showed that ASA influenced the mitochondria activity of HDPCs in a dose-dependent manner and was non-toxic to HDPCs as the mitochondria activity of all groups increased within the experimental period (Fig. 22).

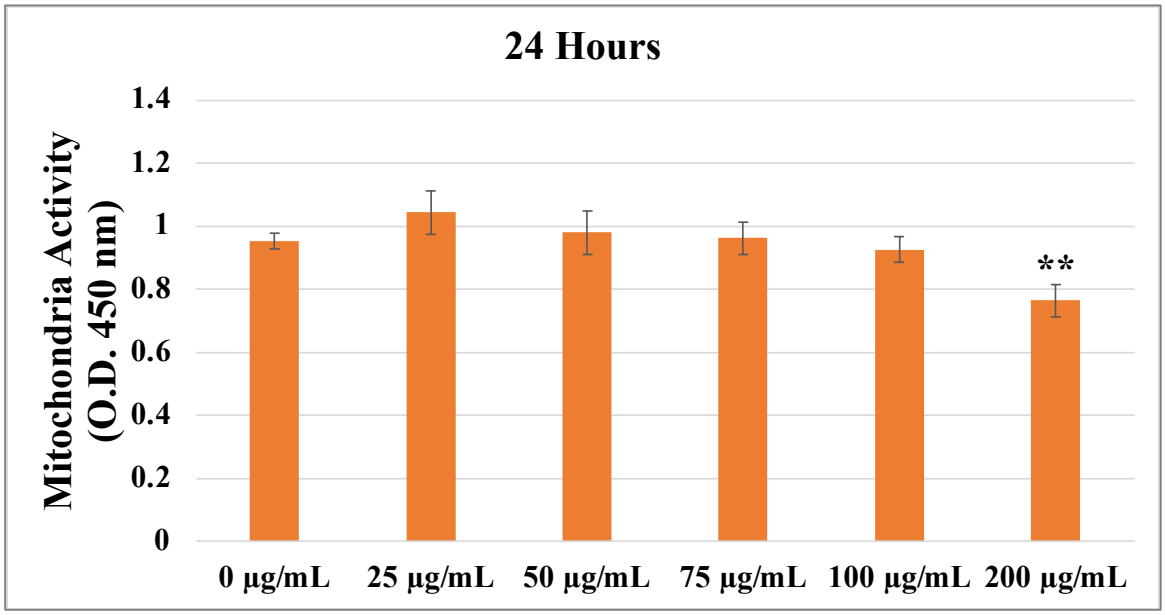


Figure 19. XTT assay at 24 hours.

XTT assay at 24 hours showed that acetylsalicylic acid (ASA) influenced the mitochondria activity of human dental pulp cells (HDPCs) in a dose-dependent manner, with 200 µg/mL ASA demonstrating significantly lower mitochondria activity than the control. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

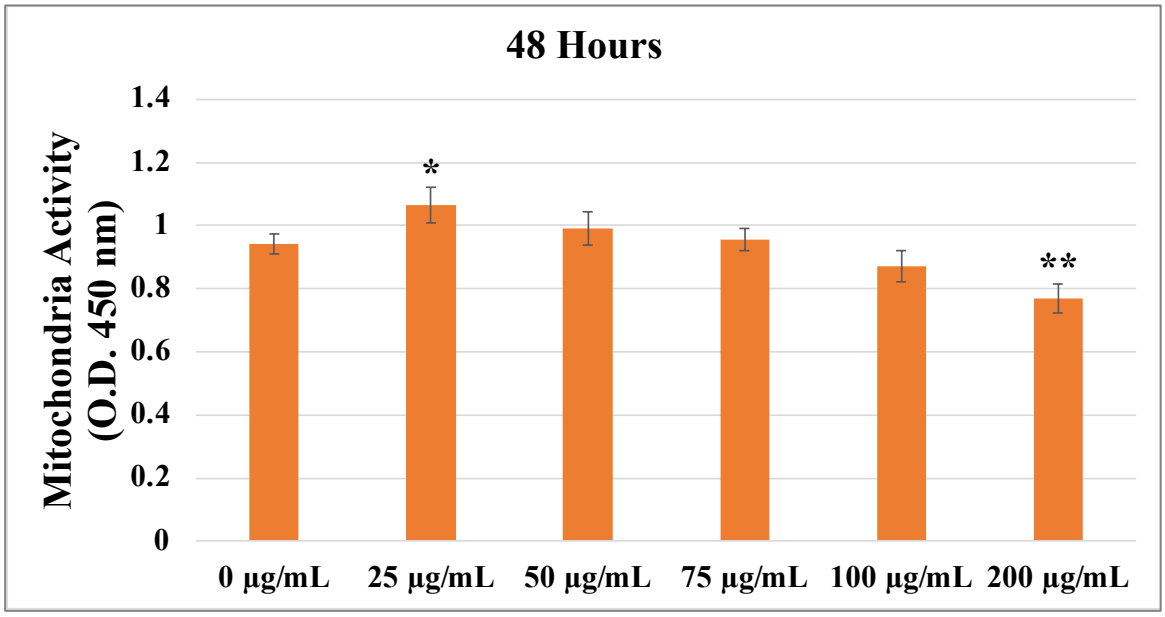


Figure 20. XTT assay at 48 hours.

XTT assay at 48 hours showed that acetylsalicylic acid (ASA) influenced the mitochondria activity of human dental pulp cells (HDPCs) in a dose-dependent manner. 25 µg/mL ASA significantly enhanced the mitochondria activity, and 200 µg/mL ASA expressed significantly lower mitochondria activity than the control. $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

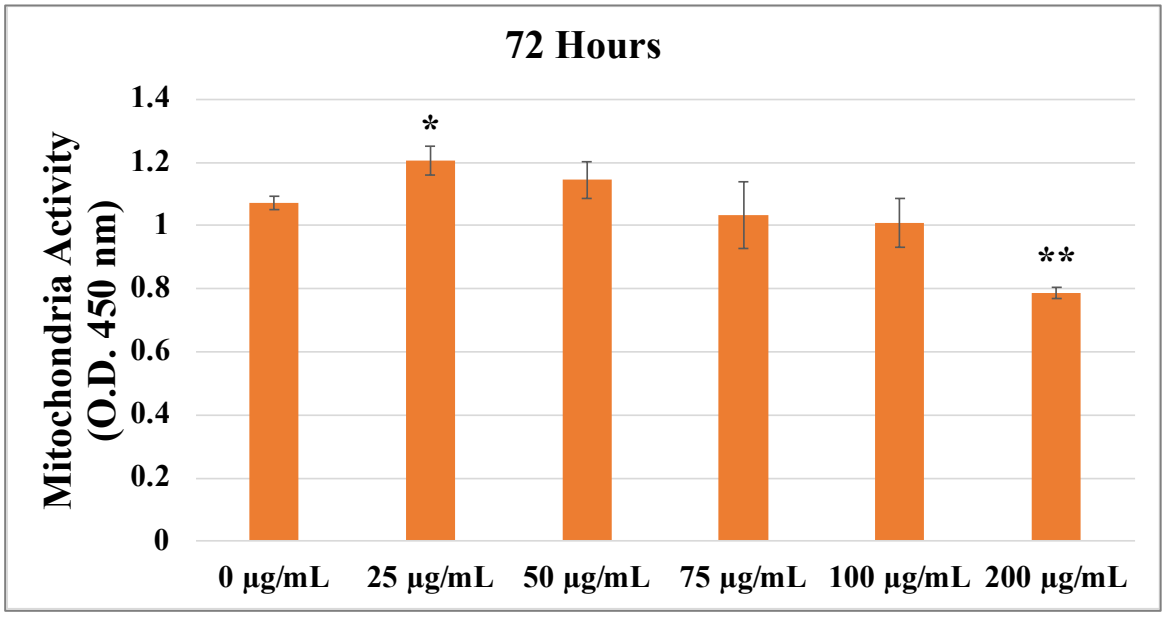


Figure 21. XTT assay at 72 hours.

XTT assay at 72 hours showed that acetylsalicylic acid (ASA) influenced the mitochondria activity of human dental pulp cells (HDPCs) in a dose-dependent manner. 25-50 µg/mL ASA slightly enhanced the mitochondria activity, and the activity was down-regulated by ASA over 75 µg/mL to the significantly bottom level at 200 µg/mL ASA. n = 6 in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

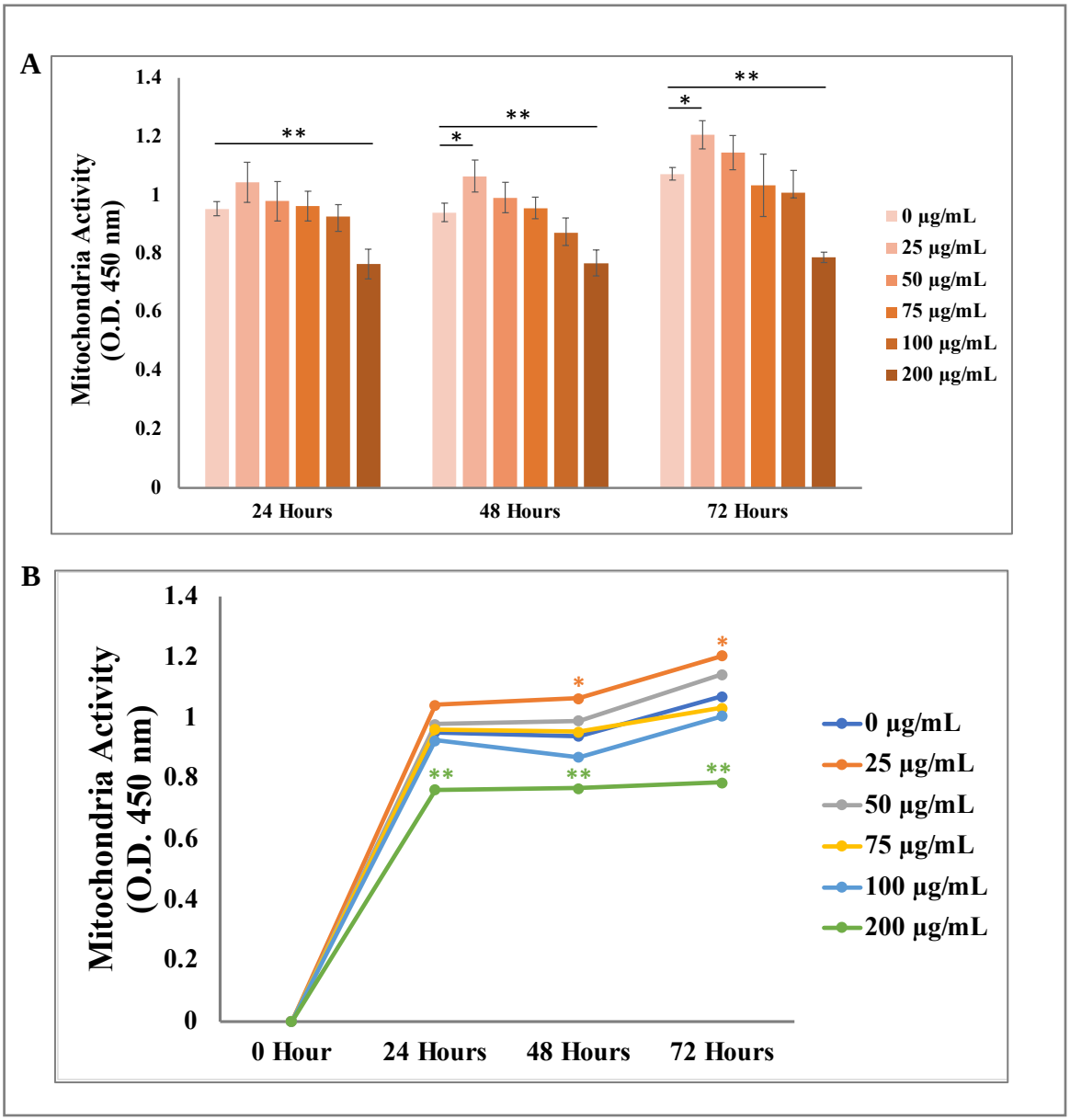


Figure 22. XTT assay overall results.

(A, B) The mitochondria activity observed with the XTT assay showed that acetylsalicylic acid (ASA) influenced the mitochondria activity of human dental pulp cells (HDPCs) in a dose-dependent manner and was non-toxic to HDPCs as the mitochondria activity of all groups increased within the experimental period. $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

6.4 Proliferation Rates

Proliferation rates were evaluated to observe the viability of HDPCs treated with 25, 50, 75, 100, 200, and 0 μg/mL ASA as a control for an extended period of 7, 14, and 21 days. The proliferation rates were calculated based on the cell attachment efficiency of each group at a 16-hour baseline.

After treating HDPCs with each concentration of ASA for 7 days, although 25-50 μg/mL ASA showed a slight increase in the proliferation rates compared to the control, and the decline occurred in the groups over 75 μg/mL ASA, no statistically significant difference in the proliferation rate was found between all the groups and the control ($P > 0.05$; Fig. 23).

However, the evaluation on day 14 demonstrated that low concentrations of ASA (25-50 μg/mL) started to show a significant enhancing effect on the proliferation rate of HDPCs ($P < 0.001$; Fig. 24). The concentrations of ASA over 75 μg/mL ASA constantly showed a down-regulating effect on the proliferation of HDPCs, but no statistically significant difference was detected ($P > 0.05$). All test conditions exhibited an increase in the proliferation rates by ten folds or over from an evaluation on day 7.

The same phenomenon of ASA on day 14 was detected on day 21 evaluation when 25-50 μg/mL ASA showed significantly higher proliferation rates of HDPCs than the control, with the peak level at 50 μg/mL ASA ($P < 0.001$; Fig. 25). The groups of 75 and 100 μg/mL ASA behaved similarly to the control and showed comparable proliferation

rates of HDPCs, whereas 200 μg/mL ASA slightly lowered the proliferation rate, but no statistically significant difference was detected ($P > 0.05$). All test conditions exhibited an increase in the proliferation rates up to two folds from an evaluation on day 14.

Overall, the proliferation rates of HDPCs treated with all concentrations of ASA increased over time, indicating that ASA was non-toxic to HDPCs. 25-50 μg/mL ASA yielded significantly higher proliferation of HDPCs than the control at 14 and 21d ($P < 0.001$), with 50 μg/mL ASA representing the peak proliferation, whereas concentrations of ASA over 75 μg/mL down-regulated the proliferation rates of HDPCs, with the lowest point at 200 μg/mL ASA (Fig. 26).

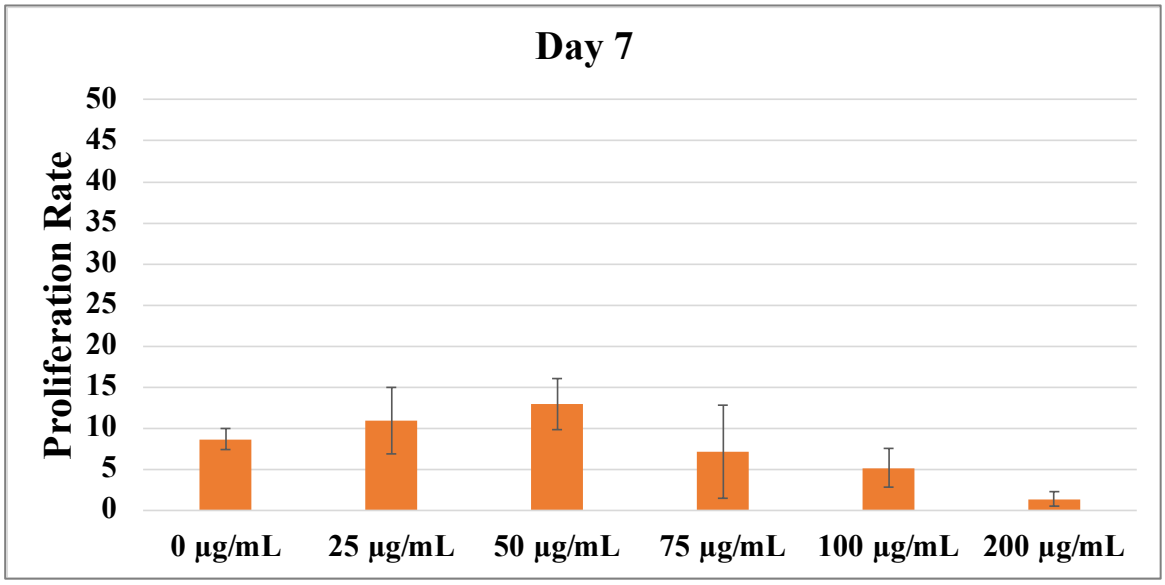


Figure 23. The proliferation rates on day 7.

The proliferation rates on day 7 showed that no statistically significant difference in the proliferation rate was found between human dental pulp cells (HDPCs) treated with each concentration of acetylsalicylic acid (ASA) and the control. $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

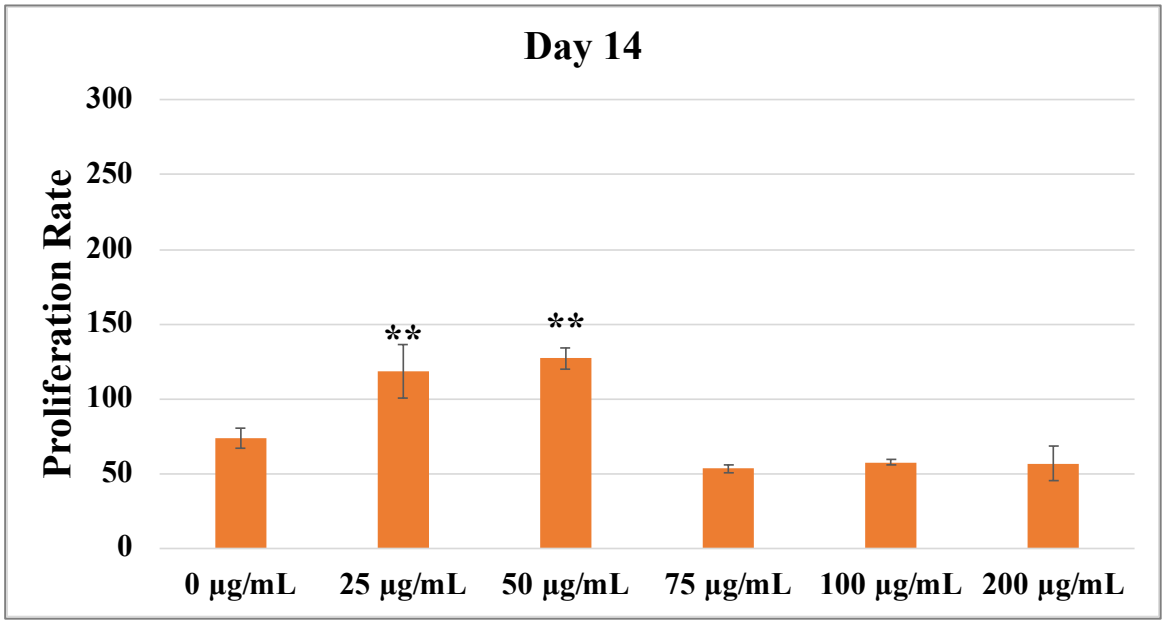


Figure 24. The proliferation rates on day 14.

The proliferation rates on day 14 showed that low concentrations of acetylsalicylic acid (ASA) started to show a significant enhancing effect on the proliferation rate of human dental pulp cells (HDPCs). No statistically significant difference was detected in HDPCs treated with ASA over 75 µg/mL compared to the control. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

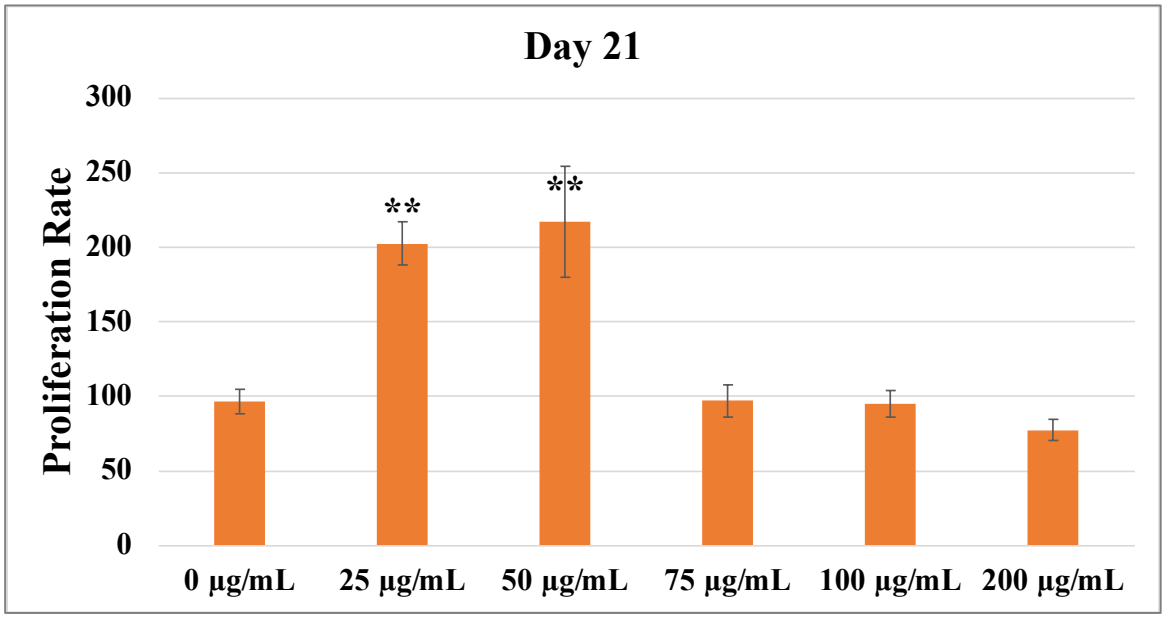


Figure 25. The proliferation rates on day 21.

