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The Effect of Curcumin and Bromelain on Osteogenesis of Gingiva Derived Stem Cells

Selin Avman, D.D.S

A Thesis Presented to the Faculty of the College of Dental Medicine of Nova Southeastern University in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

October 2018

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The Effect of Curcumin and Bromelain on Osteogenesis of Gingiva Derived Stem Cells

By

Selin Avman, D.D.S

A thesis submitted to the College of Dental Medicine of Nova Southeastern University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Pediatric Dentistry

College of Dental Medicine

Nova Southeastern University

October 2018

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DATE SUBMITTED: October 17, 2018
I certify that I am the sole author of this thesis, and that any assistance I received in its preparation has been fully acknowledged and disclosed in the thesis. I have cited any sources from which I used ideas, data, or words, and labeled as quotations any directly quoted phrases or passages, as well as providing proper documentation and citations. This thesis was prepared by me, specifically for the M.S. degree and for this assignment.

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Selin Avman, D.D.S Date

DEDICATION

"Education, actual learning--it is hard work. It's very personal. Your parents don't teach you anything. Your teachers don't teach you anything. The government doesn't teach you anything. You read it. You don't understand it; you read it again. You break a pencil and read it again."

--Dean Kamen

This body of work is dedicated to those individuals who may have taken a little longer to get there, but eventually did!

ACKNOWLEDGMENTS

First and foremost, it is with her love and dedication to the study of science that has allowed me to complete this thesis. I could not thank my mentor, Dr. Umadevi Kandalam, for the countless hours she and her staff have spent conducting the studies and analyzing data in hopes that the results will contribute to clinical application.

I would like to thank Christine Manguno for her patience while teaching me as well as all of the time she has spent conducting the experiments and reviewing the results to allow me complete this paper.

I would like to thank Shaileen Dokhte Kavir for taking the time away from her studies of dental medicine to help conduct my experiments.

I would also like to thank my committee members Dr. Ocanto and Dr. Molina for their insight.

ABSTRACT

The Effect of Curcumin and Bromelain on Osteogenesis of Gingiva Derived Stem Cells

October 2018

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Background: Mesenchymal stem cells (MSCs) are attractive cell sources for tissue engineering application because of their ability to proliferate and differentiate into mesenchymal tissues. MSCs derived from human gingiva are readily accessible and highly proliferative with the ability to differentiate into an osteogenic lineage, making them ideal sources for tissue regeneration of craniofacial defects. While dexamethasone is a traditional inducer of osteogenic differentiation, studies have shown that its prolonged presence in culture medium may have toxic effects on osteoblasts. Many antioxidants play a vital role in promoting osteogenic differentiation and offer a potential alternative to dexamethasone. It has been demonstrated that curcumin can promote osteogenesis of rat derived bone marrow mesenchymal cells, suggesting that curcumin can be used in the treatment of bone lesions. Previous studies reported that bromelain treatment relieved osteoarthritis, indicating that bromelain may be able to promote bone health, which gives a cue that bromelain can induce osteogenic differentiation in MSCs. **Objective:** The aim of this study was to investigate the effects of curcumin and

bromelain on osteogenic differentiation of human gingiva derived mesenchymal stem cells (HGMSCs) and to compare their differentiation potential to dexamethasone. **Methodology:** Stem cells were isolated from human gingival tissue samples. Surface markers were detected using flow cytometry. Guided osteogenic differentiation assay was conducted to confirm mineralization. The effects of curcumin and bromelain on HGMSCs proliferation on day1, 3 and 5 was examined using a MTT assay. Cells were treated with various concentrations of curcumin (2, 5 and 10µM) and bromelain (1, 2.5 and 5µg/ml) for two weeks and gene expression was investigated using quantitative PCR. **Results:** Our findings demonstrated that curcumin and bromelain induced osteogenic differentiation in a dose dependent manner. At 2.5µg/ml the peak up-regulation could be seen for genes Collagen, ALP, and OPG for bromelain treated cells. In curcumin the ideal concentration found was to 2µM. The maximum enhancement has been observed at 2µM for all genes. **Conclusion:** Cells treated with curcumin and bromelain induced the osteogenic differentiation, however, future *in vivo* studies need to be conducted to confirm findings.