The proliferation rates on day 21 showed that low concentrations of acetylsalicylic acid (ASA) started to show a significant enhancing effect on the proliferation rate of human dental pulp cells (HDPCs). The HDPCs treated with ASA over 75 µg/mL showed similar proliferation rates to the control, whereas 200 µg/mL ASA down-regulated the proliferation rate, but no statistically significant difference was detected. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

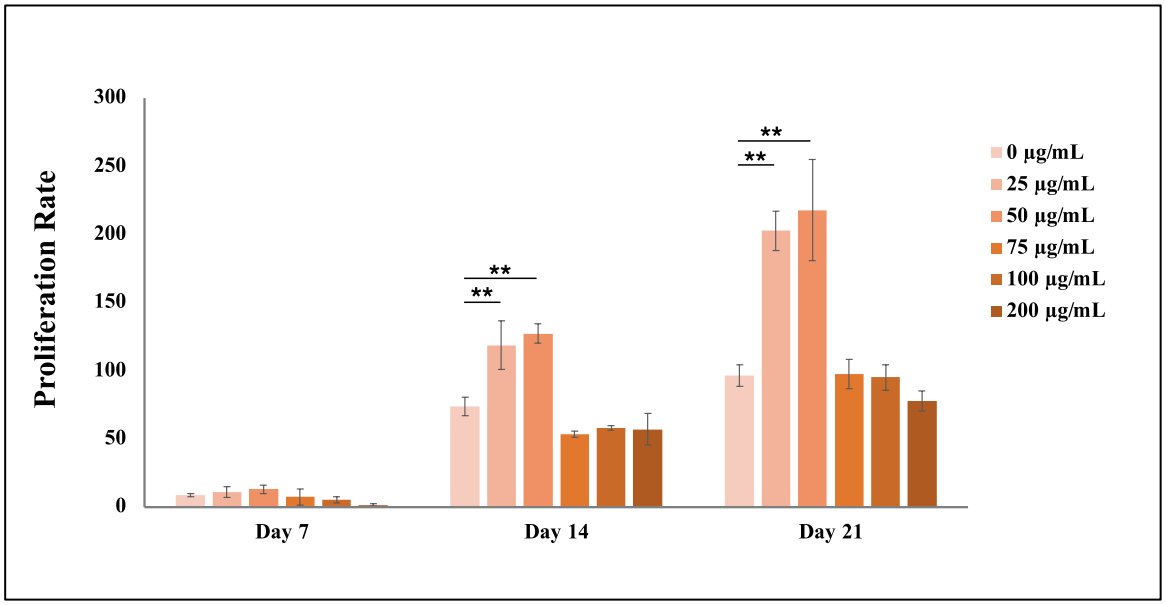


Figure 26. The proliferation rates of human dental pulp cells increased over time.

The proliferation rates evaluated by crystal violet staining solubilized by triton X-100 demonstrated that acetylsalicylic acid (ASA) was non-toxic to human dental pulp cells (HDPCs) as the proliferation rates of HDPCs treated with all concentrations of ASA increased over time. Low concentrations of ASA yielded significantly higher proliferation of HDPCs than the control at 14 and 21 days. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

6.5 Odontogenic Differentiation

6.5.1 Cell Morphology

HDPCs treated with 25, 50, 75, 100, 200, and 0 μg/mL ASA showed a flat spindle-like shape with elongated processes, and no significant differences regarding the cell morphology were detected in each group. On day 7, the observation of a single HDPC from each group was possible due to minimal numbers of proliferating cells and the loose arrangement of each cell in between an intercellular space. The oval-shaped nucleus was clearly detected in the middle of some cell bodies. On day 14, HDPCs in each group were arranged closely together in a denser format. The cell numbers increased from day 7, resulting in the establishment of cell connections to form a net, which was harder to perform an observation for a single cell. The highest confluence of HDPCs in each group was seen on day 21, specifically in 25-50 μg/mL ASA groups, leading to some cluster formations over the pack of a cell layer. More elongation of cell length was detected in all treatment groups compared to the observation on previous dates. Over time, there were no significant differences at the morphology level between the ASA-treated groups and the control (Fig. 27).

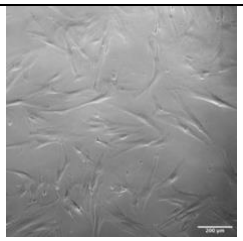
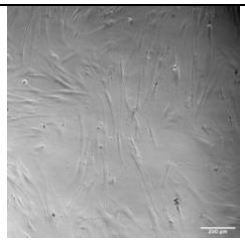
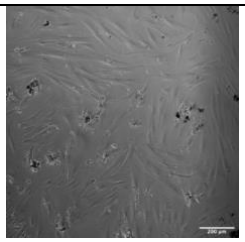
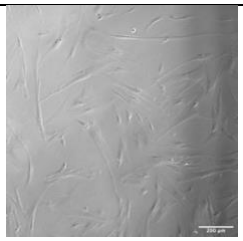
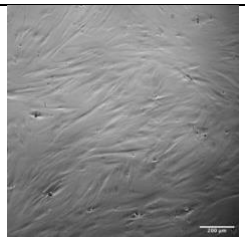
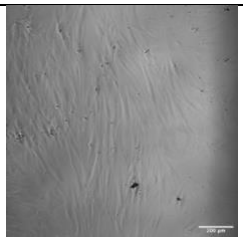
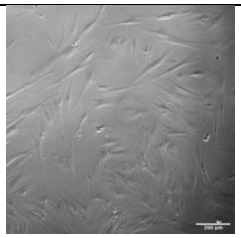
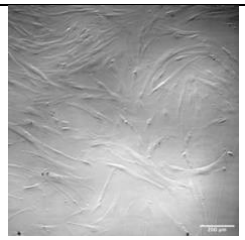
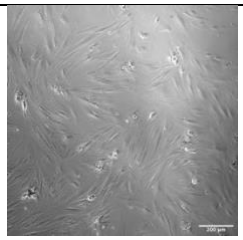
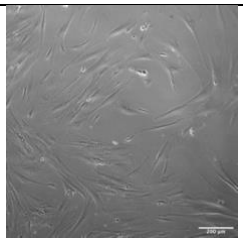
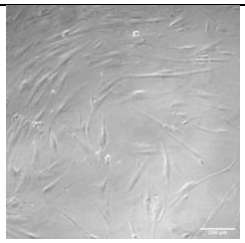
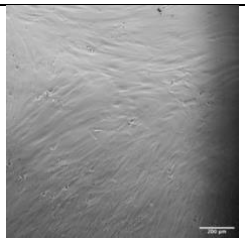
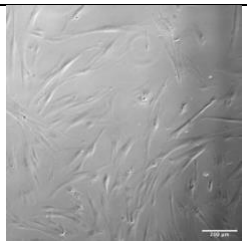
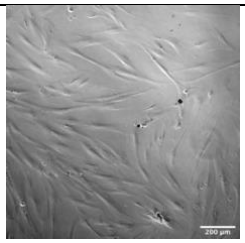
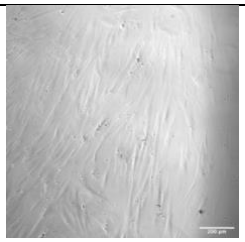
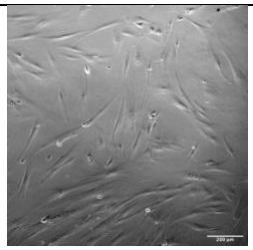
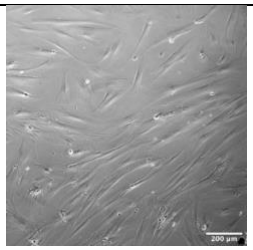
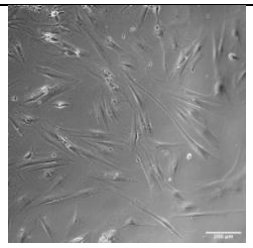
Group/ Time Point	Day 7	Day 14	Day 21
Control			
25 µg/mL ASA			
50 µg/mL ASA			
75 µg/mL ASA			
100 µg/mL ASA			
200 µg/mL ASA			

Figure 27. Microscopic images showed the morphology of human dental pulp cells (HDPCs) at each time point. Bars = 200 µm.

6.5.2 Odontogenic Biomarkers

6.5.2.1 Transcriptional Level

After odontogenic induction of HDPCs treated with 25, 50, 75, 100, 200, and 0 μg/mL ASA as a control for an extended period of 4, 7, and 14 days, quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was used to detect the odontogenic capacity of HDPCs at the transcriptional level. The relative level of the gene expression was obtained from normalizing gene expression levels of each target gene to the housekeeping gene, β-actin, then $2^{-\Delta\Delta Ct}$ was calculated to reveal the results.

Dentin Sialophosphoprotein (DSPP) Gene

The treatment of all concentrations of ASA induced higher expression of relative DSPP gene levels than the control group in a dose-dependent manner on day 4. However, the significance of the gene expression was only detected at 200 μg/mL ASA ($P < 0.05$; Fig. 28). No statistically significant differences were found between the relative DSPP gene levels of the control and 25-100 μg/mL ASA groups ($P > 0.05$).

The same pattern of ASA modulated relative DSPP gene expression level was seen on day 7 and day 14 when all concentrations of ASA increased the gene expression in a dose-dependent manner compared to the control. The statistically significant differences were detected in HDPCs treated with all concentrations of ASA on both days (Fig. 29, 30). The significance of relative DSPP gene expression levels of HDPCs treated with 100-200 μg/mL ASA later supported the DSP biomarker levels of HDPCs treated with 100-200 μg/mL ASA on day 7 and day 14.

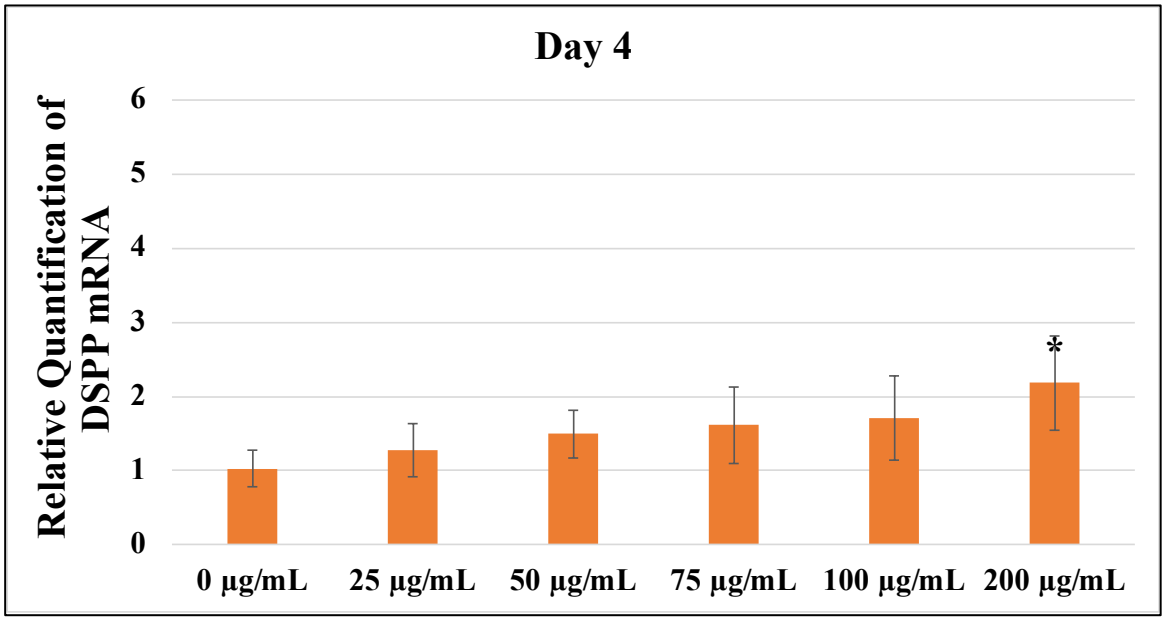


Figure 28. The relative dentin sialophosphoprotein gene expression levels of human dental pulp cells on day 4.

The relative dentin sialophosphoprotein (DSPP) gene expression levels of human dental pulp cells (HDPCs) on day 4 showed that the levels were increased by acetylsalicylic acid (ASA) treatment in a dose-dependent manner. However, the significance of the gene expression was only detected at 200 µg/mL ASA. $n = 6$ in each group. $*P < 0.05$. $**P < 0.001$. Values are presented as mean ± SD.

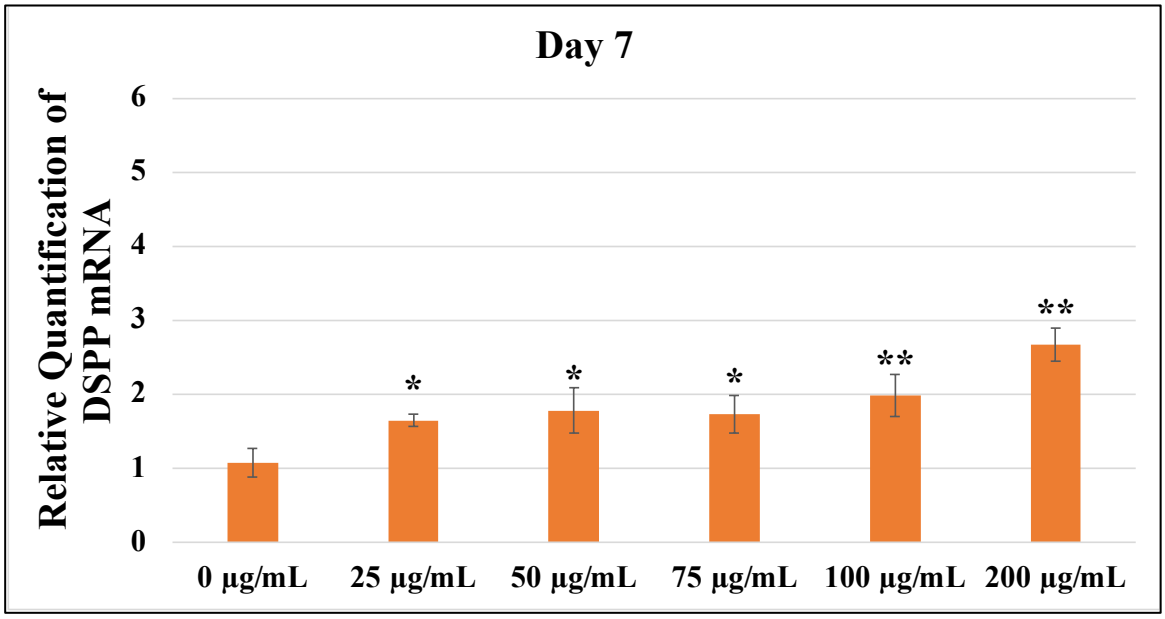


Figure 29. The relative dentin sialophosphoprotein gene expression levels of human dental pulp cells on day 7.

The relative dentin sialophosphoprotein (DSPP) gene expression levels of human dental pulp cells (HDPCs) on day 7 showed that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

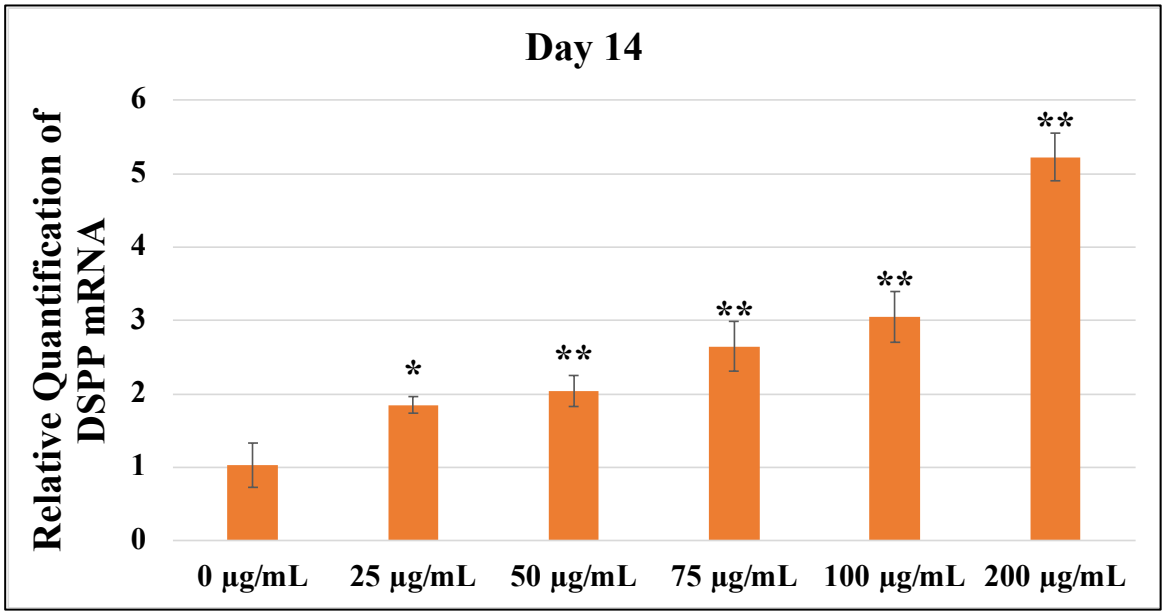


Figure 30. The relative dentin sialophosphoprotein gene expression levels of human dental pulp cells on day 14.

The relative dentin sialophosphoprotein (DSPP) gene expression levels of human dental pulp cells (HDPCs) on day 14 showed that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

Runt-Related Transcription Factor 2 (RUNX2) Gene

The treatment of all concentrations of ASA induced higher expression of relative RUNX2 gene levels than the control group in a dose-dependent manner on day 4. However, the significance of the gene expression was only detected at 100-200 μg/mL ASA ($P < 0.05$; Fig. 31). No statistically significant differences were found between the relative RUNX2 gene levels of the control and 25-75 μg/mL ASA groups ($P > 0.05$).

The same pattern of ASA modulated relative RUNX2 gene expression level was seen on day 7 and day 14 when all concentrations of ASA increased the gene expression in a dose-dependent manner compared to the control. The statistically significant differences were detected in HDPCs treated with all concentrations of ASA on both days (Fig. 32, 33). The significance of relative RUNX2 gene expression levels of HDPCs treated with 75-200 μg/mL ASA later supported the RUNX2 biomarker levels of HDPCs treated with 75-200 μg/mL ASA on day 7 and day 14.

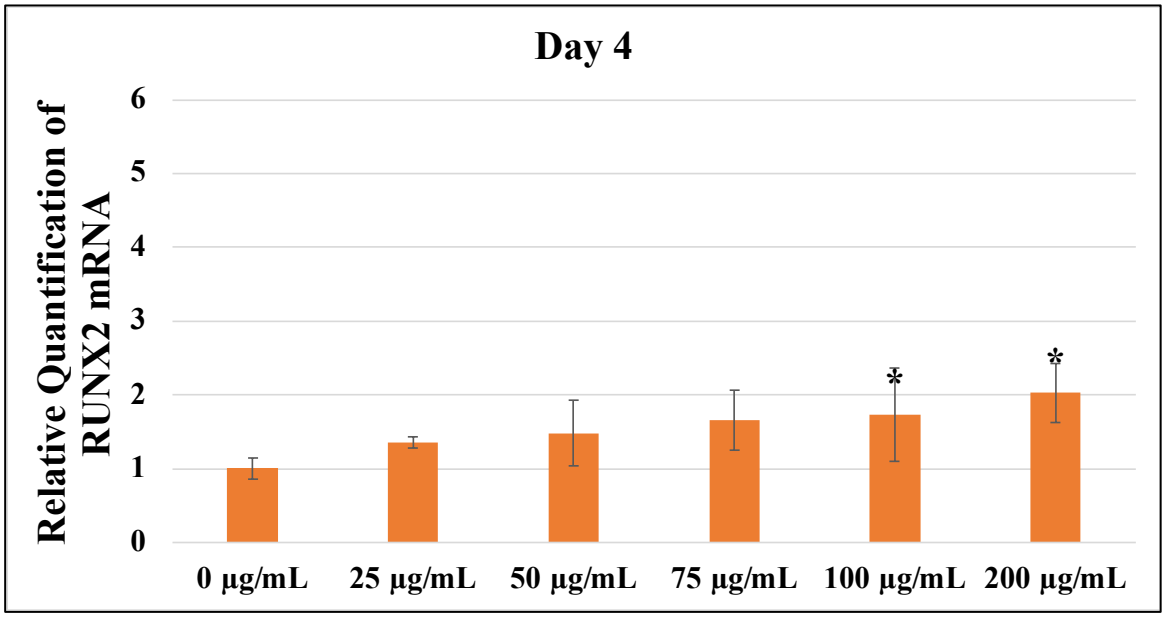


Figure 31. The relative runt-related transcription factor 2 gene expression levels of human dental pulp cells on day 4.

The relative runt-related transcription factor 2 (RUNX2) gene expression levels of human dental pulp cells (HDPCs) on day 4 showed that the levels were increased by acetylsalicylic acid (ASA) treatment in a dose-dependent manner. However, the significance of the gene expression was only detected at 100-200 µg/mL ASA. $n = 6$ in each group. $*P < 0.05$. $**P < 0.001$. Values are presented as mean ± SD.

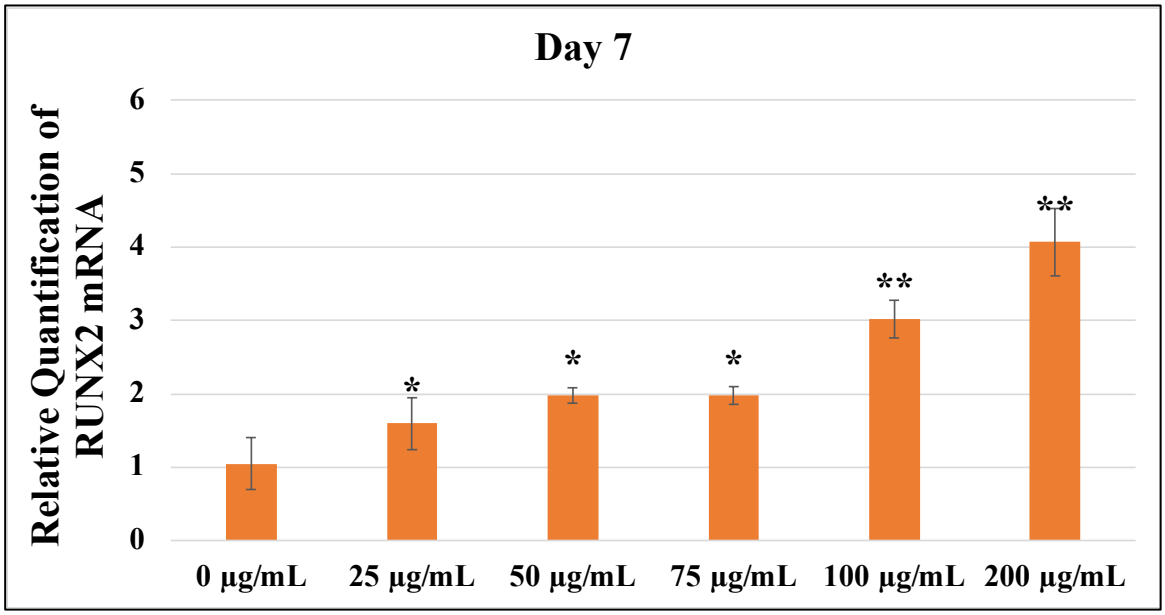


Figure 32. The relative runt-related transcription factor 2 gene expression levels of human dental pulp cells on day 7.