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LIST OF ABBREVIATIONS

HGMSCs	Human Gingiva Derived Mesenchymal Stem Cells
CM	Complete Medium
OM	Osteogenic Medium
DEX	Dexamethasone
ALP	Alkaline Phosphatase
OPN	Osteopontin
OPG	Osteoprotegerin
ONN	Osteonectin
OC	Osteocalcin
Cbfa1	Core-binding factor alpha 1
RunX2	Runt-related transcription factor 2
BMP	Bone Morphogenic Protein
MSC	Mesenchymal Stem Cell
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
DMSO	Dimethyl Sulfoxide
CO_2	Carbon Dioxide
O_2	Oxygen

CHAPTER 1. INTRODUCTION

1.1 Craniofacial Birth Defects

Craniofacial birth defects are among the most commonly occurring congenital anomalies found in newborns. Cleft lip with or without cleft palate is the most common craniofacial abnormality and the fourth most common congenital birth defect.[1] Orofacial clefts are characterized by the deficiency and displacement of soft tissues and underlying cartilaginous and bony structures of the nasal sill, lip, and soft and hard palate.[2] The cleft can vary in severity from a small notch in the vermillion border of the lip to a cleft that extends through the alveolar ridge of the maxilla to involve the floor of the nose or palate. Clefts of the lip result when the medial and lateral nasal processes, the frontonasal prominence, and the maxillary prominence fail to unite. A unilateral cleft will result when the maxillary prominence on the affected side fails to merge with the nasal prominence. If the tissues fail to unite on both sides, a bilateral cleft lip will form. Cleft palate results with failure of the fusion of the palatal shelves with the nasal septum posteriorly.[3,4]

1.2 Intramembranous Ossification

During the eighth week of normal development, after fusion of the palatal shelves occurs, intramembranous ossification of the palate begins. Ossification will extend from the posterior part of the palate anteriorly, leaving the most posterior portion to become the region of the soft palate.[5] During intramembranous ossification, neural crest derived mesencymal cells will proliferate and condense into compact nodules. Some of the mesencymal cells will differentiate into osteoblasts, bone precursor cells. These cells

will secrete osteoid, a collagen-proteoglycan matrix, that binds to calcium salts to become calcifed. As the calcification process continues, spicules of bone will radiate from the center of ossification and will become surrounded by compact mesenchymal cells that will form the periosteum (membrane surrounding the bone). The mesenchymal cells lining the periosteum will differentiate into osteoblasts that will deposit osteoid matrix parallel to the existing spicules, creating one of the many layers of bone.[6]