The relative runt-related transcription factor 2 (RUNX2) gene expression levels of human dental pulp cells (HDPCs) on day 7 showed that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

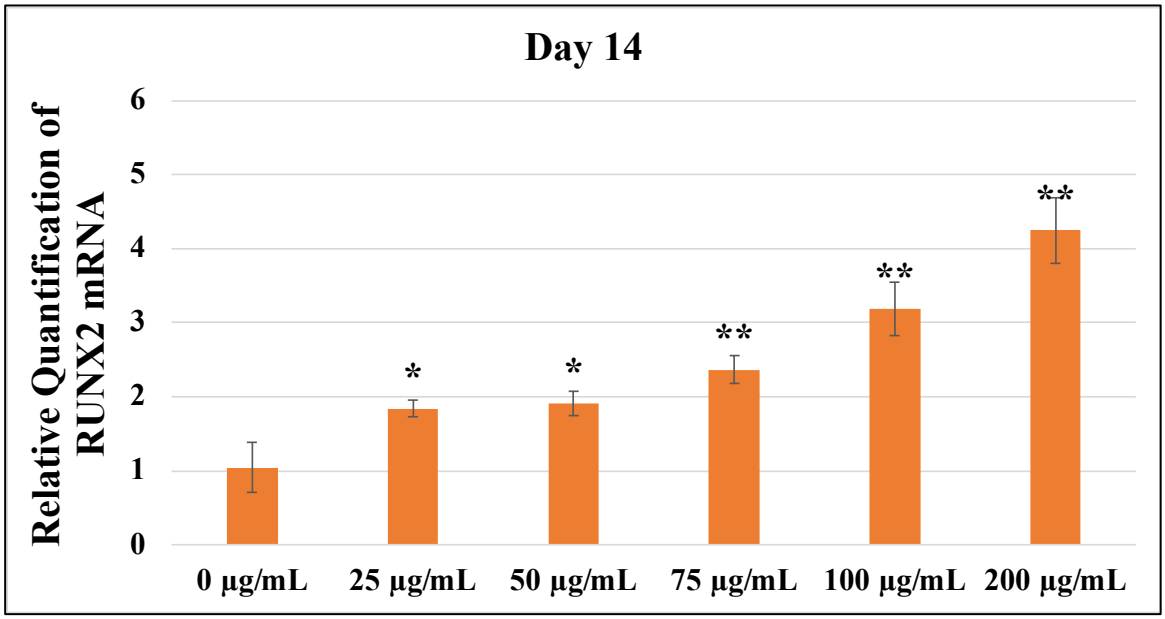


Figure 33. The relative runt-related transcription factor 2 gene expression levels of human dental pulp cells on day 14.

The relative runt-related transcription factor 2 (RUNX2) gene expression levels of human dental pulp cells (HDPCs) on day 14 showed that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

The relative DSPP and RUNX2 gene expression levels of HDPCs detected after 4, 7, and 14 days of ASA concentrations treatment displayed a fairly consistent pattern of a dose-dependent inducing effect of ASA compared to the control group. This effect was in line with the odontogenic biomarker level found in later experiments. The results could also underpin the influence of high concentrations of ASA (100-200 µg/mL) on the gene expression levels starting to exhibit significance on day 4 and were maintained until the last observation on day 14. On the other hand, 25-75 µg/mL ASA started to show a significant inducing effect on the relative DSPP and RUNX2 gene expression levels on day 7 and continued until day 14.

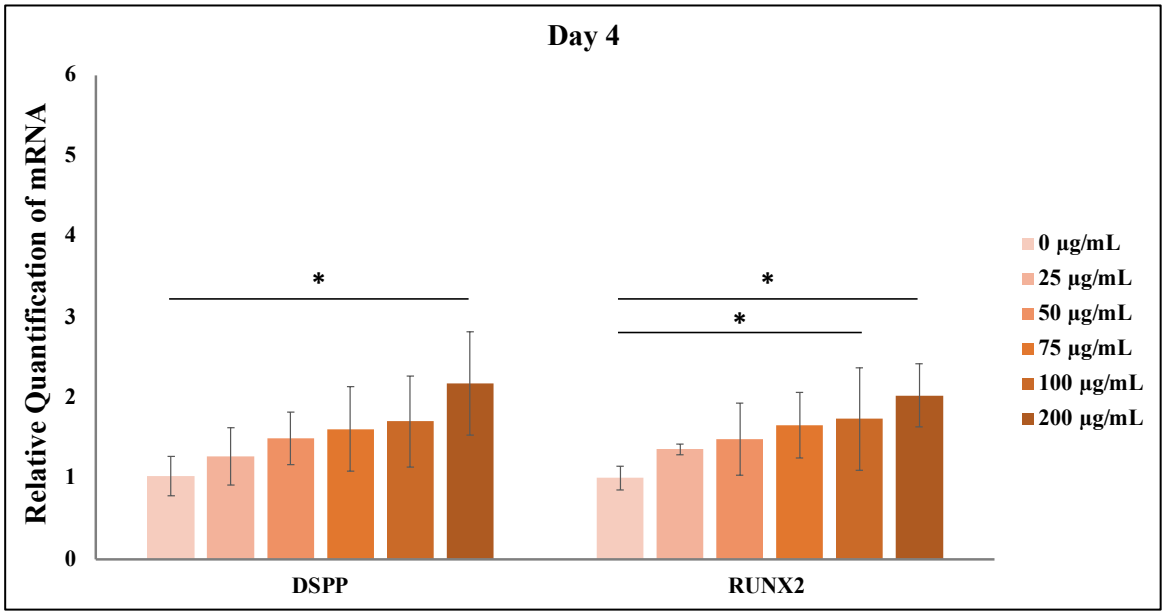


Figure 34. The relative gene expression levels of dentin sialophosphoprotein and runt related transcription factor 2 of human dental pulp cells evaluated on day 4.

The relative gene expression levels of dentin sialophosphoprotein (DSPP) and runt-related transcription factor 2 (RUNX2) of human dental pulp cells (HDPCs) evaluated on day 4 illustrated a consistent pattern of a dose-dependent effect of acetylsalicylic acid (ASA) and significance in the inducing effect exhibited by 100-200 µg/mL ASA. $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

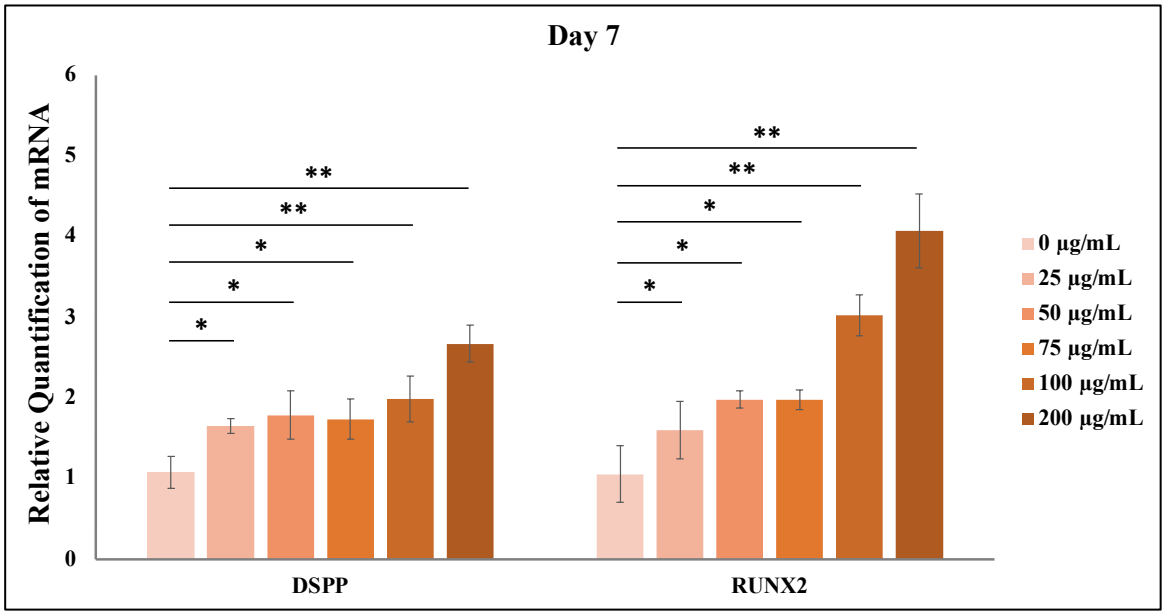


Figure 35. The relative gene expression levels of dentin sialophosphoprotein and runt related transcription factor 2 of human dental pulp cells evaluated on day 7.

The relative gene expression levels of dentin sialophosphoprotein (DSPP) and runt-related transcription factor 2 (RUNX2) of human dental pulp cells (HDPCs) evaluated on day 7 illustrated that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner compared to the control. The pattern of DSPP and RUNX2 relative gene expressions aligned with each other. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

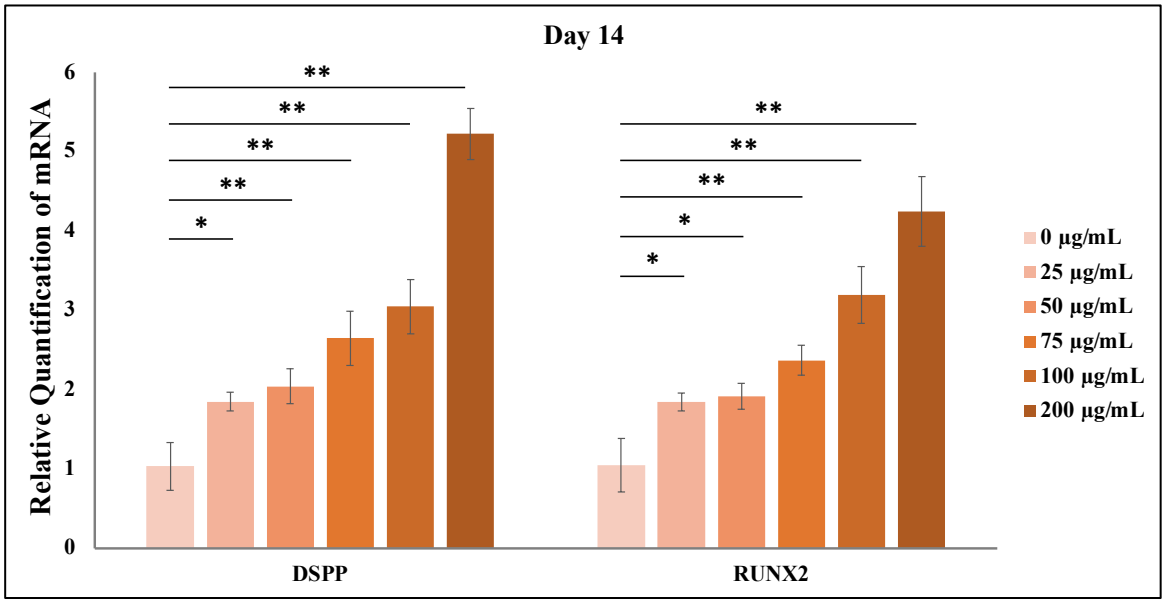


Figure 36. The relative gene expression levels of dentin sialophosphoprotein and runt related transcription factor 2 of human dental pulp cells evaluated on day 14.

The relative gene expression levels of dentin sialophosphoprotein (DSPP) and runt-related transcription factor 2 (RUNX2) of human dental pulp cells (HDPCs) evaluated on day 14 illustrated that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner compared to the control. The pattern of DSPP and RUNX2 relative gene expressions aligned with each other. $n = 6$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

6.5.2.2 Post-Transcriptional Level

The supernatant of HDPCs treated with 0, 25, 50, 75, 100, and 200 μg/mL ASA was harvested after 7, 14, and 21 days of odontogenic induction. The biomarker levels were assessed using alkaline phosphatase (ALP) fluorometric assay along with dentin sialoprotein (DSP) and RUNX2 enzyme-linked immunosorbent assay (ELISA) to elucidate the odontogenic differentiation of HDPCs at the post-transcriptional level. The detected biomarker levels were normalized to cell numbers per million cells.

ALP Activity

HDPCs treated with all concentrations of ASA exhibited increased ALP activities per cell than the control group in a dose-dependent manner on day 7. However, the significance of the ALP activities was only detected at 75-200 μg/mL ASA, with 200 μg/mL ASA showing the peak ALP activity, which was dramatically higher than the rest of the treatment conditions and also significantly higher than the control ($P < 0.001$; Fig. 37). No statistically significant differences were found between the ALP activities of control and 25-50 μg/mL ASA groups ($P > 0.05$).

On day 14, ASA consecutively affected the ALP activities per cell of HDPCs in a dose-dependent manner compared to the control. The significance was detected in the groups of 50-200 μg/mL ASA, with 200 μg/mL continuing to exhibit higher ALP activities per cell than the rest of the treatment conditions and the control by an estimate of three to five folds ($P < 0.001$; Fig. 38). No statistically significant differences were found between

the ALP activities of the control and 25 μg/mL ASA groups ($P > 0.05$). All test conditions exhibited a decline in the ALP activities per cell compared to an evaluation on day 7.

The evaluation on day 21 showed a subtle dose-dependent effect of ASA on the ALP activities per cell of HDPCs, but no statistically significant differences were found between the ALP activities of the control and 25-100 μg/mL ASA groups ($P > 0.05$). The treatment of HDPCs with 25 μg/mL ASA exhibited a relatively comparable level of ALP activity to the control group, whereas 200 μg/mL ASA continued to show statistically significant enhanced ALP activity compared to the control ($P < 0.001$; Fig. 39). All test conditions exhibited a decline in the ALP activities per cell compared to an evaluation on day 14.

Overall results illustrated that ASA exerted a dose-dependent effect on the ALP activities of HDPCs, with 200 μg/mL ASA markedly enhancing ALP activities at every time point ($P < 0.001$; Fig. 40). The level of ALP activities per cell of HDPCs decreased over time.

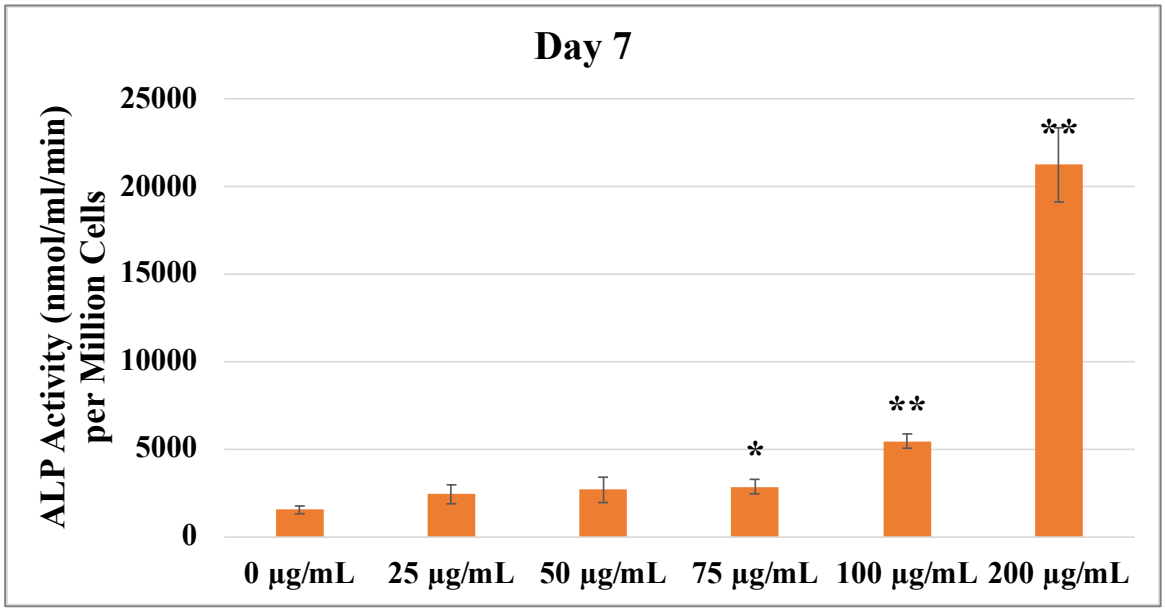


Figure 37. The alkaline phosphatase activities of human dental pulp cells on day 7.

The alkaline phosphatase (ALP) activities of human dental pulp cells (HDPCs) on day 7 showed that acetylsalicylic acid (ASA) exhibited increased ALP activities per cell than the control group in a dose-dependent manner. However, the significance of the ALP activities was only detected at 75-200 µg/mL ASA, with 200 µg/mL ASA markedly enhancing ALP activity of HDPCs compared to the control. $n = 4$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

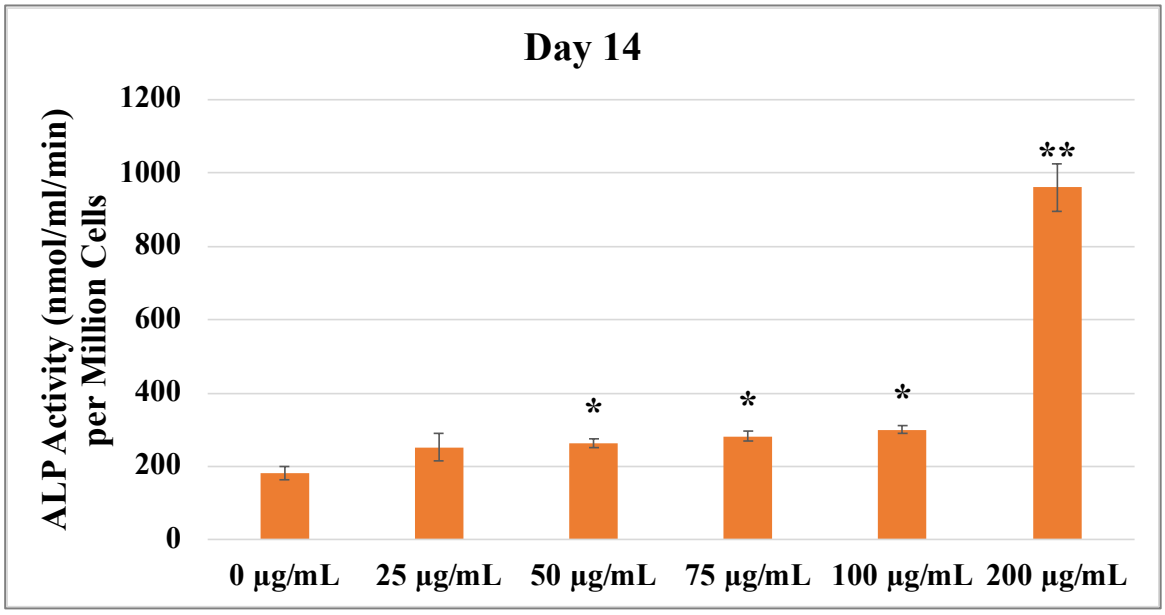


Figure 38. The alkaline phosphatase activities of human dental pulp cells on day 14.

The alkaline phosphatase (ALP) activities of human dental pulp cells (HDPCs) on day 14 showed that acetylsalicylic acid (ASA) exhibited increased ALP activities per cell than the control group in a dose-dependent manner. However, the significance of the ALP activities was only detected at 50-200 µg/mL ASA, with 200 µg/mL ASA markedly enhancing ALP activity of HDPCs compared to the control. $n = 4$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

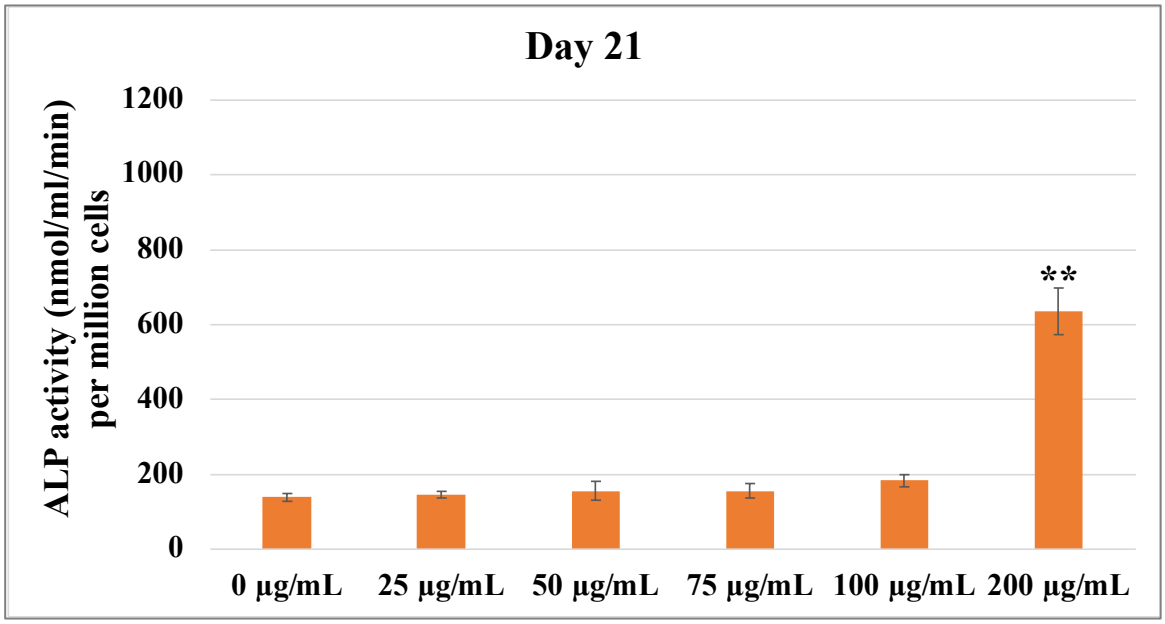


Figure 39. The alkaline phosphatase activities of human dental pulp cells on day 21.

The alkaline phosphatase (ALP) activities of human dental pulp cells (HDPCs) on day 21 showed a subtle dose-dependent effect of acetylsalicylic acid (ASA) on the ALP activities per cell of HDPCs. 25 µg/mL ASA exhibited a relatively comparable level of ALP activity to the control group, whereas 200 µg/mL ASA markedly enhanced ALP activity of HDPCs compared to the control. $n = 4$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

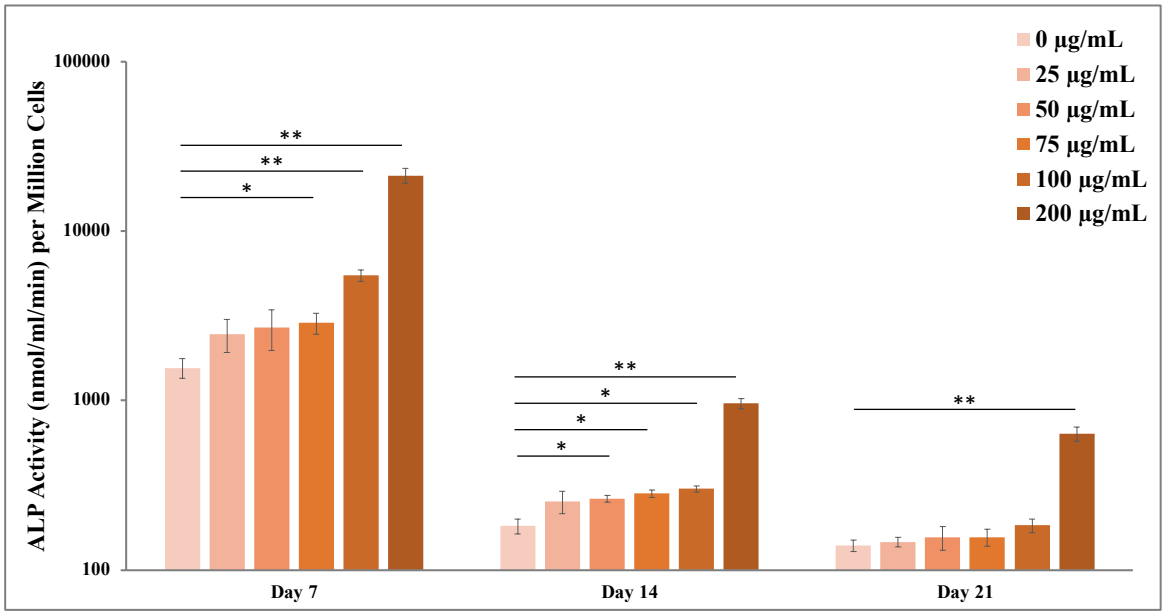


Figure 40. The acetylsalicylic acid exerted a dose-dependent effect on the alkaline phosphatase activities of human dental pulp cells.

The alkaline phosphatase (ALP) activities of human dental pulp cells (HDPCs), assessed by the fluorometric assay, demonstrated that acetylsalicylic acid (ASA) exerted a dose-dependent effect on the ALP activities of HDPCs. 200 µg/mL ASA markedly enhanced ALP activities at every time point, and the level of ALP activities per cell of HDPCs treated with each concentration of ASA decreased over time. $n = 4$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

DSP Level

HDPCs treated with all concentrations of ASA exhibited significantly increased DSP levels per cell than the control group in a dose-dependent manner on day 7, with 200 μg/mL ASA showing the peak DSP level, which was dramatically higher than the rest of the treatment conditions and the control by an estimate of over ten folds ($P < 0.001$; Fig. 41). The results were in line with the relative gene expression levels of DSPP shown on day 7.

The DSP levels per cell of HDPCs evaluated after 14 days of ASA treatment demonstrated that ASA influenced the DSP levels in a dose-dependent manner compared to the control group. However, the significance was detected in the groups of 100-200 μg/mL ASA, with 200 μg/mL continuing to exhibit higher DSP level per cell than the rest of the treatment conditions and the control by an estimate of seven folds ($P < 0.001$; Fig. 42). No statistically significant differences were found between the DSP levels of the control group and 25-75 μg/mL ASA groups ($P > 0.05$). All test conditions exhibited a decline in the DSP levels per cell compared to an evaluation on day 7.

The DSP levels per cell on day 21 did not follow the same pattern as found at other time points, with 25-100 μg/mL ASA groups exhibiting significantly lower DSP levels than the control groups ($P < 0.001$; Fig. 43). However, 200 μg/mL ASA showed a relatively comparable level of DSP per cell to the control group with no statistically significant differences ($P > 0.05$), and the overall production of DSP levels per cell declined from a markedly distinct elevation on day 14 to the lower level on day 21 by an estimate of ten

folds except the control group that the DSP level per cell remained fairly similar to day 14 evaluation.

Overall results illustrated that ASA exerted a dose-dependent effect on the DSP levels per cell of HDPCs on day 7 and day 14, with 200 μg/mL ASA markedly enhanced DSP levels ($P < 0.001$). On day 21, the DSP levels did not follow the same trend. However, the overall production of the DSP levels per cell of HDPCs declined from markedly distinct elevation on day 7 to the lowest level on day 21 by several folds (Fig. 44).

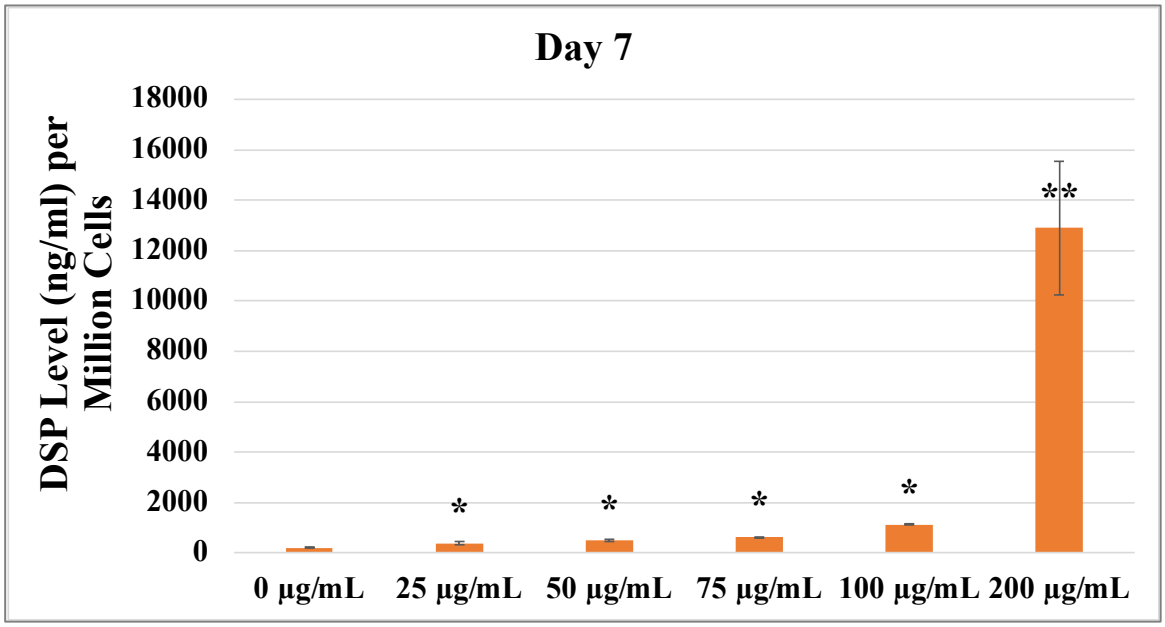


Figure 41. The dentin sialoprotein levels per cell of human dental pulp cells on day 7.

The dentin sialoprotein (DSP) levels per cell of human dental pulp cells (HDPCs) on day 7 showed that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner, with 200 µg/mL ASA markedly enhancing DSP level per cell of HDPCs compared to the control. $n = 4$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

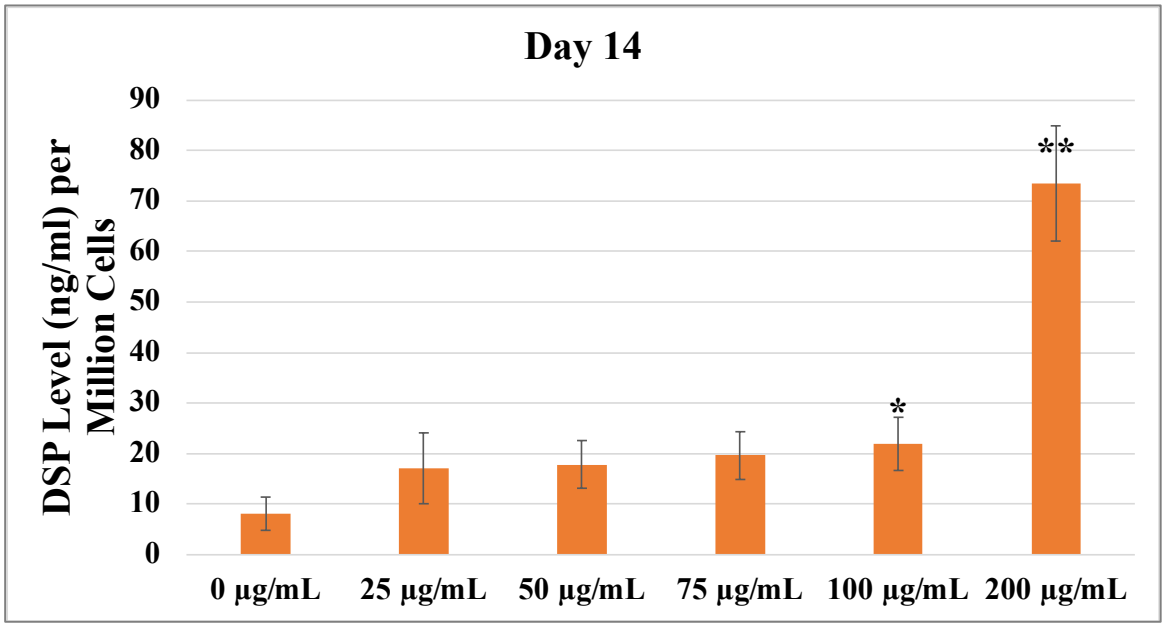


Figure 42. The dentin sialoprotein levels per cell of human dental pulp cells on day 14.

The dentin sialoprotein (DSP) levels per cell of human dental pulp cells (HDPCs) on day 14 showed that acetylsalicylic acid (ASA) exhibited increased DSP levels than the control group in a dose-dependent manner. However, the significance of the DSP levels per cell was only detected at 100-200 µg/mL ASA, with 200 µg/mL ASA markedly enhancing the DSP level of HDPCs compared to the control. $n = 4$ in each group. $*P < 0.05$. $**P < 0.001$. Values are presented as mean ± SD.

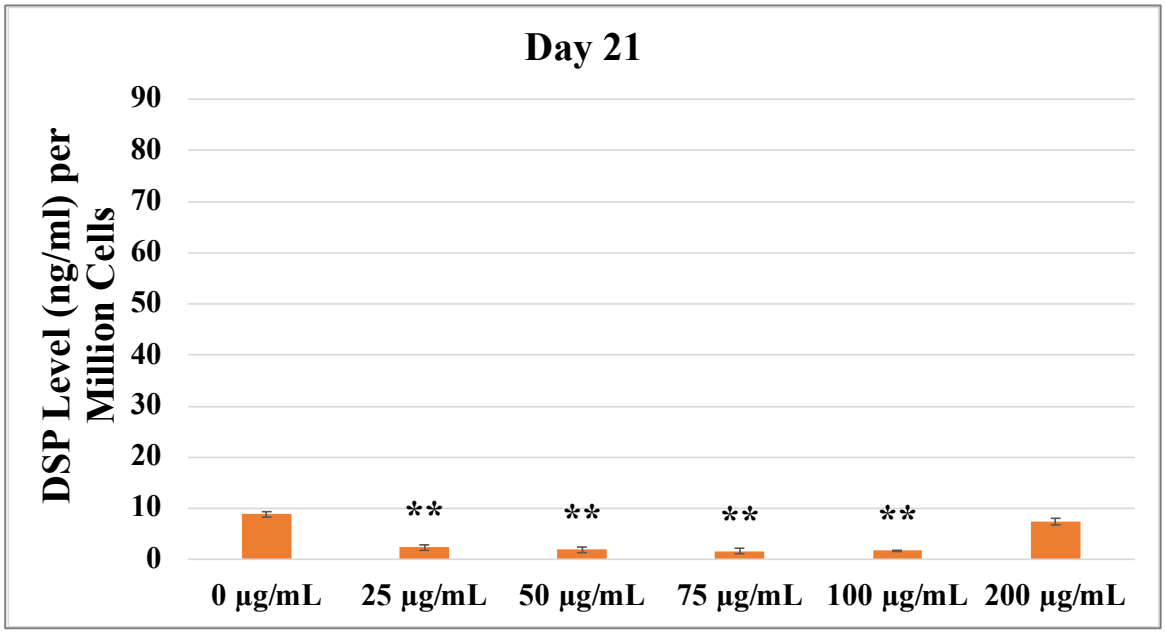


Figure 43. The dentin sialoprotein levels per cell of human dental pulp cells on day 21.

The dentin sialoprotein (DSP) levels per cell of human dental pulp cells (HDPCs) treated with acetylsalicylic acid (ASA) on day 21 showed that the DSP levels did not follow the same pattern as seen in other time points, with 25-100 µg/mL ASA groups exhibiting significantly lower DSP levels than the control groups, and 200 µg/mL ASA showed a relatively comparable level of DSP per cell to the control group. $n = 4$ in each group. $*P < 0.05$. $**P < 0.001$. Values are presented as mean ± SD.

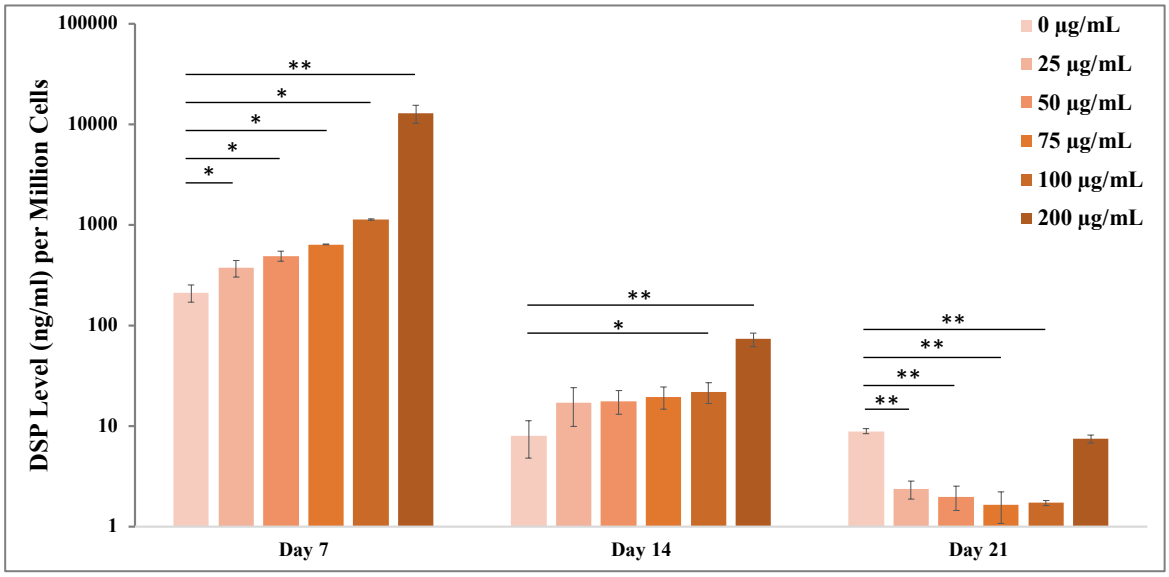


Figure 44. The overall production of the dentin sialoprotein levels per cell of human dental pulp cells treated with acetylsalicylic acid.

The dentin sialoprotein (DSP) levels per cell of human dental pulp cells (HDPCs), assessed by enzyme-linked immunosorbent assays (ELISA), illustrated that acetylsalicylic acid (ASA) exerted a dose-dependent effect on the DSP levels per cell of HDPCs on day 7 and day 14, with 200 µg/mL ASA markedly enhancing DSP levels. On day 21, the DSP levels did not follow the same trend. However, the overall production of the DSP levels per cell of HDPCs treated with each concentration of ASA declined from markedly distinct elevation on day 7 to the lowest level on day 21 by several folds. $n = 4$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

RUNX2 Level

HDPCs treated with all concentrations of ASA exhibited significantly increased RUNX2 levels per cell than the control group in a dose-dependent manner on day 7, with 200 μg/mL ASA showing the peak RUNX2 level, which was dramatically higher than the rest of the treatment conditions and the control by an estimate over ten folds ($P < 0.001$; Fig. 45). The results were in line with the relative gene expression levels of RUNX2 shown on day 7.

The RUNX2 levels per cell of HDPCs evaluated after 14 days of ASA treatment demonstrated that ASA influenced the RUNX2 levels in a dose-dependent manner compared to the control group. However, the significances were detected in the groups of 75-200 μg/mL ASA, with 200 μg/mL continuing to exhibit higher RUNX2 level per cell than the rest of the treatment conditions and the control by an estimate of eight folds ($P < 0.001$; Fig. 46). No statistically significant differences were found between the RUNX2 levels of the control group and 25-50 μg/mL ASA groups ($P > 0.05$). All test conditions exhibited a decline in the RUNX2 levels per cell compared to an evaluation on day 7.

The dose-dependent influence of ASA concentrations on the RUNX2 levels of HDPCs was also seen on day 21 evaluation, but no statistically significant differences were found between the RUNX2 levels of the control and 25-100 μg/mL ASA groups ($P > 0.05$). 200 μg/mL ASA continued to show statistically significant enhanced RUNX2 level compared to the control ($P < 0.001$; Fig. 47). All test conditions exhibited a decline in the RUNX2 levels per cell compared to an evaluation on day 14.

Overall results illustrated that ASA exerted a dose-dependent effect on the RUNX2 levels of HDPCs, with 200 μg/mL ASA markedly enhancing RUNX2 levels at every time point (P < 0.001; Fig. 48). The levels of RUNX2 per cell of HDPCs decreased over time.

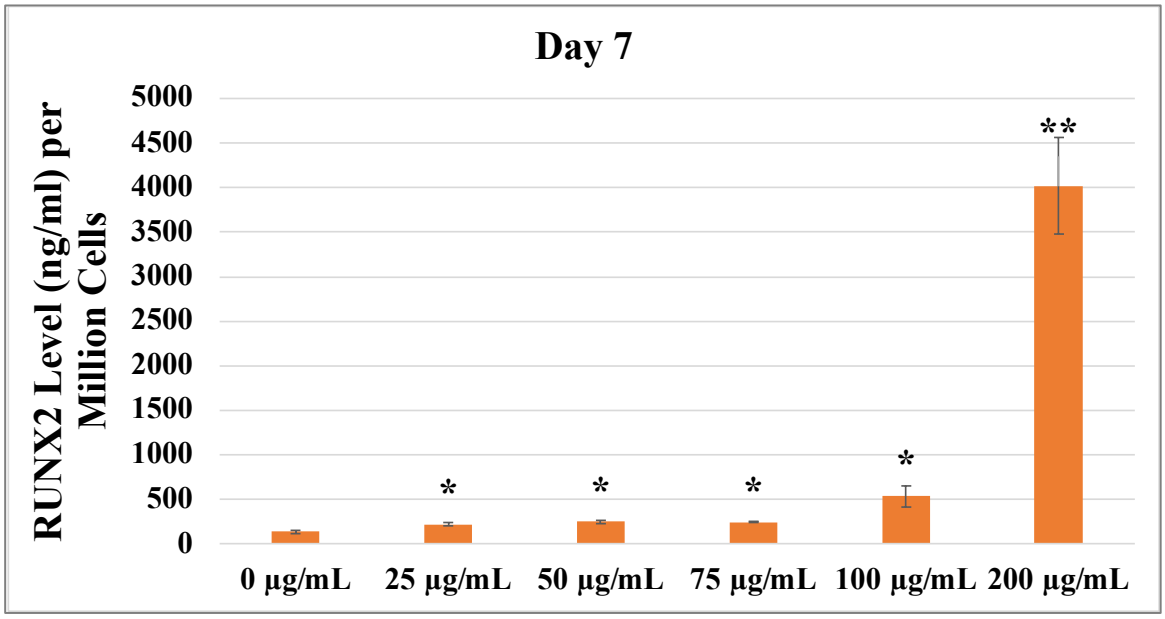


Figure 45. The runt-related transcription factor 2 levels per cell of human dental pulp cells on day 7.

The runt-related transcription factor 2 (RUNX2) levels per cell of human dental pulp cells (HDPCs) on day 7 showed that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner, with 200 µg/mL ASA markedly enhancing the RUNX2 level per cell of HDPCs compared to the control. $n = 4$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

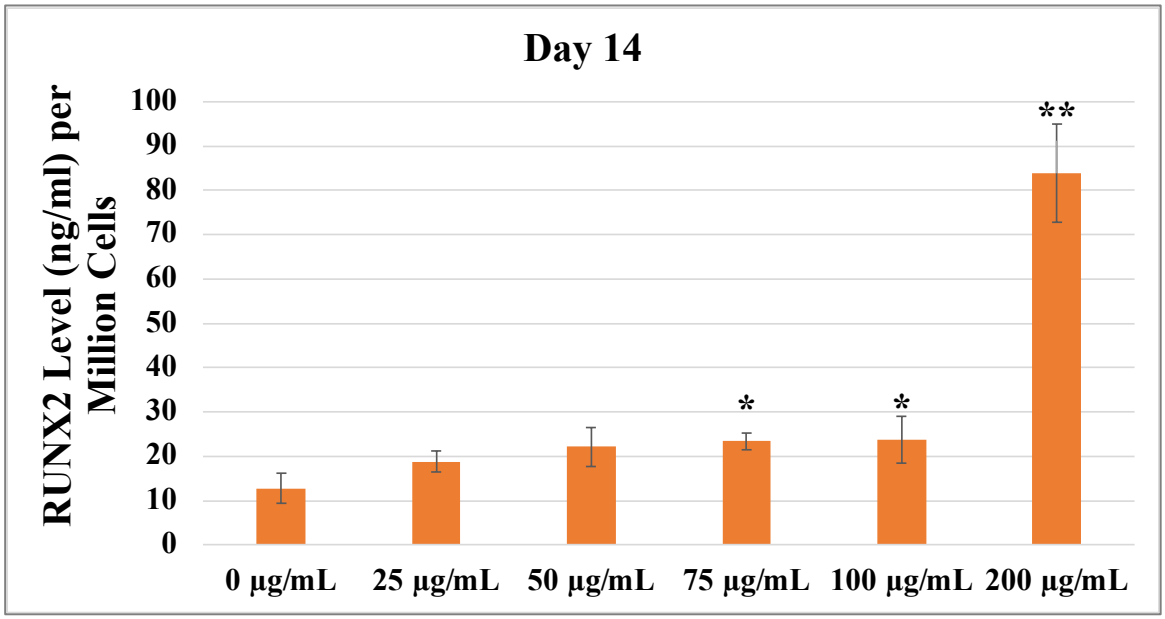


Figure 46. The runt-related transcription factor 2 levels per cell of human dental pulp cells on day 14.