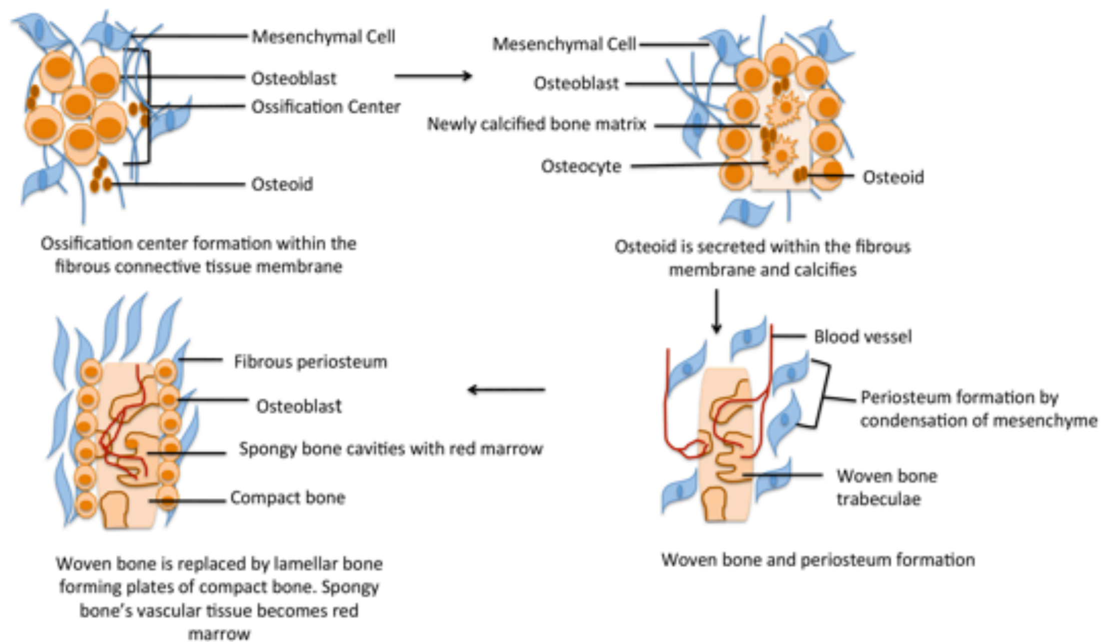


Figure 1.1 Steps of Intramembranous Ossification

1.3 Proteins and Genes Involved in Osteogenesis

Bone tissue is composed of cells (osteoblasts and osteoclasts) and a matrix. Ninety percent of the bone matrix contains type I collagen with the other ten percent composing a large number of noncollagenous proteins such as osteocalcin, osteonectin, bone sialoproteins, and proteoglycans. These non-collagenous proteins participate in matrix

maturation, mineralization and perhaps the regulation of the activity of bone cells. Hormones, growth factors, mechanical stimuli, and cell to cell and cell to matrix interactions, act by signaling pathways to activate transcription factors that effect the expression of genes, thereby regulating osteoblast differentiation and commitment.[7]

Intramembranous ossification involves bone morphogenic proteins and the activation of transcription factor Cbfa1 (RunX2). It is believed that bone morphogenic proteins activate the Cbfa1 gene in neural crest derived mesenchymal cells to transform them into osteoblasts (Figure 1.2). [6,8]

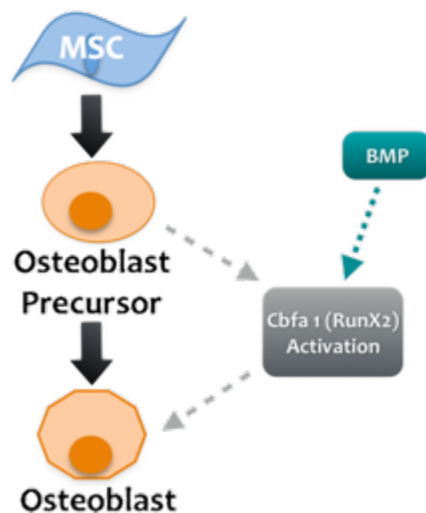


Figure 1.2 Influence of BMP and RunX2 on Osteoblast Differentiation

Studies have found that the mRNA for Cbfa1 in mice is restricted to the mesenchyme that forms bone and is limited to the lineage of osteoblasts. This protein activates genes for osteopontin, osteocalcin, bone sialoprotein, alkaline phosphatase, collagen type I and other extracellular matrix proteins that are bone specific (Figure 1.3).[6,8]

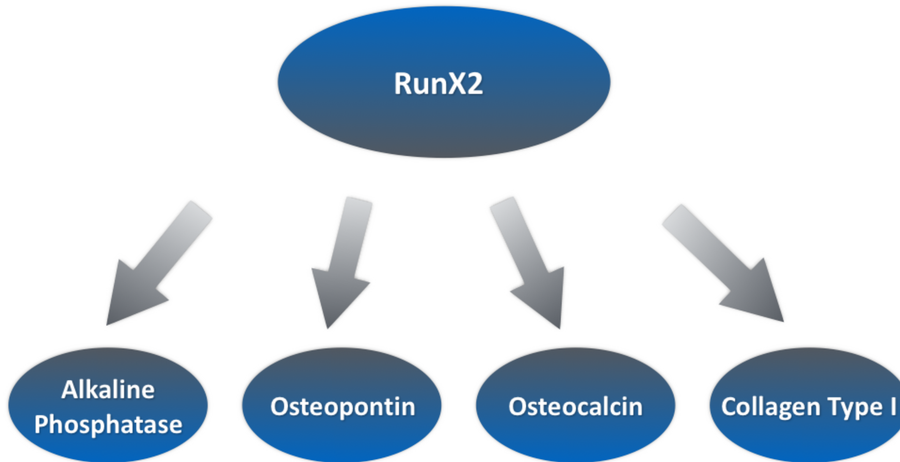


Figure 1.3 Gene Activation by RunX2

1.4 Phenotypic Characteristics During Osteogenic Differentiation of Mesenchymal Stem Cells

Osteoblasts (bone forming cells) are differentiated from precursor cells present within the bone marrow stroma (non-hematopoietic compartment of bone marrow). These fibroblast-like stem cells with osteogenic differentiation potential are referred to as mesenchymal stem cells.[7] Mesenchymal stem cells (MSCs) are multipotent* adult stem cells with the capability of differentiating into various tissue types, including bone. During osteogenic differentiation of MSCs, there are many changes in gene expression, most importantly the up-regulation of osteopontin, osteocalcin, alkaline phosphatase, collagen type I and core binding factor α1. When expressed, these markers are indicative of osteogenic differentiation with their level of expression being equivocal to the stage of differentiation.[9]

---

* Multipotent Stem Cell- Cells that can differentiate into a number of cells from a germ layer.[9]

There are three distinct phases of osteogenic differentiation: Proliferation, Extracellular matrix maturation and Matrix mineralization.[9] During days one to four, the proliferative stage takes place, where the MSCs are highly mitotic creating a peak in the number of cells.[9,10] At this phase, there is a high expression of H4 histone and c-fos as well as a peak level of genes associated with extracellular matrix production, such as collagen type I, fibronectin and transforming growth factor β.[9] This stage is followed by early cell differentiation, days 5-14, where alkaline phosphatase activity will be at maximal level. Also found at this stage is the expression of collagen type I matrix, onto which the mineral is deposited. With the final stage, days 14- 28, there is an increased expression of osteocalcin and osteopontin, followed by the deposition of phosphate and calcium.[10]

Alkaline phosphatase expression is low as osteoprogenitor cells undergo proliferation. Its activity rises during differentiation and maturation, followed by a decline and absence as osteoblasts turn into osteocytes. Alkaline phosphatase activity generally takes place prior to osteocalcin, implying that it is an early osteoblast marker, while osteocalcin indicates a more mature phenotype as seen with osteocytes. Their expression has been used as identifying markers for osteoblasts. Another marker for osteogenic differentiation is osteopontin, which is expressed during the early stages of osteogenesis prior to mineralization or osteocalcin expression (Figure 1.4). Therefore, this marker is an identifier of osteoprogenitor cells.[10]