The runt-related transcription factor 2 (RUNX2) levels per cell of human dental pulp cells (HDPCs) on day 14 showed that acetylsalicylic acid (ASA) exhibited increased RUNX2 levels than the control group in a dose-dependent manner. However, the significance of the RUNX2 levels per cell was only detected at 75-200 µg/mL ASA, with 200 µg/mL ASA markedly enhancing the RUNX2 level per cell of HDPCs compared to the control. $n = 4$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

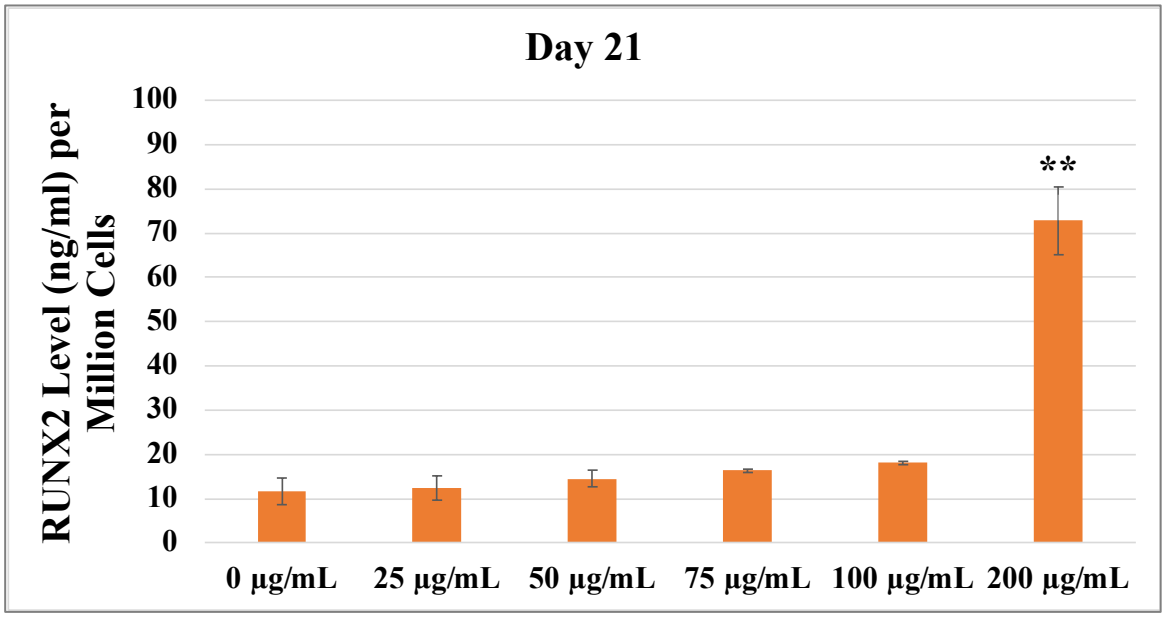


Figure 47. The runt-related transcription factor 2 levels per cell of human dental pulp cells on day 21.

The runt-related transcription factor 2 (RUNX2) levels per cell of human dental pulp cells (HDPCs) on day 21 showed that acetylsalicylic acid (ASA) exhibited increased RUNX2 levels than the control group in a dose-dependent manner. However, the significance of the RUNX2 levels per cell was only detected at 200 µg/mL ASA, which markedly enhanced the RUNX2 level per cell of HDPCs compared to the control. $n = 4$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

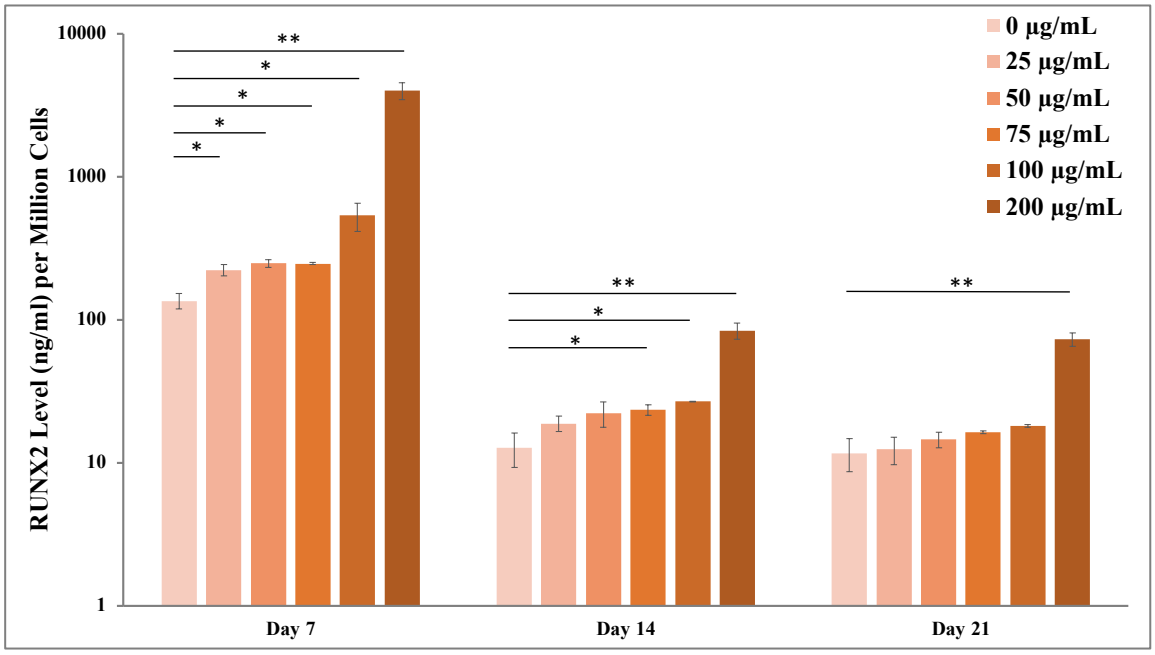


Figure 48. The acetylsalicylic acid exerted a dose-dependent effect on the runt-related transcription factor 2 levels of human dental pulp cells.

The runt-related transcription factor 2 (RUNX2) levels per cell of human dental pulp cells (HDPCs), assessed by enzyme-linked immunosorbent assays (ELISA), illustrated that acetylsalicylic acid (ASA) exerted a dose-dependent effect on the RUNX2 levels of HDPCs, with 200 µg/mL ASA markedly enhanced RUNX2 levels at every time point. The levels of RUNX2 per cell of HDPCs treated with each ASA concentration decreased over time. $n = 4$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

6.6 Mineralization

After 7, 14, and 21 days of odontogenic induction of HDPCs treated with 0, 25, 50, 75, 100, and 200 μg/mL ASA, the supernatant was harvested to evaluate odontogenic biomarkers, and the cells were fixed with 10% neutral buffered formalin before staining with alizarin red S solution (ARS) and solubilized by 10% acetic acid to quantify the mineralized nodule formation. The detected alizarin red levels were normalized to cell numbers per million cells.

The alizarin red levels per cell of HDPCs evaluated on day 7 demonstrated that concentrations of ASA significantly influenced the mineralized nodule formation of the HDPCs in a dose-dependent manner, with 200 μg/mL ASA showing markedly enhanced alizarin red level per cell compared to the rest of the treatment conditions and the control by an estimate over ten folds ($P < 0.001$; Fig. 49).

The alizarin red levels per cell of HDPCs evaluated after 14 days of ASA treatment demonstrated that ASA influenced the mineralized nodule formation of the HDPCs in a dose-dependent manner compared to the control group. However, the significance was detected in the groups of 50-200 μg/mL ASA, with 200 μg/mL continuing to exhibit higher alizarin red level per cell than the rest of the treatment conditions and the control ($P < 0.001$; Fig. 50). No statistically significant differences were found between the alizarin red levels of the control group and 25 μg/mL ASA groups ($P > 0.05$). All test conditions exhibited a decline in the alizarin red levels per cell compared to an evaluation on day 7.

The evaluation on day 21 showed a subtle dose-dependent effect of ASA on the mineralized nodule formation of HDPCs, but no statistically significant differences were found between the alizarin red levels of the control and 25-75 μg/mL ASA groups ($P > 0.05$). The treatment of HDPCs with 25 μg/mL ASA exhibited a relatively comparable level of alizarin red per cell to the control group, whereas 100-200 μg/mL ASA continued to show statistically significant enhanced alizarin red levels per cell compared to the control (Fig. 51). All test conditions exhibited a decline in the alizarin red levels per cell compared to an evaluation on day 14.

Overall results illustrated that ASA exerted a dose-dependent effect on the mineralized nodule formation of HDPCs, with 200 μg/mL ASA markedly enhancing alizarin red levels at every time point ($P < 0.001$; Fig. 52). The mineralized nodule production per cell of HDPCs decreased over time.

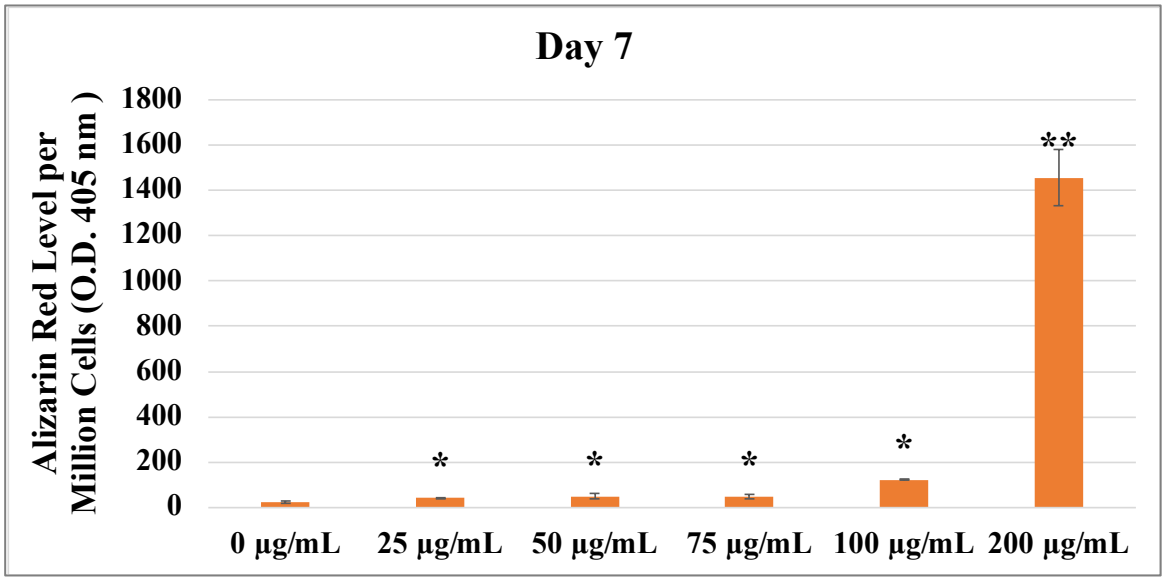


Figure 49. The alizarin red levels per cell of human dental pulp cells on day 7.

The alizarin red levels per cell of human dental pulp cells (HDPCs) on day 7 showed that the levels were significantly elevated by acetylsalicylic acid (ASA) treatment in a dose-dependent manner, with 200 µg/mL ASA markedly enhancing alizarin red level per cell of HDPCs compared to the control. $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

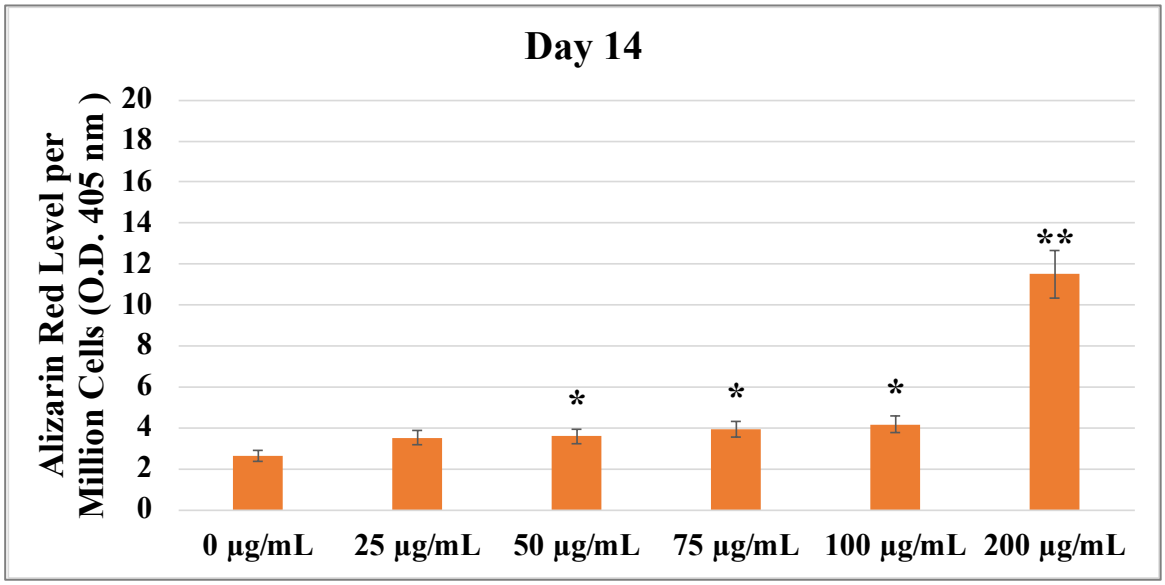


Figure 50. The alizarin red levels per cell of human dental pulp cells on day 14.

The alizarin red levels per cell of human dental pulp cells (HDPCs) on day 14 showed that acetylsalicylic acid (ASA) exhibited increased alizarin red levels than the control group in a dose-dependent manner. However, the significance of the alizarin red levels per cell was only detected at 50-200 µg/mL ASA, with 200 µg/mL ASA markedly enhancing alizarin red levels of HDPCs compared to the control. $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

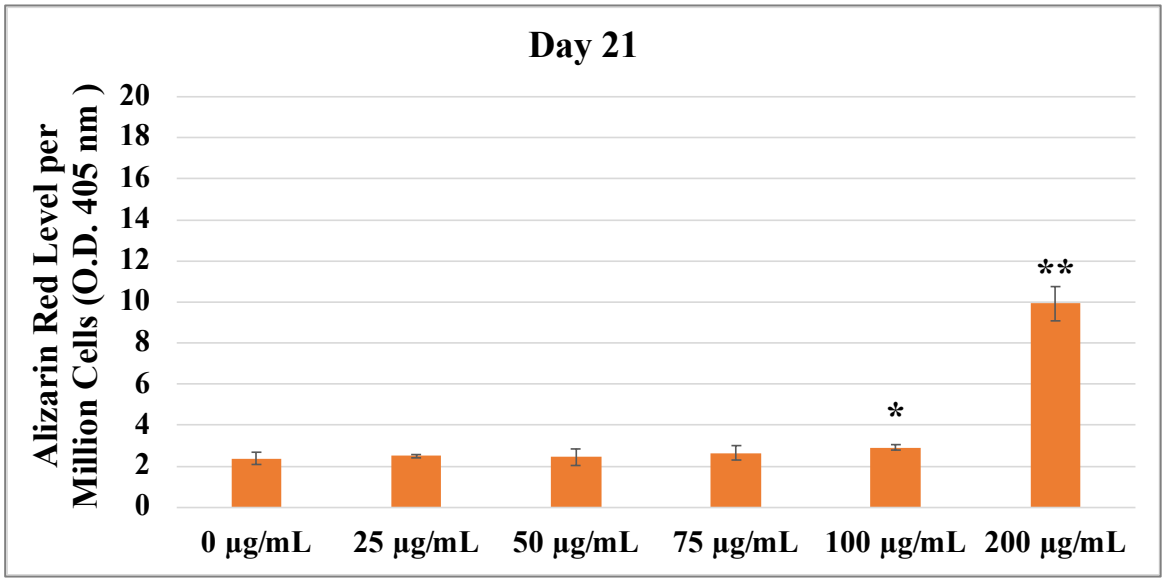


Figure 51. The alizarin red levels per cell of human dental pulp cells on day 21.

The alizarin red levels per cell of human dental pulp cells (HDPCs) on day 21 showed that acetylsalicylic acid (ASA) exhibited a subtle increased alizarin red level than the control group in a dose-dependent manner. However, the significance of the alizarin red levels per cell was only detected at 100-200 µg/mL ASA, with 200 µg/mL ASA markedly enhancing alizarin red levels of HDPCs compared to the control. $n = 6$ in each group. $*P < 0.05$. $**P < 0.001$. Values are presented as mean ± SD.

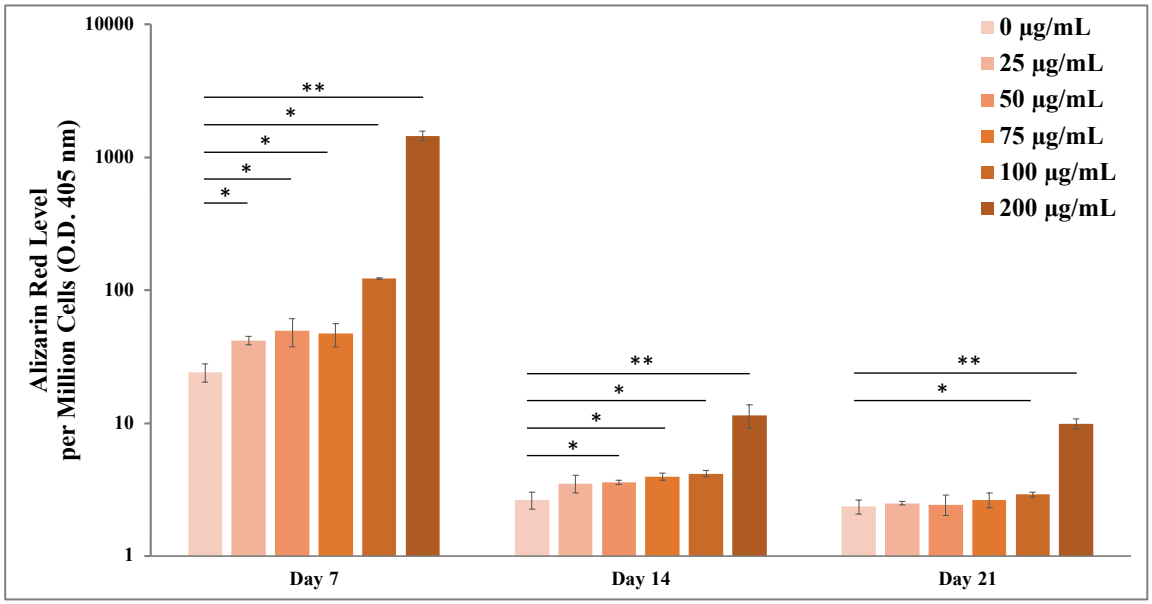


Figure 52. The alizarin red levels per cell of human dental pulp cells illustrated that acetylsalicylic acid exerted a dose-dependent effect on the mineralized nodule formation of human dental pulp cells.

The alizarin red levels per cell of human dental pulp cells (HDPCs), assessed by alizarin red staining and solubilized by 10% acetic acid, illustrated that acetylsalicylic acid (ASA) exerted a dose-dependent effect on the mineralized nodule formation of HDPCs, with 200 µg/mL ASA markedly enhancing alizarin red levels at every time point. The mineralized nodule production per cell of HDPCs treated with each ASA concentration decreased over time. $n = 6$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

6.7 Evaluation of Liberated Transforming Growth Factor-ß1 from Dentin Slices

To elucidate the potential of ASA in liberating the transforming growth factor-ß1 (TGF-ß1) from dentin, dentin slices were prepared and conditioned with phosphate-buffered saline (PBS; negative control), 17% ethylenediaminetetraacetic acid (EDTA; positive control), 25 and 200 μg/mL for 5 or 10 minutes. The supernatant was collected and quantified for the TGF-ß1 level using an ELISA kit.

After 5 minutes of conditioning dentin slices, the results showed that no TGF-ß1 was found in the supernatant collected from the PBS group as opposed to the significantly highest level of TGF-ß1 detected in the 17% EDTA group ($P < 0.001$), confirming the negative and positive control subsequently. The supernatant collected from conditioning the dentin slices with 25 and 200 μg/mL ASA exhibited dose-dependent levels of TGF-ß1, which were both significantly higher than the negative control ($P < 0.001$; Fig. 53).

The increase in the TGF-ß1 levels was detected in all groups, including PBS, after 10 minutes of dentin conditioning compared to the levels seen at 5 minutes. At this point of the evaluation, the supernatant from dentin slices conditioned with 25 μg/mL ASA showed no statistical significance compared to the negative control. However, the group of 200 μg/mL expressed a relatively comparable level of TGF-ß1 to the positive control, which was considered significantly higher than the negative control group ($P < 0.05$; Fig. 54).

The overall data suggested that ASA had the potential to release TGF-ß1 from dentin with ASA enhanced the TGF-ß1 liberation in a dose-dependent manner, and the levels increased over time (Fig. 55). ASA 200 μg/mL demonstrated a comparable potential to the positive control (17 % EDTA) after a 10 minute-conditioning. At 5 minutes, there was no TGF-ß1 detected in the supernatant after PBS conditioning. The TGF-ß1 concentration increased at 10 minute-PBS conditioning, although the level was significantly lower than the level released by EDTA ($P < 0.001$) and ASA 200 μg/mL ($P < 0.05$).

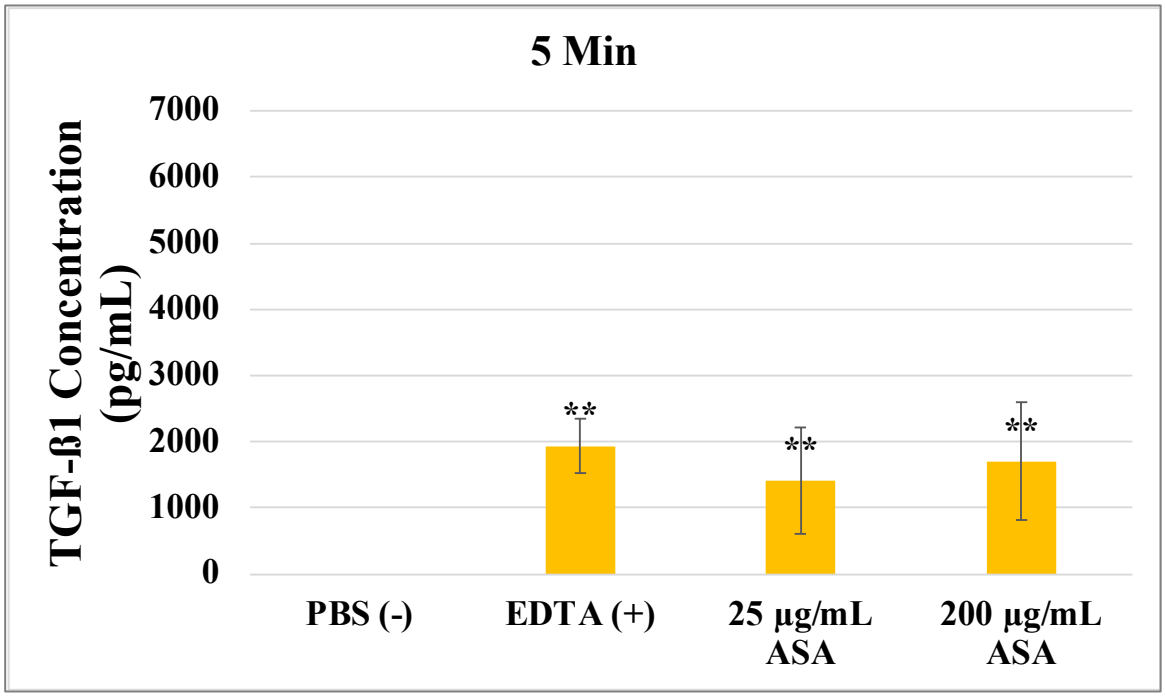


Figure 53. The transforming growth factor-ß1 concentrations released after 5 minutes of dentin slice conditioning.

The transforming growth factor-ß1 (TGF-ß1) concentrations released after 5 minutes of dentin slice conditioning showed that the acetylsalicylic acid (ASA) concentrations affected the TGF-ß1 level in a dose-dependent manner. The TGF-ß1 levels were significantly detected in the supernatant of dentin slices conditioned with the 17% ethylenediaminetetraacetic acid (EDTA; positive control) and the ASA in both concentrations compared to the phosphate-buffered saline (PBS; negative control). $n = 5$ in each group. $*P < 0.05$. $**P < 0.001$. Values are presented as mean ± SD.

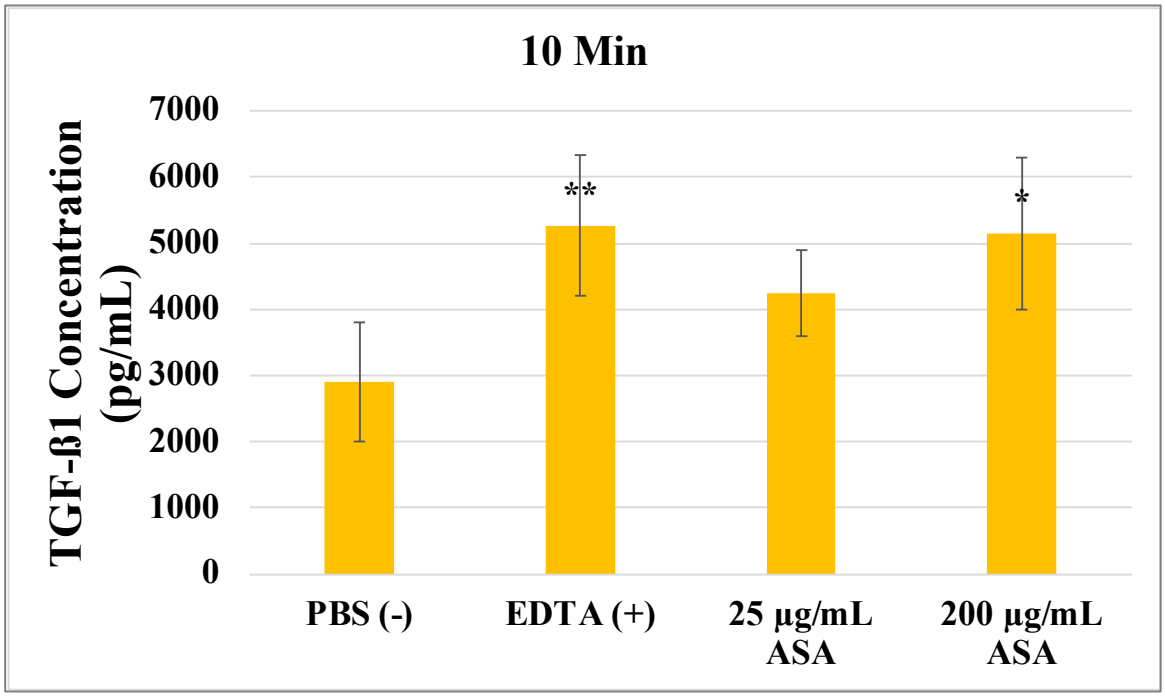


Figure 54. The transforming growth factor-ß1 concentrations released after 10 minutes of dentin slice conditioning.

The transforming growth factor-ß1 (TGF-ß1) concentrations released after 10 minutes of dentin slice conditioning showed that the acetylsalicylic acid (ASA) concentrations affected the TGF-ß1 level in a dose-dependent manner. The TGF-ß1 levels were significantly detected in the supernatant of dentin slices conditioned with the 17% ethylenediaminetetraacetic acid (EDTA; positive control) and the 200 µg/mL ASA compared to the phosphate-buffered saline (PBS; negative control). $n = 5$ in each group. *$P < 0.05$. **$P < 0.001$. Values are presented as mean ± SD.

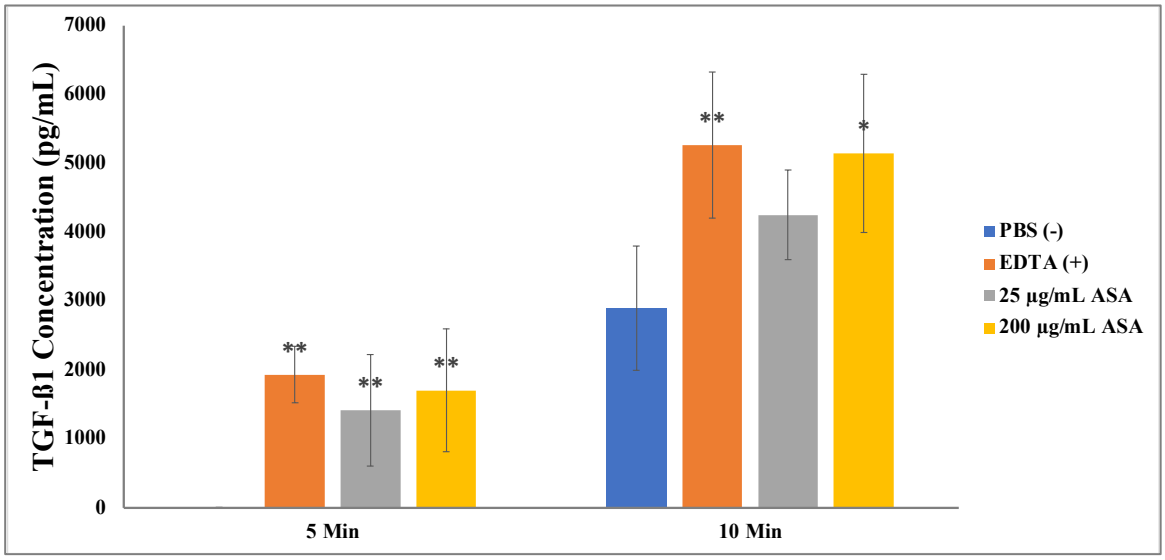


Figure 55. Transforming growth factor-ß1 concentration in the supernatant following 5 minutes and 10 minutes of dentin slice conditioning.

Transforming growth factor-ß1 (TGF-ß1) concentration in the supernatant following 5 minutes and 10 minutes of dentin slice conditioning with phosphate-buffered saline (PBS; negative control), 17% ethylenediaminetetraacetic acid (EDTA; positive control), or concentrations of acetylsalicylic acid (ASA) showed that ASA enhanced TGF-ß1 release from dentin in a dose-dependent manner, and the TGF-ß1 concentrations increased over time in all treatment conditions. $n = 5$ in each group. $^{*}P < 0.05$. $^{**}P < 0.001$. Values are presented as mean ± SD.

CHAPTER SEVEN: DISCUSSION

Chapter Seven: Discussion

Recently, acetylsalicylic acid (ASA) has been reported to show positive outcomes in osteogenic induction in cell therapy and bone tissue engineering (39, 149, 159). Previous studies on the roles of ASA on the osteogenic and odontogenic potentials were conducted on many cell types, such as bone marrow stem cells (BMSCs), stem cells from the apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), and stem cells from human exfoliated deciduous teeth (SHED). However, the knowledge of the influence of ASA on the odontogenic capacity of human dental pulp cells (HDPCs) is yet to be elucidated.

We speculated in this study that ASA, with its chemical and therapeutic properties, could potentially enhance the odontogenesis of HDPCs and might be another promising element to develop novel pulp capping materials. This study aimed to evaluate the effect of ASA and its concentrations on the HDPCs *in vitro* in terms of attachment efficiency, proliferation rate, odontogenic differentiation at the transcriptional and post-transcriptional levels, and mineralization. The subsidiary aim was to investigate the possibility of ASA to liberate the transforming growth factor-ß1 (TGF-ß1) from dentin.

HDPCs compose a heterogeneous population, including postnatal mesenchymal stem cells (MSCs) with multipotentiality confined within the pulp chamber (42). The MSCs, or multipotent cells, were firstly characterized in 2000 from the cells isolated from the impacted third molar (62). The MSCs preserve the capacity to differentiate into the cell types that will generate mesenchymal derived tissues, including odontoblast, osteoblasts, chondrocytes, adipocytes, and neural cells, indicating the promising potential in

regenerative medicine (43, 160). When the teeth are subject to an injury, HDPCs play a crucial role in sustaining the damage by generating the tertiary dentin, namely reparative and reactionary dentin, to keep the pulp tissue away from the injurious site (64, 151).

In the present study, the HDPCs were obtained without a pre-selection of dental pulp stem cells (DPSCs) or a picked colony culturing method for lineage-specific differentiation. It is because the purpose of the study is to evaluate the potential of ASA to be utilized in the future development of materials for a clinical setting, such as dental pulp capping procedure, and that the chance for the material to be directly exposed to HDPCs in the pulp tissue is high. Hence, heterogenous HDPCs would be suitable to provide more clinically relevant results. Also, recent studies have demonstrated the multilineage differentiation potential of HDPCs, and no significant difference was found between multi-colony derived and picked colony cultures regarding stem cell capacity and cell expansion (49, 154, 159).

To determine the therapeutic potential and the possibility of ASA utilization in dental tissue regeneration, investigation of biocompatibility and the influence of ASA and its concentrations on HDPCs are crucial. According to Drug and Chemical Blood-Level Data 2001 (21), the concentration of ASA present in the blood following the therapeutically effective dosage in humans, defined as a therapeutic blood level, ranges between 20-250 μg/mL. Serious toxic symptoms of ASA could start at the blood concentration level of 150-300 μg/mL, and the 500 μg/mL blood level of ASA is considered lethal or reported to cause death in humans (21, 161). This information is supported by the Food and Drug

Administration (FDA), Code of Federal Regulations (CFR) Title 21 CFR 343, stating that ASA at plasma concentrations approximating over 300 μg/mL are clearly toxic, and over 400 μg/mL could potentially relate to severe toxic effects in humans (20). The maximum concentration of ASA in this study was 200 μg/mL, which was declared by FDA to be the minimal plasma concentration that early salicylism could occur, and other concentrations (25-100 μg/mL ASA) were within the *in vivo* therapeutic range (15, 162).

Before introducing new materials to the cell culture, it is critical to be aware of the susceptibility of the cells to changes in culture medium pH. The pH plays an important role relating to the homeostasis regulation of the living. Therefore, an alternation in the pH value beyond the acceptable limit may cause detrimental effects on the biological process of the organism at a cellular, tissue, and whole-body level (163). Previous studies have reported that the extracellular pH influenced by the culture medium could affect the reprogramming of somatic cells and the differentiation of pluripotent stem cells (164). It has been reported that the proper pH conditioned medium for human cell cultures ranges from 7.0 to 7.8, with the most optimal pH of 7.2 for human osteoblasts and pH of 7.8 for HDPCs (93, 165). The activity of human osteoblast function, such as lactate production, alkaline phosphatase (ALP) activity, and DNA synthesis, was enhanced with the increasing pH within the range of pH 7.0 to 7.6 and markedly suppressed by pH 7.8 and over (165). Another study investigating the difference between mineralization levels of HDPCs cultured in pH 7.2 and 7.8 conditioned medium found that an alkaline tendency could better promote calcified nodule formation (93).

In this study, the end concentrations of ASA in the culture medium manifested a pH of 7.5 to 7.7, which was within the optimal range for HDPCs. The acidity in concentrations of ASA lowered the pH value of the culture medium in a dose-dependent manner. To our surprise, this study also detected increased levels of odontogenic differentiation and mineralization in HDPCs treated with high concentrations of ASA. The results contrasted with the previous finding that reported the enhanced mineralization in an alkaline environment, suggesting that ASA may exert other mechanisms on the differentiation and mineralization of HDPCs apart from the pH variation.

In terms of cell viability, preceding evidence indicated that concentrations of nonsteroidal anti-inflammatory drugs (NSAIDs) largely influenced their effects on MSCs (162, 166). Treatment of MSCs with therapeutically relevant concentrations of NSAIDs did not show to have negative effects on cell growth and proliferation, especially in low concentrations (0.01–50 μM) that even exhibited some viability-improving effects (166-168), whereas at higher concentrations above therapeutic levels (100-1000 μM) could exert negative effects on cell viability and hinder the cell survival (166, 169). Also, the influence of 0.5 mM ASA, equivalent to a low therapeutic plasma concentration, on the cell viability and proliferation of endothelial cells was undetectable. On the other hand, notable apoptotic levels were found well above the therapeutic plasma concentration of ASA at 5 mM (162). PDLSCs treated with different concentrations of ASA showed declined proliferation rates in a dose-dependent manner over time (37). Low concentrations of ASA (< 100 μg/mL) demonstrated no cytotoxic effects on BMSCs, while higher concentrations at 150 μg/mL

showed an inhibitory effect on BMSC proliferation (149, 170). However, a previous study proved that administration of any doses of ASA within the range of 1-1000 μM could reduce percentages of cell apoptosis dramatically in murine bone marrow stromal cells (170).

This present study is in line with the previous reports on other cell types that, within the therapeutic range of concentrations used in the study (25-200 μg/mL), ASA was non-toxic to HDPCs but may manifest a down-regulating effect on cell proliferation in high concentrations of ASA. Interestingly, another contradicted report showed decreased DPSCs viability by ASA dose over 100 μg/mL (159). However, the repeatability of our cell viability results was confirmed with several repeated experiments of methylthiazolyldiphenyl-tetrazolium bromide (MTT) and XTT assays accompanying the crystal violet staining and triton X-100 for an extended observation of proliferation rates. The MTT and XTT assays are quantitative methods of cytotoxicity testing (ISO 10993-5), which is currently the only *in vitro* test required by the FDA to evaluate the biocompatibility of medical devices and materials (171). The crystal violet staining solubilized by triton X-100 is also a reliable indirect quantification of cell death and differences in proliferation by detecting adherence of cells using crystal violet solution, which binds to proteins and DNA (172).

Both cell viability and proliferation rates found in this study consistently showed the enhancing effect of low concentrations of ASA (25-50 μg/mL) on HDPCs. Although higher concentrations of ASA manifested some down-regulating effect, no significant cell

damage or cell death was detected in the observation at cell morphology or cell viability levels, and HDPCs treated with all concentrations of ASA proliferated over time.

The significance found in this study could support the hypothesis that ASA could enhance the odontogenesis of HDPCs at both gene expression and biomarker protein levels suggesting the potential of ASA in dental pulp regeneration. ALP is considered a marker for odontoblast-like differentiation and is associated with the mineral deposition of connective tissues (92, 173). Runt-related transcription factor 2 (RUNX2), a critical transcription factor, usually presents and plays an important role at the onset of odontoblast differentiation as well as the ALP (173-176). An expression of a phosphorylated protein dentin sialophosphoprotein (DSPP) is reportedly transcriptionally induced by RUNX2 (94, 101). The DSPP, which is also known as a mature odontoblast marker that will later be cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), plays a part in odontogenic differentiation and dentin formation (89, 94, 97, 101). The final stage of odontogenic differentiation is the mineralized nodule formation prompted by odontoblast to form dentin (177-179).

This study proved that ASA, especially in high concentrations (200 μg/mL), markedly promoted the gene expression levels of RUNX2 and DSPP in HDPCs. The results were consistent with the mineralization and the odontogenic biomarkers of ALP, DSP, and RUNX2, especially on day 7 and day 14, in which the levels were increased by ASA in a dose-dependent manner. Considering that the proliferation rate of HDPCs declined as the ASA concentration rose, we speculate that high concentrations of ASA caused the cells to

ease down the proliferation and proceed into differentiation. The same phenomenon has been reported in the ASA-induced osteogenic differentiation in DPSCs and PDLSCs (37, 159).

Regarding the mineralization, we also speculated that there was a possibility that the calcium accumulation could be influenced by one of the main functional groups of ASA, the carboxyl group (-COOH), consisting of a carbonyl group (C=O) and a hydroxyl group (-OH). The -COOH group has contributed to several biological processes of an organism as it is an element contained in the amino acid (180, 181). Evidence showing the binding strength of the -COOH group to different cations has been reported and showed the highest bond strength with the calcium ion (Ca^{2+}) (182). For instance, a Ca^{2+} could bind to a negatively charged carboxyl group ($-COO^{-}$) of a clotting factor leading to blood clotting in the hemostasis process (183). For this reason, the influence of the -COOH group on the mineralized nodule production of HDPCs treated with ASA is another possible factor to be considered apart from the enhancing effect of ASA on biomarker production. Therefore, further investigation on this matter needs to be conducted in the future.

This study also found that the peak biomarker and mineralization levels per cell of all groups were detected on day 7 and substantially declined over time, which might suggest an early differentiation of HDPCs influenced by ASA, as found in the previous study (170). The supposition of the early differentiation phenomenon of ASA-treated HDPCs concurred with the evidence of gene expression levels of RUNX2 and DSPP detected on day 4, which too manifested a dose-dependent pattern relating to ASA concentrations. The phenomenon

was supported by a previous study that suggested the same trend of an odontogenic induction of 100 μg/mL ASA-treated SCAPs (38). A report of ASA effects on murine bone marrow stromal cells showed peak ALP and RUNX2 levels on day 3 before declining. The highest dose of ASA used in the study, 1000 μM, significantly arrested the cell cycle of murine bone marrow stromal cells in the resting phase G_0/G_1 after 48 hours of ASA treatment, indicating a sign of the cell exiting the cell cycle to enter the differentiation phase (170).

Due to the limitation of the equipment, we were not able to compare the trend of the gene expression over time since the gene expression level of extracted mRNA had to be evaluated on the same day as the harvest to preserve the stability of mRNA. Hence, it would be inappropriate to compare the relative gene levels obtained from different time points since the calculation was based on the gene level of the control group evaluated on each day which differed, not from the standard value as in the assay kits. Nevertheless, the relative gene expression levels of DSPP and RUNX2 detected at each time point exhibited a dose-dependent effect of ASA on HDPCs, which supported the finding of biomarker protein production of DSP and RUNX2 at the same treatment period. This present study firstly demonstrated that 200 μg/mL ASA had the potential to enhance HDPC's odontogenesis at both transcriptional and post-transcriptional levels, which is opposed to some previous findings that found ASA over 100 μg/mL cytotoxic on other cell types (36, 149, 159).

Supporting evidence on the mechanisms associated with ASA and the enhanced osteogenic differentiation has been proposed for other cell types. A low dose of ASA significantly enhanced telomerase activity in telomerase reverse transcriptase (TERT)/Wnt/β-catenin cascade, leading to an improved stem cell function and bone formation (34, 36). The treatment of ASA showed immunomodulatory capacity by reducing interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in MSC-based tissue regeneration (184, 185). In addition, an elevated expression of growth factor-associated genes, including FGFs and BMPs, was found in PDLSCs after treatment with 180 μg/mL of ASA for 24 hours (37). Another study also showed the relation between ASA treatment and the activation of the phosphoinositide 3-kinase–AKT signaling pathway (PI3K-AKT), leading to the odontogenic differentiation of SCAPs (38). It is suspected that these mechanisms could apply to ASA treated-HDPCs, but further investigations and *in vivo* studies should be addressed.

This study is the first discovery that ASA significantly enhanced TGF-ß1 liberation from dentin which supports another hypothesis of this study. The property could be beneficial for vital pulp treatments as the TGF-ß1 was found primarily in tertiary dentin, suggesting their crucial roles in dental tissue response to injury (80). During the restorative procedure, the dentinal tubules serve as channels between the capping material and pulp. Once the sequestrated TGF-ß1 is released from dentin, it may commute through the dentinal tubules into the pulp and promote odontoblast-like cell differentiation from progenitor cells for dentin repair (66, 74). Many studies have been trying to isolate the TGF-ß1 from dentin demineralization to utilize its repairing ability to develop novel

regenerative materials (74, 186-189). The TGF-ß1 extraction from dentin powder is the property found in calcium hydroxide ($Ca(OH)_2$) and tricalcium silicate cement (TSC). But TGF-ß1 extracted from dentin slice condition is minimal (85, 86). An attempt to liberate the bioactive molecules from dentin slices using dental etchants/conditioners was reported, but the solutions were unable to extract TGF-ß1 (86).

In this study, 200 μg/mL ASA could successfully release TGF-ß1 into an aqueous environment, and the TGF-ß1 concentrations increased over time to the comparable amount as the positive control at 10 minutes. The ability to solubilize the dentin matrix component may be due to hydrogen ion concentration in the acidic environment reported in the previous studies (129, 190, 191). Although further investigation on the mechanism is required, this is an interesting finding to see the increased amount of TGF-ß1 over time since the previous study reported the denature of bioactive molecules in a prolonged treatment period (86). It is acknowledged that the conditioning times used in this study may not be feasible in the clinical setting. However, the finding might provide fundamentals for the potential application of ASA in pulp capping materials and protocols.