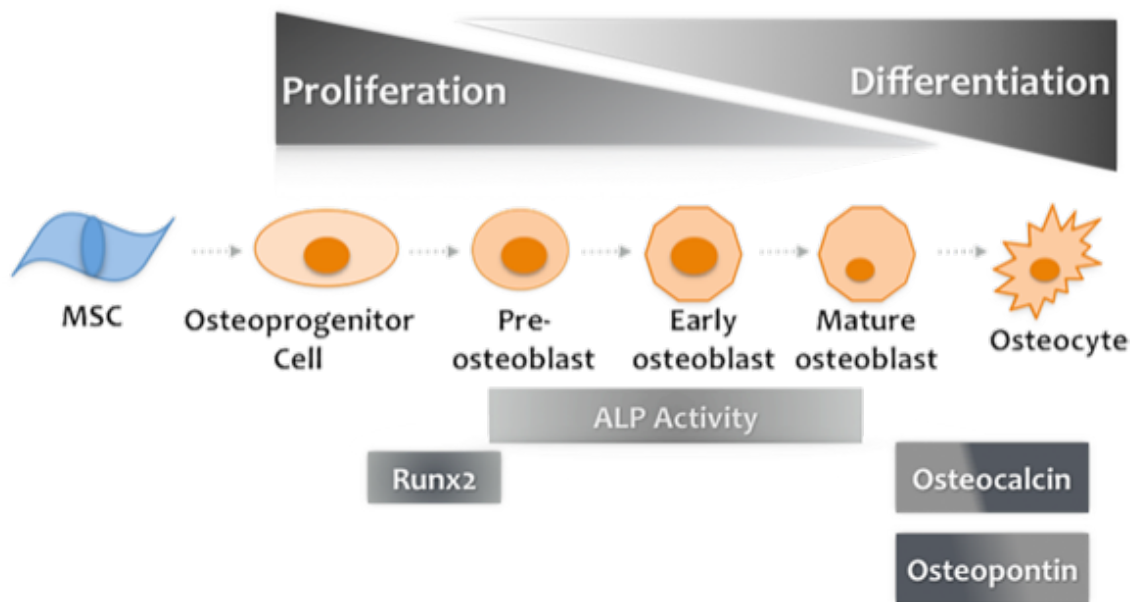


Figure 1.4 Phenotypic Characteristics During Osteogenic Differentiation of Mesenchymal Stem Cells

1.5 Therapeutic Application of Mesenchymal Stem Cells

Mesenchymal stem cells have become an important field of study for tissue regeneration and regenerative medicine.[11] They have illustrated the ability to differentiate into several types of cells including, osteocytes, chondrocytes, neurons, and cardiomyoctes, making them a promising resource for regenerative cell therapy for various diseases.[12] Although most mesenchymal stem cells are derived from bone marrow, a study by Tomar et al. has demonstrated that mesenchymal stem cells derived from human gingiva are far more superior. These cells are easy to isolate, are uniformly homogenous, proliferate faster, are not tumorigenic, display stable phenotype, and maintain normal karyotype and telomerase activity in long term cultures, making them ideal sources for cell therapy in regenerative medicine.[13]

1.6 Osteogenic Differentiation Role of Dexamethasone

Several *in vitro* studies have been conducted utilizing a mixture of dexamethasone, calcitriol, ascorbic acid, and β-glycerophosphate to demonstrate the differentiation of bone marrow derived mesenchymal cells into osteoblasts. This differentiation has been confirmed by illustrating the induction of osteoblast specific gene expression and proteins.[7]

Dexamethasone supports osteogenic differentiation by binding to regulatory proteins and activating transcription of osteoblast specific genes within cells. Evidence has also shown that dexamethasone functions at multiple points of the osteoblastic differentiation process. *In vitro,* constant treatment with the corticosteroid would increase alkaline phosphatase activity, which is necessary for matrix mineralization and morphological changes of mesenchymal stem cells. Constant presence of dexamethasone in the culture medium is required to achieve maximal osteoblastic differentiation of mesenchymal cells, however, a prolonged presence in differentiation media would have toxic effects on osteoblasts and cause lysis of cells.[9]

1.7 Osteogenic Differentiation Role of Antioxidants

Studies have shown that antioxidants play a vital role in stem cell proliferation and differentiation.[14,15] Curcumin, a natural phenol derived from Curcuma longa (turmeric), has been shown to exhibit anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties. It can inhibit osteoclastogenesis, induce apoptosis in osteoclasts, and can directly interact with adiopocytes to suppress their differentiation.[16-18] A study

conducted by Gu et al., demonstrated that curcumin can promote osteogenic differentiation of rat derived bone marrow mesenchymal cells, suggesting that curcumin can be used in the treatment of bone defects.[19] Bromelain, a known antioxidant derived from pineapple extract, has also demonstrated anti-inflammatory and analgesic properties. Bromelain has also shown to have the ability to inhibit adipocyte differentiation by reducing adipogenic gene expression and to induce apoptosis and lipolysis in mature adipocytes. Furthermore, its wound healing ability and tissue regenerating capability indicate that bromelain may assist in the bone tissue repair process and promote tissue regeneration.[20-22]

1.8 Objective

The aim of this study was to investigate the effects of curcumin and bromelain on osteogenic differentiation of human gingiva derived mesenchymal stem cells (HGMSCs) and to compare their differentiation potential to dexamethasone.

1.9 Long Term Goal

Due to the limitation of bone grafting procedures on the treatment of bone defects, an alternative approach to treatment is necessary. The long-term goal of this study is to repair craniofacial defects using cell based tissue engineering techniques. Tissue engineering involves the implantation of a synthetic scaffold seeded with mesenchymal stem cells and molecular signals at the bone defect site. Harvesting cells from bone marrow is the classical approach to this technique, but is invasive. The need for a more available source of mesenchymal stem cells has lead to the investigation of other

tissues.[7] Human gingiva derived tissue is a readily accessible source of neural crest derived MSC and can be easily isolated making them an ideal source for cell therapy in regenerative medicine.[13] Furthermore, their low toxicity, anti-inflammatory property, wound healing ability, and capability to influence the differentiation of mesenchymal stem cells, indicate that antioxidants (bromelain and curcumin) may assist in bone tissue repair and promote tissue regeneration in individuals with craniofacial defects.[16-22]

1.10 Innovation

To the best of our knowledge, this was the first study to determine the effects of antioxidants (bromelain and curcumin) on the osteogenic differentiation of human gingiva derived mesenchymal stem cells (HGMSCs).

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), Penicillin/Streptomycin, Von Kossa Stain, Alizarin Red Stain, Ascorbic Acid, β-glycerophosphate, Dexamethasone, Dimethyl Sulfoxide (DMSO), Collagenase, Dispase, L-glutamine, Curcumin, and Bromelain were purchased from Sigma Aldrich (St. Louis, MO). Trypsin, Gene Expression Assay Mix and cDNA kit were purchased from Life Technology (Carlsbad, CA). MTT reagent and Alkaline Phosphate Assay kit was purchased from Sciencell (Carlsbad, CA). The Mesenchymal Stem Cell Phenotyping kit, Human was purchased from Miltenyi Biotec (Auburn, CA). Tissue culture flasks, micropipettes, tissue culture plates and other lab supplies were obtained from VWR International Company (Atlanta, GA).

2.2 Overall Study Design

Human gingival tissue samples from three healthy individuals were collected under the approved IRB protocol at NSU College of Dental Medicine. Stem cells were isolated and incubated under standard culture conditions for expansion until confluency was reached. Cells were then cryopreserved. Cryopreserved human gingival stem cells (HGMSCs) were all revived at the same time and maintained under standard expansion culture conditions until confluent. Mesenchymal surface markers were detected via Flow Cytometry. Cells were induced with osteogenic medium (DMEM, 10% FBS, 1% antibiotic/antimycotic solution, 10Mm β-glycerophosphate, and 50μg/mL ascorbic acid) supplemented with 100nM Dexamethasone and monitored at 1,2,3, and 4 week intervals

to determine their osteogenic potential. At day 21, cells were stained with Alizarin Red S and Von Kossa to examine the presence of mineralization. To test the effect of bromelain and curcumin on cell proliferation, cells were treated with either antioxidant at different concentrations and incubated for 1,3, and 5 days. Cells cultured in complete medium (DMEM, 10% FBS, 1% antibiotic/antimycotic solution) were used as control. At each time point, cells were analyzed for proliferation. For gene expression analysis, cells cultured in OM (DMEM, 10% FBS, antibiotic/antimycotic solution, β-glycerophosphate, and ascorbic acid) were designated as the control group. Experimental groups included cells cultured in OM + DEX as well as cells cultured in OM with their assigned concentration of either bromelain or curcumin as found in Table 2.2 After two weeks of treatment, cells were harvested and RNA was extracted using Trizol method. cDNA was then prepared via the reverse transcription method. Gene expression analysis of selected osteogenic markers (ALP, OPN, OPG, ONN, Collagen) was measured by PCR method to determine cell differentiation and osteogenesis (Figure 2.1).