CHAPTER EIGHT: CONCLUSIONS AND CLINICAL CONSIDERATIONS

Chapter Eight: Conclusions and Clinical Considerations

8.1 Conclusions

The present study demonstrated that acetylsalicylic acid (ASA) at concentrations of 25-200 μg/mL had no cytotoxic effect on human dental pulp cells (HDPCs) after 21 days of treatment. Also, ASA at concentrations of 25-200 μg/mL could significantly promote the proliferation rates of HDPCs and enhance HDPCs' odontogenesis at transcriptional and post-transcriptional levels, as well as transforming growth factor-ß1 (TGF-ß1) liberation from dentin in a dose-dependent manner. The results indicate a possible approach to developing a novel pulp capping material containing ASA for dental tissue regeneration.

8.2 Clinical Considerations

The pulp capping materials commonly used in vital pulp therapy procedures nowadays, such as calcium hydroxide ($Ca(OH)_2$) and tricalcium silicate cement (TSC), show no odontogenic potential. Their mechanisms rely upon pulpal irritation to induce an inflammatory response resulting in unsatisfactory success rates and compromised mechanical properties of the dentin in the long run. Although the alkaline pH of $Ca(OH)_2$ and TSC enables their potential to liberate the bioactive molecules from dentin, the amount of released growth factor is not enough to support the natural healing of the pulp.

This study demonstrated that ASA at the optimal concentrations has the odontogenic property by inducing the proliferation of HDPCs at low doses (25-50 μg/mL ASA) along with the enhanced effects on the differentiation and mineralization in a dose-dependent manner. The acidic property of ASA may benefit in neutralizing the pH of the pulp capping materials and help reduce the irritation, which may expedite the healing process of the pulp. Moreover, this study is the first to show that not only 200 μg/mL could induce odontogenesis, but it can also release the TGF-ß1 at a comparable level to the 17% ethylenediaminetetraacetic acid (EDTA). This knowledge may be usefully implemented in regenerative dentinogenesis. On this account, ASA preserves the potential to be a promising element to the pulp capping materials that may help improve the therapeutic property as well as increase the successful clinical outcome of vital pulp therapy and repair.

8.3 Future Studies

The results of this *in vitro* study revealed that ASA at a maximum concentration of 200 μg/mL could exhibit enhancing effects on the odontogenesis of HDPCs without showing cytotoxicity at the morphology level despite the acidity. This finding contrasted with some previous studies on other cell types, stating that ASA at concentrations over 100 μg/mL could exert cytotoxic effects on cells. The underlying mechanism and pathway leading to this odontogenic effect of ASA on HDPCs are yet unclear and worth investigating to expand the knowledge in the regeneration research for future applications.

Additional studies are also required to investigate the degradation rate of ASA incorporated pulp capping materials to determine the effective dose for an *in vivo* study. Furthermore, the dentin conditioning times conducted in this study to liberate the TGF-ß1 may not be convenient in the clinical setting. The adjustment in dosage and duration are needed to be improved to develop more suitable protocols.

Lastly, this *in vitro* study is merely the initiation of the concept of utilizing ASA as an element for pulp capping materials. Future *in vivo* study is imperative to take a further step toward the development of novel biomaterials for dental tissue regeneration.

LIST OF ABBREVIATED JOURNAL TITLES

Adv Clin Chem……..Advances in clinical chemistry

Adv Dent Res…… Advances in dental research

Adv Exp Med Biol……... Advances in experimental medicine and biology

Altern Lab Anim…….….Alternatives to laboratory animals

Am J Med…….. American journal of medicine

Ann Intern Med……. Annals of internal medicine

Arch Oral Biol……. Archives of oral biology

Aust Dent J…….…… Australian dental journal

Aust Endod J……..… Australian endodontic journal

Biochem Pharmacol…….. Biochemical pharmacology

Braz Oral Res……..... Brazilian oral research

Br J Clin Pharmacol…….. British journal of clinical pharmacology

Br J Haematol…….…… British journal of haematology

Calcif Tissue Int…….……..….. Calcified tissue international

Cell Biol Int…….……Cell biology international

Cell Tissue Res……Cell and tissue research

Clin Oral Investig……Clinical oral investigations

Cold Spring Harb Protoc……Cold Spring Harbor protocols

Connect Tissue Res…….Connective tissue research

Crit Rev Oncol Hematol……...Critical reviews in oncology/hematology

Crit Rev Oral Biol Med……Critical reviews in oral biology and medicine

Curr Opin HIV AIDS……Current opinion in HIV and AIDS

Curr Stem Cell Res Ther……..Current stem cell research & therapy

Dent Clin North Am……Dental clinics of North America

Dent Mater……Dental materials

Dent Res J (Isfahan)……..Dental research journal

Drug Des Devel Ther……Drug design, development and therapy

EMBO Mol Med…….EMBO molecular medicine

Endod Dent Traumatol……...Endodontics & dental traumatology

Eur Cell Mater……European cells & materials

Eur J Clin Pharmacol…….European journal of clinical pharmacology

Expert Rev Med Devices……..Expert review of medical devices

Faseb j……FASEB journal

Forensic Sci Int……Forensic science international

Genes Dev……...Genes & development

Histochem J……..The Histochemical journal

Indian J Clin Biochem……..Indian journal of clinical biochemistry

Int Endod J……..International endodontic journal

Int J Dev Biol……The International journal of developmental biology

Int J Mol Med……International journal of molecular medicine

Iran Endod J…….Iranian endodontic journal

J Adhes Dent……...The journal of adhesive dentistry

J Am Dent Assoc…….Journal of the American Dental Association

J Biol Buccale……..Journal de biologie buccale

J Biol Chem……The Journal of biological chemistry

J Bone Miner Metab……..Journal of bone and mineral metabolism

J Bone Miner Res……Journal of bone and mineral research

J Cell Biochem……Journal of cellular biochemistry

J Cell Sci Suppl…….Journal of cell science. Supplement

J Clin Invest……..The Journal of clinical investigation

J Clin Pediatr Dent……The Journal of clinical pediatric dentistry

J Dent……...Journal of dentistry

J Dent Educ…….Journal of dental education

J Dent Res…….Journal of dental research

J Dent Sci……..Journal of dental sciences

J Endod……...Journal of endodontics

J Neurol Neurosurg Psychiatry……..Journal of neurology, neurosurgery, and psychiatry

J Oral Maxillofac Surg……..Journal of oral and maxillofacial surgery

J Periodontol…… Journal of periodontology

J Phys Chem A…….The journal of physical chemistry. A

J Physiol Pharmacol…….Journal of physiology and pharmacology

J Prosthet Dent……The Journal of prosthetic dentistry

Kidney Int…….Kidney international

Matrix Biol…….Matrix biology

Mech Dev……Mechanisms of development

Mol Biol Cell…… ……Molecular biology of the cell

Nat Genet……..Nature Genetics

Nat Med……...….Nature medicine

Nat Rev Cardiol…… …….Nature reviews. Cardiology

N Engl J Med…. ……The New England journal of medicine

Oper Dent…….Operative dentistry

Oral Dis…….Oral diseases

Oral Surg Oral Med Oral Pathol Oral Radiol Endod……..Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics

Orthod Craniofac Res……...Orthodontics & craniofacial research

Osteoporos Int……..Osteoporosis international

Pharmacol Res……...Pharmacological research

Proc Natl Acad Sci U S A……Proceedings of the National Academy of Sciences of the United States of America

Prog Biophys Mol Biol……..Progress in biophysics and molecular biology

Quintessence Int……..Quintessence international

Reprod Med Biol……Reproductive medicine and biology

Stem Cells Dev……..Stem cells and development

Stem Cell Res Ther……Stem cell research & therapy

Stem Cell Rev Rep……..Stem cell reviews and reports

Thromb Res……...Thrombosis research

Tissue Eng……Tissue engineering

Transpl Immunol……..Transplant immunology

REFERENCES

1. Fuster V, Sweeny JM. Aspirin: a historical and contemporary therapeutic overview. Circulation. 2011;123(7):768-78.

2. Jeffreys D. Aspirin: The Remarkable Story of a Wonder Drug. 1st ed ed. New York, NY: Bloomsbury; 2004.

3. Desborough MJR, Keeling DM. The aspirin story – from willow to wonder drug. British Journal of Haematology. 2017;177(5):674-83.

4. Lévesque H, Lafont O. L'aspirine à travers les siècles: Rappel historique. La Revue de Médecine Interne. 2000;21:S8-S17.

5. Zundorf U. Aspirin 100 Years: The Future Has Just Begun: Bayer AG, Consumer Care Business Group; 1997.

6. Campbell CL, Smyth S, Montalescot G, Steinhubl SR. Aspirin dose for the prevention of cardiovascular disease: a systematic review. Jama. 2007;297(18):2018-24.

7. Botting RM. Inhibitors of cyclooxygenases: mechanisms, selectivity and uses. J Physiol Pharmacol. 2006;57 Suppl 5:113-24.

8. Hemler M, Lands WE. Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. J Biol Chem. 1976;251(18):5575-9.

9. Vane JR, Botting RM. The mechanism of action of aspirin. Thromb Res. 2003;110(5-6):255-8.

10. Bala M, Chin CN, Logan AT, Amin T, Marnett LJ, Boutaud O, et al. Acetylation of prostaglandin H2 synthases by aspirin is inhibited by redox cycling of the peroxidase. Biochem Pharmacol. 2008;75(7):1472-81.

11. Patrono C, Baigent C, Hirsh J, Roth G. Antiplatelet drugs: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest. 2008;133(6 Suppl):199s-233s.

12. DeWitt DL, el-Harith EA, Kraemer SA, Andrews MJ, Yao EF, Armstrong RL, et al. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. J Biol Chem. 1990;265(9):5192-8.

13. Roth GJ, Majerus PW. The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein. J Clin Invest. 1975;56(3):624-32.

14. Sweeny JM, Gorog DA, Fuster V. Antiplatelet drug 'resistance'. Part 1: mechanisms and clinical measurements. Nat Rev Cardiol. 2009;6(4):273-82.

15. Awtry EH, Loscalzo J. Aspirin. Circulation. 2000;101(10):1206-18.

16. Patrono C, García Rodríguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med. 2005;353(22):2373-83.

17. Farrell B, Godwin J, Richards S, Warlow C. The United Kingdom transient ischaemic attack (UK-TIA) aspirin trial: final results. J Neurol Neurosurg Psychiatry. 1991;54(12):1044-54.

18. Peters RJ, Mehta SR, Fox KA, Zhao F, Lewis BS, Kopecky SL, et al. Effects of aspirin dose when used alone or in combination with clopidogrel in patients with acute coronary syndromes: observations from the Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) study. Circulation. 2003;108(14):1682-7.

19. Steinhubl SR, Bhatt DL, Brennan DM, Montalescot G, Hankey GJ, Eikelboom JW, et al. Aspirin to prevent cardiovascular disease: the association of aspirin dose and clopidogrel with thrombosis and bleeding. Ann Intern Med. 2009;150(6):379-86.

20. Administration FaD. Code of Federal Regulations Title 21. In: Services HaH, editor. Silver Spring, MD 2019. p. 2.

21. Winek CL, Wahba WW, Winek CL, Jr., Balzer TW. Drug and chemical blood-level data 2001. Forensic Sci Int. 2001;122(2-3):107-23.

22. ISIS-2 SISoISCG. Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. Lancet. 1988;2(8607):349-60.

23. Patrono C, Dunn MJ. The clinical significance of inhibition of renal prostaglandin synthesis. Kidney Int. 1987;32(1):1-12.

24. Roderick PJ, Wilkes HC, Meade TW. The gastrointestinal toxicity of aspirin: an overview of randomised controlled trials. Br J Clin Pharmacol. 1993;35(3):219-26.

25. Sandler DP, Burr FR, Weinberg CR. Nonsteroidal anti-inflammatory drugs and the risk for chronic renal disease. Ann Intern Med. 1991;115(3):165-72.

26. Lisowska B, Kosson D, Domaracka K. Lights and shadows of NSAIDs in bone healing: the role of prostaglandins in bone metabolism. Drug Des Devel Ther. 2018;12:1753-8.

27. Raisz LG. Potential impact of selective cyclooxygenase-2 inhibitors on bone metabolism in health and disease. Am J Med. 2001;110 Suppl 3A:43s-5s.

28. Raisz LG, Pilbeam CC, Fall PM. Prostaglandins: mechanisms of action and regulation of production in bone. Osteoporos Int. 1993;3 Suppl 1:136-40.

29. Copeland RA, Williams JM, Giannaras J, Nurnberg S, Covington M, Pinto D, et al. Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase. Proc Natl Acad Sci U S A. 1994;91(23):11202-6.

30. Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, et al. Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(8):3228-32.

31. Tai H, Miyaura C, Pilbeam CC, Tamura T, Ohsugi Y, Koishihara Y, et al. Transcriptional induction of cyclooxygenase-2 in osteoblasts is involved in interleukin-6-induced osteoclast formation. Endocrinology. 1997;138(6):2372-9.

32. Bauer DC, Orwoll ES, Fox KM, Vogt TM, Lane NE, Hochberg MC, et al. Aspirin and NSAID use in older women: effect on bone mineral density and fracture risk. Study of Osteoporotic Fractures Research Group. J Bone Miner Res. 1996;11(1):29-35.

33. Carbone LD, Tylavsky FA, Cauley JA, Harris TB, Lang TF, Bauer DC, et al. Association between bone mineral density and the use of nonsteroidal anti-inflammatory drugs and aspirin: impact of cyclooxygenase selectivity. J Bone Miner Res. 2003;18(10):1795-802.

34. Yamaza T, Miura Y, Bi Y, Liu Y, Akiyama K, Sonoyama W, et al. Pharmacologic stem cell based intervention as a new approach to osteoporosis treatment in rodents. PLoS One. 2008;3(7):e2615.

35. Liu H, Li W, Liu Y, Zhang X, Zhou Y. Co-administration of aspirin and allogeneic adipose-derived stromal cells attenuates bone loss in ovariectomized rats through the anti-inflammatory and chemotactic abilities of aspirin. Stem Cell Res Ther. 2015;6:200.

36. Liu Y, Chen C, Liu S, Liu D, Xu X, Chen X, et al. Acetylsalicylic acid treatment improves differentiation and immunomodulation of SHED. J Dent Res. 2015;94(1):209-18.

37. Abd Rahman F, Mohd Ali J, Abdullah M, Abu Kasim NH, Musa S. Aspirin Enhances Osteogenic Potential of Periodontal Ligament Stem Cells (PDLSCs) and Modulates the Expression Profile of Growth Factor-Associated Genes in PDLSCs. J Periodontol. 2016;87(7):837-47.

38. Tanaka Y, Sonoda S, Yamaza H, Murata S, Nishida K, Kyumoto-Nakamura Y, et al. Acetylsalicylic Acid Treatment and Suppressive Regulation of AKT Accelerate Odontogenic Differentiation of Stem Cells from the Apical Papilla. J Endod. 2019;45(5):591-8 e6.

39. Rankin R, Lundy FT, Schock BC, Zhang SD, Al-Natour B, About I, et al. A connectivity mapping approach predicted acetylsalicylic acid (aspirin) to induce osteo/odontogenic differentiation of dental pulp cells. Int Endod J. 2020;53(6):834-45.

40. Malcomson B, Wilson H, Veglia E, Thillaiyampalam G, Barsden R, Donegan S, et al. Connectivity mapping (ssCMap) to predict A20-inducing drugs and their antiinflammatory action in cystic fibrosis. Proc Natl Acad Sci U S A. 2016;113(26):E3725-34.

41. Zhang SD, Gant TW. sscMap: an extensible Java application for connecting small-molecule drugs using gene-expression signatures. BMC Bioinformatics. 2009;10:236.

42. Kobayashi T, Torii D, Iwata T, Izumi Y, Nasu M, Tsutsui TW. Characterization of proliferation, differentiation potential, and gene expression among clonal cultures of human dental pulp cells. Hum Cell. 2020;33(3):490-501.

43. Nuti N, Corallo C, Chan BM, Ferrari M, Gerami-Naini B. Multipotent Differentiation of Human Dental Pulp Stem Cells: a Literature Review. Stem Cell Rev Rep. 2016;12(5):511-23.

44. Piva E, Tarlé SA, Nör JE, Zou D, Hatfield E, Guinn T, et al. Dental Pulp Tissue Regeneration Using Dental Pulp Stem Cells Isolated and Expanded in Human Serum. J Endod. 2017;43(4):568-74.

45. Yu C, Abbott PV. An overview of the dental pulp: its functions and responses to injury. Aust Dent J. 2007;52(1 Suppl):S4-16.

46. Moraleda JM, Blanquer M, Bleda P, Iniesta P, Ruiz F, Bonilla S, et al. Adult stem cell therapy: dream or reality? Transpl Immunol. 2006;17(1):74-7.

47. Suchánek J, Soukup T, Ivancaková R, Karbanová J, Hubková V, Pytlík R, et al. Human dental pulp stem cells--isolation and long term cultivation. Acta Medica (Hradec Kralove). 2007;50(3):195-201.

48. Bozdağ SC, Yüksel MK, Demirer T. Adult Stem Cells and Medicine. Adv Exp Med Biol. 2018;1079:17-36.

49. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. J Dent Res. 2002;81(8):531-5.

50. Harrington J, Sloan AJ, Waddington RJ. Quantification of clonal heterogeneity of mesenchymal progenitor cells in dental pulp and bone marrow. Connect Tissue Res. 2014;55 Suppl 1:62-7.

51. Menicanin D, Bartold PM, Zannettino AC, Gronthos S. Identification of a common gene expression signature associated with immature clonal mesenchymal cell populations derived from bone marrow and dental tissues. Stem Cells Dev. 2010;19(10):1501-10.

52. Berardi AC, Wang A, Levine JD, Lopez P, Scadden DT. Functional isolation and characterization of human hematopoietic stem cells. Science. 1995;267(5194):104-8.

53. Owen M. Marrow stromal stem cells. J Cell Sci Suppl. 1988;10:63-76.

54. Liu SS, Zhang C, Zhang X, Chen XH. Human umbilical cord blood-derived stromal cells: A new source of stromal cells in hematopoietic stem cell transplantation. Crit Rev Oncol Hematol. 2014;90(2):93-8.

55. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7(2):211-28.

56. Davies OG, Cooper PR, Shelton RM, Smith AJ, Scheven BA. A comparison of the in vitro mineralisation and dentinogenic potential of mesenchymal stem cells derived from adipose tissue, bone marrow and dental pulp. J Bone Miner Metab. 2015;33(4):371-82.

57. Huang GT, Garcia-Godoy F. Missing Concepts in De Novo Pulp Regeneration. J Dent Res. 2014;93(8):717-24.

58. Zuk PA. The adipose-derived stem cell: looking back and looking ahead. Mol Biol Cell. 2010;21(11):1783-7.

59. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.

60. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411):143-7.

61. Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S. The efficacy of mesenchymal stem cells to regenerate and repair dental structures. Orthod Craniofac Res. 2005;8(3):191-9.

62. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000;97(25):13625-30.

63. Liu J, Yu F, Sun Y, Jiang B, Zhang W, Yang J, et al. Concise reviews: Characteristics and potential applications of human dental tissue-derived mesenchymal stem cells. Stem Cells. 2015;33(3):627-38.

64. Mitsiadis TA, Rahiotis C. Parallels between tooth development and repair: conserved molecular mechanisms following carious and dental injury. J Dent Res. 2004;83(12):896-902.

65. Smith AJ. Vitality of the dentin-pulp complex in health and disease: growth factors as key mediators. J Dent Educ. 2003;67(6):678-89.

66. Tziafas D, Smith AJ, Lesot H. Designing new treatment strategies in vital pulp therapy. J Dent. 2000;28(2):77-92.

67. Thesleff I. Developmental biology and building a tooth. Quintessence Int. 2003;34(8):613-20.

68. Lesot H, Lisi S, Peterkova R, Peterka M, Mitolo V, Ruch JV. Epigenetic signals during odontoblast differentiation. Adv Dent Res. 2001;15:8-13.

69. Ruch JV, Lesot H, Bègue-Kirn C. Odontoblast differentiation. Int J Dev Biol. 1995;39(1):51-68.

70. Boskey AL. The role of extracellular matrix components in dentin mineralization. Crit Rev Oral Biol Med. 1991;2(3):369-87.

71. Linde A. Dentin mineralization and the role of odontoblasts in calcium transport. Connect Tissue Res. 1995;33(1-3):163-70.

72. Simon S, Cooper, P., Lumley, P., Berdal, A., Tomson, P., & Smith, A. . Understanding pulp biology for routine clinical practice. ENDO Endodontic Practice Today. 2009;3(3):14.

73. Gonçalves LF, Fernandes AP, Cosme-Silva L, Colombo FA, Martins NS, Oliveira TM, et al. Effect of EDTA on TGF-β1 released from the dentin matrix and its influence on dental pulp stem cell migration. Braz Oral Res. 2016;30(1):e131.

74. Smith AJ, Tobias RS, Murray PE. Transdentinal stimulation of reactionary dentinogenesis in ferrets by dentine matrix components. J Dent. 2001;29(5):341-6.

75. Tomson PL, Grover LM, Lumley PJ, Sloan AJ, Smith AJ, Cooper PR. Dissolution of bio-active dentine matrix components by mineral trioxide aggregate. J Dent. 2007;35(8):636-42.

76. Smith AJ, Cassidy N, Perry H, Bègue-Kirn C, Ruch JV, Lesot H. Reactionary dentinogenesis. Int J Dev Biol. 1995;39(1):273-80.

77. Goldberg M, Smith AJ. CELLS AND EXTRACELLULAR MATRICES OF DENTIN AND PULP: A BIOLOGICAL BASIS FOR REPAIR AND TISSUE ENGINEERING. Crit Rev Oral Biol Med. 2004;15(1):13-27.

78. Smith AJ, Lesot H. Induction and regulation of crown dentinogenesis: embryonic events as a template for dental tissue repair? Crit Rev Oral Biol Med. 2001;12(5):425-37.

79. Baker SM, Sugars RV, Wendel M, Smith AJ, Waddington RJ, Cooper PR, et al. TGF-beta/extracellular matrix interactions in dentin matrix: a role in regulating sequestration and protection of bioactivity. Calcif Tissue Int. 2009;85(1):66-74.

80. Sloan AJ, Perry H, Matthews JB, Smith AJ. Transforming growth factor-beta isoform expression in mature human healthy and carious molar teeth. Histochem J. 2000;32(4):247-52.

81. Sloan AJ, Moseley R, Dobie K, Waddington RJ, Smith AJ. TGF-beta latency-associated peptides (LAPs) in human dentin matrix and pulp. Connect Tissue Res. 2002;43(2-3):381-6.

82. Sloan AJ, Smith AJ. Stimulation of the dentine-pulp complex of rat incisor teeth by transforming growth factor-beta isoforms 1-3 in vitro. Arch Oral Biol. 1999;44(2):149-56.