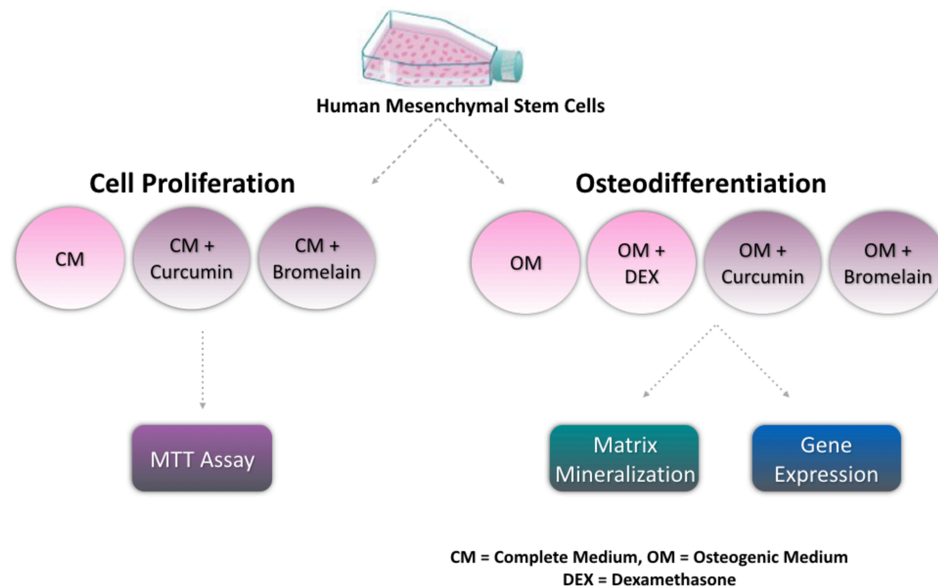


Figure 2.1 Overall Study Design

Table 2.1 Experimental Groups for Cell Proliferation

Group	Media	
Group 1 (Control)	Complete Medium (CM)	
Group 2	CM + 1μM Curcumin	CM + 1μg/ml Bromelain
Group 3	CM + 2.5μM Curcumin	CM + 2.5μg/ml Bromelain
Group 4	CM + 5μM Curcumin	CM + 5μg/ml Bromelain
Group 5	CM + 7.5μM Curcumin	CM + 7.5μg/ml Bromelain
Group 6	CM + 10μM Curcumin	CM + 10μg/ml Bromelain
Group 7	CM + 15μM Curcumin	CM + 15μg/ml Bromelain

Table 2.2 Experimental Groups for Gene Expression

Group	Media	
	Bromelain	Curcumin
Group 1 (Control)	Osteogenic Medium (OM)	Osteogenic Medium (OM) + DMSO (used as carrier)
Group 2	OM + 100nM Dexamethasone (DEX)	OM + 100nM Dexamethasone (DEX)
Group 3	OM + 1μg/ Bromelain	OM + 2μM Curcumin
Group 4	OM + 2.5μg/ml Bromelain	OM + 5μM Curcumin
Group 5	OM + 5μg/ml Bromelain	OM + 10μM Curcumin

2.3 Sample Size Determination

Cells from three donors were randomly selected for this study. For the study of cell proliferation, there was 1 control group and 12 experimental groups (6 Curcumin and 6 Bromelain). The control group consisted of cells cultured in a complete medium (DMEM, 10% FBS, 1% antibiotic/antimycotic solution) and the experimental group cells were cultured in a complete medium with an assigned antioxidant dose as found in Table 2.1. The cells were treated at day intervals of 1,3,5 and analyzed for cell viability and proliferation via the MTT Assay. The sample size was calculated as follows: 3

donors x 1 control group x 12 experimental groups (6 Curcumin and 6 Bromelain) x 3 treatment days = 108 samples.