83. Smith AJ, Tobias RS, Plant CG, Browne RM, Lesot H, Ruch JV. In vivo morphogenetic activity of dentine matrix proteins. J Biol Buccale. 1990;18(2):123-9.

84. Tziafas D, Alvanou A, Papadimitriou S, Gasic J, Komnenou A. Effects of recombinant basic fibroblast growth factor, insulin-like growth factor-II and transforming growth factor-beta 1 on dog dental pulp cells in vivo. Arch Oral Biol. 1998;43(6):431-44.

85. Huang XQ, Camba J, Gu LS, Bergeron BE, Ricucci D, Pashley DH, et al. Mechanism of bioactive molecular extraction from mineralized dentin by calcium hydroxide and tricalcium silicate cement. Dent Mater. 2018;34(2):317-30.

86. Sadaghiani L, Gleeson HB, Youde S, Waddington RJ, Lynch CD, Sloan AJ. Growth Factor Liberation and DPSC Response Following Dentine Conditioning. J Dent Res. 2016;95(11):1298-307.

87. Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. 2010;5(6):463-6.

88. Brizuela C, Meza G, Mercadé M, Inostroza C, Chaparro A, Bravo I, et al. Inflammatory biomarkers in dentinal fluid as an approach to molecular diagnostics in pulpitis. Int Endod J. 2020;53(9):1181-91.

89. Ching HS, Luddin N, Rahman IA, Ponnuraj KT. Expression of Odontogenic and Osteogenic Markers in DPSCs and SHED: A Review. Curr Stem Cell Res Ther. 2017;12(1):71-9.

90. Sharma U, Pal D, Prasad R. Alkaline phosphatase: an overview. Indian J Clin Biochem. 2014;29(3):269-78.

91. Vimalraj S. Alkaline phosphatase: Structure, expression and its function in bone mineralization. Gene. 2020;754:144855.

92. Emilia E, Neelakantan P. Biomarkers in the dentin-pulp complex: role in health and disease. J Clin Pediatr Dent. 2015;39(2):94-9.

93. Okabe T, Sakamoto M, Takeuchi H, Matsushima K. Effects of pH on mineralization ability of human dental pulp cells. J Endod. 2006;32(3):198-201.

94. Chen S, Rani S, Wu Y, Unterbrink A, Gu TT, Gluhak-Heinrich J, et al. Differential regulation of dentin sialophosphoprotein expression by Runx2 during odontoblast cytodifferentiation. J Biol Chem. 2005;280(33):29717-27.

95. Lim D, Wu KC, Lee A, Saunders TL, Ritchie HH. DSPP dosage affects tooth development and dentin mineralization. PLoS One. 2021;16(5):e0250429.

96. George A, Bannon L, Sabsay B, Dillon JW, Malone J, Veis A, et al. The carboxyl-terminal domain of phosphophoryn contains unique extended triplet amino acid repeat sequences forming ordered carboxyl-phosphate interaction ridges that may be essential in the biomineralization process. J Biol Chem. 1996;271(51):32869-73.

97. D'Souza RN, Cavender A, Sunavala G, Alvarez J, Ohshima T, Kulkarni AB, et al. Gene expression patterns of murine dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions in vivo. J Bone Miner Res. 1997;12(12):2040-9.

98. Xiao S, Yu C, Chou X, Yuan W, Wang Y, Bu L, et al. Dentinogenesis imperfecta 1 with or without progressive hearing loss is associated with distinct mutations in DSPP. Nat Genet. 2001;27(2):201-4.

99. Zhang X, Zhao J, Li C, Gao S, Qiu C, Liu P, et al. DSPP mutation in dentinogenesis imperfecta Shields type II. Nat Genet. 2001;27(2):151-2.

100. Karsenty G. The genetic transformation of bone biology. Genes Dev. 1999;13(23):3037-51.

101. Chen S, Gluhak-Heinrich J, Wang YH, Wu YM, Chuang HH, Chen L, et al. Runx2, osx, and dspp in tooth development. J Dent Res. 2009;88(10):904-9.

102. Yamashiro T, Aberg T, Levanon D, Groner Y, Thesleff I. Expression of Runx1, -2 and -3 during tooth, palate and craniofacial bone development. Mech Dev. 2002;119 Suppl 1:S107-10.

103. Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res. 2010;339(1):189-95.

104. D'Souza RN, Aberg T, Gaikwad J, Cavender A, Owen M, Karsenty G, et al. Cbfa1 is required for epithelial-mesenchymal interactions regulating tooth development in mice. Development. 1999;126(13):2911-20.

105. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell. 1997;89(5):755-64.

106. Dammaschke T, Leidinger J, Schäfer E. Long-term evaluation of direct pulp capping--treatment outcomes over an average period of 6.1 years. Clin Oral Investig. 2010;14(5):559-67.

107. Cohenca N, Paranjpe A, Berg J. Vital pulp therapy. Dent Clin North Am. 2013;57(1):59-73.

108. Cox CF, Keall CL, Keall HJ, Ostro E, Bergenholtz G. Biocompatibility of surface-sealed dental materials against exposed pulps. J Prosthet Dent. 1987;57(1):1-8.

109. Yong D, Cathro P. Conservative pulp therapy in the management of reversible and irreversible pulpitis. Aust Dent J. 2021;66 Suppl 1:S4-s14.

110. Al-Hiyasat AS, Barrieshi-Nusair KM, Al-Omari MA. The radiographic outcomes of direct pulp-capping procedures performed by dental students: a retrospective study. J Am Dent Assoc. 2006;137(12):1699-705.

111. Schwendicke F, Stolpe M. Direct pulp capping after a carious exposure versus root canal treatment: a cost-effectiveness analysis. J Endod. 2014;40(11):1764-70.

112. Calişkan MK. Pulpotomy of carious vital teeth with periapical involvement. Int Endod J. 1995;28(3):172-6.

113. Matsuo T, Nakanishi T, Shimizu H, Ebisu S. A clinical study of direct pulp capping applied to carious-exposed pulps. J Endod. 1996;22(10):551-6.

114. Mejàre I, Cvek M. Partial pulpotomy in young permanent teeth with deep carious lesions. Endod Dent Traumatol. 1993;9(6):238-42.

115. Lin LM, Ricucci D, Saoud TM, Sigurdsson A, Kahler B. Vital pulp therapy of mature permanent teeth with irreversible pulpitis from the perspective of pulp biology. Aust Endod J. 2020;46(1):154-66.

116. Ghoddusi J, Forghani M, Parisay I. New approaches in vital pulp therapy in permanent teeth. Iran Endod J. 2014;9(1):15-22.

117. Schwendicke F, Dörfer CE, Paris S. Incomplete caries removal: a systematic review and meta-analysis. J Dent Res. 2013;92(4):306-14.

118. Gruythuysen RJ, van Strijp AJ, Wu MK. Long-term survival of indirect pulp treatment performed in primary and permanent teeth with clinically diagnosed deep carious lesions. J Endod. 2010;36(9):1490-3.

119. Duncan HF, Galler KM, Tomson PL, Simon S, El-Karim I, Kundzina R, et al. European Society of Endodontology position statement: Management of deep caries and the exposed pulp. Int Endod J. 2019;52(7):923-34.

120. Aguilar P, Linsuwanont P. Vital pulp therapy in vital permanent teeth with cariously exposed pulp: a systematic review. J Endod. 2011;37(5):581-7.

121. AAE AAoE. Glossary of endodontic terms. 10th ed. Chicago, IL: American Association of Endodontists; 2020.

122. Simon S, Perard M, Zanini M, Smith AJ, Charpentier E, Djole SX, et al. Should pulp chamber pulpotomy be seen as a permanent treatment? Some preliminary thoughts. Int Endod J. 2013;46(1):79-87.

123. Cvek M. A clinical report on partial pulpotomy and capping with calcium hydroxide in permanent incisors with complicated crown fracture. J Endod. 1978;4(8):232-7.

124. Kiatwateeratana T, Kintarak S, Piwat S, Chankanka O, Kamaolmatyakul S, Thearmontree A. Partial pulpotomy on caries-free teeth using enamel matrix derivative or calcium hydroxide: a randomized controlled trial. Int Endod J. 2009;42(7):584-92.

125. Nosrat IV, Nosrat CA. Reparative hard tissue formation following calcium hydroxide application after partial pulpotomy in cariously exposed pulps of permanent teeth. Int Endod J. 1998;31(3):221-6.

126. Asgary S, Eghbal MJ, Fazlyab M, Baghban AA, Ghoddusi J. Five-year results of vital pulp therapy in permanent molars with irreversible pulpitis: a non-inferiority multicenter randomized clinical trial. Clin Oral Investig. 2015;19(2):335-41.

127. Eren B, Onay EO, Ungor M. Assessment of alternative emergency treatments for symptomatic irreversible pulpitis: a randomized clinical trial. Int Endod J. 2018;51 Suppl 3:e227-e37.

128. Galani M, Tewari S, Sangwan P, Mittal S, Kumar V, Duhan J. Comparative Evaluation of Postoperative Pain and Success Rate after Pulpotomy and Root Canal Treatment in Cariously Exposed Mature Permanent Molars: A Randomized Controlled Trial. J Endod. 2017;43(12):1953-62.

129. Ferracane JL, Cooper PR, Smith AJ. Dentin matrix component solubilization by solutions of pH relevant to self-etching dental adhesives. J Adhes Dent. 2013;15(5):407-12.

130. Holland R, Filho JA, de Souza V, Nery MJ, Bernabé PF, Junior ED. Mineral trioxide aggregate repair of lateral root perforations. J Endod. 2001;27(4):281-4.

131. Schröder U. Effects of calcium hydroxide-containing pulp-capping agents on pulp cell migration, proliferation, and differentiation. J Dent Res. 1985;64 Spec No:541-8.

132. Hilton TJ. Keys to clinical success with pulp capping: a review of the literature. Oper Dent. 2009;34(5):615-25.

133. Lan WH, Lan WC, Wang TM, Lee YL, Tseng WY, Lin CP, et al. Cytotoxicity of conventional and modified glass ionomer cements. Oper Dent. 2003;28(3):251-9.

134. Andelin WE, Shabahang S, Wright K, Torabinejad M. Identification of hard tissue after experimental pulp capping using dentin sialoprotein (DSP) as a marker. J Endod. 2003;29(10):646-50.

135. Sawyer AN, Nikonov SY, Pancio AK, Niu LN, Agee KA, Loushine RJ, et al. Effects of calcium silicate-based materials on the flexural properties of dentin. J Endod. 2012;38(5):680-3.

136. Tran XV, Gorin C, Willig C, Baroukh B, Pellat B, Decup F, et al. Effect of a calcium-silicate-based restorative cement on pulp repair. J Dent Res. 2012;91(12):1166-71.

137. Yassen GH, Platt JA. The effect of nonsetting calcium hydroxide on root fracture and mechanical properties of radicular dentine: a systematic review. Int Endod J. 2013;46(2):112-8.

138. Aeinehchi M, Eslami B, Ghanbariha M, Saffar AS. Mineral trioxide aggregate (MTA) and calcium hydroxide as pulp-capping agents in human teeth: a preliminary report. Int Endod J. 2003;36(3):225-31.

139. Mente J, Hufnagel S, Leo M, Michel A, Gehrig H, Panagidis D, et al. Treatment outcome of mineral trioxide aggregate or calcium hydroxide direct pulp capping: long-term results. J Endod. 2014;40(11):1746-51.

140. Nair PN, Duncan HF, Pitt Ford TR, Luder HU. Histological, ultrastructural and quantitative investigations on the response of healthy human pulps to experimental capping with mineral trioxide aggregate: a randomized controlled trial. Int Endod J. 2008;41(2):128-50.

141. Ford TR, Torabinejad M, Abedi HR, Bakland LK, Kariyawasam SP. Using mineral trioxide aggregate as a pulp-capping material. J Am Dent Assoc. 1996;127(10):1491-4.

142. Ford TR, Torabinejad M, McKendry DJ, Hong CU, Kariyawasam SP. Use of mineral trioxide aggregate for repair of furcal perforations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1995;79(6):756-63.

143. Barbosa Silva MJ, Vieira LQ, Sobrinho AP. The effects of mineral trioxide aggregates on cytokine production by mouse pulp tissue. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008;105(5):e70-6.

144. Rezende TM, Vieira LQ, Sobrinho AP, Oliveira RR, Taubman MA, Kawai T. The influence of mineral trioxide aggregate on adaptive immune responses to endodontic pathogens in mice. J Endod. 2008;34(9):1066-71.

145. Paranjpe A, Zhang H, Johnson JD. Effects of mineral trioxide aggregate on human dental pulp cells after pulp-capping procedures. J Endod. 2010;36(6):1042-7.

146. Marciano MA, Costa RM, Camilleri J, Mondelli RF, Guimarães BM, Duarte MA. Assessment of color stability of white mineral trioxide aggregate angelus and bismuth oxide in contact with tooth structure. J Endod. 2014;40(8):1235-40.

147. Parirokh M, Torabinejad M. Mineral trioxide aggregate: a comprehensive literature review--Part I: chemical, physical, and antibacterial properties. J Endod. 2010;36(1):16-27.

148. Nowicka A, Lipski M, Parafiniuk M, Sporniak-Tutak K, Lichota D, Kosierkiewicz A, et al. Response of human dental pulp capped with biodentine and mineral trioxide aggregate. J Endod. 2013;39(6):743-7.

149. Cao Y, Xiong J, Mei S, Wang F, Zhao Z, Wang S, et al. Aspirin promotes bone marrow mesenchymal stem cell-based calvarial bone regeneration in mini swine. Stem Cell Res Ther. 2015;6:210.

150. Chen ZW, Wu ZX, Sang HX, Qin GL, Wang LS, Feng J, et al. [Effect of aspirin administration for the treatment of osteoporosis in ovariectomized rat model]. Zhonghua Yi Xue Za Zhi. 2011;91(13):925-9.

151. Sloan AJ, Smith AJ. Stem cells and the dental pulp: potential roles in dentine regeneration and repair. Oral Dis. 2007;13(2):151-7.

152. Hilton TJ, Ferracane JL, Mancl L. Comparison of CaOH with MTA for direct pulp capping: a PBRN randomized clinical trial. J Dent Res. 2013;92(7 Suppl):16s-22s.

153. Kundzina R, Stangvaltaite L, Eriksen HM, Kerosuo E. Capping carious exposures in adults: a randomized controlled trial investigating mineral trioxide aggregate versus calcium hydroxide. Int Endod J. 2017;50(10):924-32.

154. Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, et al. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell Tissue Res. 2013;353(1):65-78.

155. Raoof M, Yaghoobi MM, Derakhshani A, Kamal-Abadi AM, Ebrahimi B, Abbasnejad M, et al. A modified efficient method for dental pulp stem cell isolation. Dent Res J (Isfahan). 2014;11(2):244-50.

156. Alkhattab O. Cytotoxicity and genotoxicity induced by silica nanoparticles in human dental pulp cell culture [dissertation]. Boston (MA): Boston University; 2020.

157. Alsenan J. Silicon, calcium and phosphate effect on human dental pulp cell cultures [dissertation] Boston (MA): Boston University; 2019.

158. Alwafi H. Effect of thymoquinone on odontogensis of human dental pulp cells [dissertation] Boston (MA): Boston University; 2017.

159. Yuan M, Zhan Y, Hu W, Li Y, Xie X, Miao N, et al. Aspirin promotes osteogenic differentiation of human dental pulp stem cells. Int J Mol Med. 2018;42(4):1967-76.

160. Koyama N, Okubo Y, Nakao K, Bessho K. Evaluation of pluripotency in human dental pulp cells. J Oral Maxillofac Surg. 2009;67(3):501-6.

161. Ekwall B, Clemedson C, Crafoord B, Ekwall B, Hallander S, Walum E, et al. MEIC Evaluation of Acute Systemic Toxicity: Part V. Rodent and Human Toxicity Data for the 50 Reference Chemicals. Altern Lab Anim. 1998;26 Suppl 2:571-616.

162. Borthwick GM, Johnson AS, Partington M, Burn J, Wilson R, Arthur HM. Therapeutic levels of aspirin and salicylate directly inhibit a model of angiogenesis through a Cox-independent mechanism. Faseb j. 2006;20(12):2009-16.

163. Occhipinti R, Boron WF. Mathematical modeling of acid-base physiology. Prog Biophys Mol Biol. 2015;117(1):43-58.

164. Kim N, Minami N, Yamada M, Imai H. Immobilized pH in culture reveals an optimal condition for somatic cell reprogramming and differentiation of pluripotent stem cells. Reprod Med Biol. 2017;16(1):58-66.

165. Kaysinger KK, Ramp WK. Extracellular pH modulates the activity of cultured human osteoblasts. J Cell Biochem. 1998;68(1):83-9.

166. Müller M, Raabe O, Addicks K, Wenisch S, Arnhold S. Effects of non-steroidal anti-inflammatory drugs on proliferation, differentiation and migration in equine mesenchymal stem cells. Cell Biol Int. 2011;35(3):235-48.

167. Clemett D, Goa KL. Celecoxib: a review of its use in osteoarthritis, rheumatoid arthritis and acute pain. Drugs. 2000;59(4):957-80.

168. Depré M, Ehrich E, Van Hecken A, De Lepeleire I, Dallob A, Wong P, et al. Pharmacokinetics, COX-2 specificity, and tolerability of supratherapeutic doses of rofecoxib in humans. Eur J Clin Pharmacol. 2000;56(2):167-74.

169. Beretta C, Garavaglia G, Cavalli M. COX-1 and COX-2 inhibition in horse blood by phenylbutazone, flunixin, carprofen and meloxicam: an in vitro analysis. Pharmacol Res. 2005;52(4):302-6.

170. Du M, Pan W, Duan X, Yang P, Ge S. Lower dosage of aspirin promotes cell growth and osteogenic differentiation in murine bone marrow stromal cells. J Dent Sci. 2016;11(3):315-22.

171. Reeve L, Baldrick P. Biocompatibility assessments for medical devices - evolving regulatory considerations. Expert Rev Med Devices. 2017;14(2):161-7.

172. Feoktistova M, Geserick P, Leverkus M. Crystal Violet Assay for Determining Viability of Cultured Cells. Cold Spring Harb Protoc. 2016;2016(4):pdb.prot087379.

173. Väkevä L, Mackie E, Kantomaa T, Thesleff I. Comparison of the distribution patterns of tenascin and alkaline phosphatase in developing teeth, cartilage, and bone of rats and mice. The Anatomical Record. 1990;228(1):69-76.

174. Gaikwad JS, Hoffmann M, Cavender A, Bronckers AL, D'Souza RN. Molecular insights into the lineage-specific determination of odontoblasts: the role of Cbfa1. Adv Dent Res. 2001;15:19-24.

175. Sonoda S, Mei Y-f, Atsuta I, Danjo A, Yamaza H, Hama S, et al. Exogenous nitric oxide stimulates the odontogenic differentiation of rat dental pulp stem cells. Scientific Reports. 2018;8(1):3419.

176. Widbiller M, Lindner SR, Buchalla W, Eidt A, Hiller KA, Schmalz G, et al. Three-dimensional culture of dental pulp stem cells in direct contact to tricalcium silicate cements. Clin Oral Investig. 2016;20(2):237-46.

177. Chaudhary SC, Kuzynski M, Bottini M, Beniash E, Dokland T, Mobley CG, et al. Phosphate induces formation of matrix vesicles during odontoblast-initiated mineralization in vitro. Matrix Biol. 2016;52-54:284-300.

178. Liu J, Taocong J, Ritchie HH, Smith AJ, Brian HC. In vitro Differentiation and Mineralization of Human Dental Pulp Cells Induced by Dentin Extract. In Vitro Cellular & Developmental Biology Animal. 2005;41(7):232-8.

179. Phung S, Lee C, Hong C, Song M, Yi JK, Stevenson RG, et al. Effects of Bioactive Compounds on Odontogenic Differentiation and Mineralization. J Dent Res. 2017;96(1):107-15.

180. Hoare DG, Koshland DE. A Procedure for the Selective Modification of Carboxyl Groups in Proteins. Journal of the American Chemical Society. 1966;88(9):2057-8.

181. Sawyer L, James MNG. Carboxyl–carboxylate interactions in proteins. Nature. 1982;295(5844):79-80.

182. Bala T, Prasad BL, Sastry M, Kahaly MU, Waghmare UV. Interaction of different metal ions with carboxylic acid group: a quantitative study. J Phys Chem A. 2007;111(28):6183-90.

183. Winter WE, Greene DN, Beal SG, Isom JA, Manning H, Wilkerson G, et al. Clotting factors: Clinical biochemistry and their roles as plasma enzymes. Adv Clin Chem. 2020;94:31-84.

184. Chen C, Akiyama K, Yamaza T, You YO, Xu X, Li B, et al. Telomerase governs immunomodulatory properties of mesenchymal stem cells by regulating FAS ligand expression. EMBO Mol Med. 2014;6(3):322-34.

185. Liu Y, Wang L, Kikuiri T, Akiyama K, Chen C, Xu X, et al. Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-γ and TNF-α. Nat Med. 2011;17(12):1594-601.

186. Cassidy N, Fahey M, Prime SS, Smith AJ. Comparative analysis of transforming growth factor-beta isoforms 1-3 in human and rabbit dentine matrices. Arch Oral Biol. 1997;42(3):219-23.

187. Finkelman RD, Mohan S, Jennings JC, Taylor AK, Jepsen S, Baylink DJ. Quantitation of growth factors IGF-I, SGF/IGF-II, and TGF-beta in human dentin. J Bone Miner Res. 1990;5(7):717-23.

188. Smith AJ, Duncan HF, Diogenes A, Simon S, Cooper PR. Exploiting the Bioactive Properties of the Dentin-Pulp Complex in Regenerative Endodontics. J Endod. 2016;42(1):47-56.

189. Widbiller M, Eidt A, Lindner SR, Hiller KA, Schweikl H, Buchalla W, et al. Dentine matrix proteins: isolation and effects on human pulp cells. Int Endod J. 2018;51 Suppl 4:e278-e90.

190. Galler KM, Buchalla W, Hiller KA, Federlin M, Eidt A, Schiefersteiner M, et al. Influence of root canal disinfectants on growth factor release from dentin. J Endod. 2015;41(3):363-8.

191. Smith EL, Colombo JS, Sloan AJ, Waddington RJ. TGF-beta1 exposure from bone surfaces by chemical treatment modalities. Eur Cell Mater. 2011;21:193-201.

CURRICULUM VITAE