For the study of gene expression, there were 2 control groups, 2 dexamethasone groups and 6 experimental groups (3 Bromelain and 3 Curcumin). The control groups consisted of cells cultured in an osteogenic medium (DMEM, 10% FBS, antibiotic/antimycotic solution, β-glycerophosphate, and ascorbic acid), the dexamethasone groups consisted of cells cultures in an osteogenic medium supplemented with 100nM dexamethasone and the experimental group cells were cultured in an osteogenic medium with an assigned antioxidant dose as found in Table 2.2. The cells were treated for two weeks and analyzed for osteogenic differentiation via PCR. The sample size was calculated as follows: 3 donors x 2 control groups x 2 dexamethasone groups x 6 experimental groups (3 Curcumin and 3 Bromelain) x 1 treatment day = 72 samples. Overall, sample size was 72, which includes three biological replicas and for each biological replica two technical replicas have been used.

2.4 Methodology

Under the approved Institutional Review Board protocol (#2018-241), samples of gingival tissue were obtained from discarded or remnant tissues from three random healthy individuals who were undergoing procedures at Nova Southeastern University College of Dental Medicine. Mesenchymal stem cells were isolated from these tissues using established methods from previous studies.[13,23] The tissues were de-epithelialized and washed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%

Fetal Bovine Serum (FBS), 400 mmol/ml L-glutamine, 100 1g/ml streptomycin, 1% amphotericin, and 100 U/ml penicillin. Tissues were then minced, placed in a medium containing 0.1% collagenase and 0.2% dispase and incubated at 37°C for 15 minutes. Tissues were again treated with 0.1% collagenase and 0.2% dispase and incubated at 37°C for 5, 10 and 15 minute increments and cell suspensions were pooled. The pooled cell suspension was centrifuged and the pellet was re-suspended in DMEM supplemented with 10% FBS and seeded ($1x2x10^4$ cells cm^2) in a tissue culture flask. Plated cells were expanded in a medium containing DMEM supplemented with 10% FBS, and 100 U/ml penicillin/100μg/ml streptomycin and cultured at 37°C in a humidified tissue culture incubator with 5% CO_2 and 95% O_2. After 48 hours, non-adherent cells were removed. Remaining cells were fed with culture medium every 2-3 days until 70-80% confluency was reached, then cryopreserved.

2.5 Cell Culture

Cryopreserved cells were revived and re-suspended in DMEM containing 10% FBS and seeded ($2x10^4$ cells cm^2) in a tissue culture flask. The cells plated in the culture flask were then expanded in a medium containing DMEM, 10% FBS, and 1% antibiotic/antimycotic solution at 37°C and 5% CO_2 and 95% O_2 for 48 hours. After 48 hours, non-adherent cells were removed and the medium was changed. Cells were continuously sub-cultured and maintained in culture medium. Cells from fourth and fifth cell passages were used for all experiments.

2.6 Cell Surface Marker Detection via Flow Cytometry

Utilizing the Human Mesenchymal Stem Cell Phenotyping kit, samples of cells at the concentration of $1x10^6$ were used for the detection of mesenchymal stem cell surface markers via flow cytometric analysis. Samples were prepared according to manufacturer protocol. Positive markers include: CD90, CD73, CD105 and negative markers include: CD45, CD34, CD11b, CD79A, and HLA-DR as determined by the criteria set forth by the International Society for Cellular Therapy.[24] Fluorescence intensity of the cell samples was evaluated using the FACScan flow cytometer (BD Biosciences; San Jose, CA) at the University of Miami. Data analysis was completed using CellQuest Software (BD Biosciences; San Jose, CA).

2.7 *In-Vitro* Osteoblast Differentiation Assays

Mesenchymal stem cells were incubated either in CM (DMEM, 10% FBS, 1% antibiotic/antimycotic solution) or CM supplemented with 10% FBS, 10mM β-glycerophosphate, 50μg/mL ascorbic acid, and 100nM dexamethasone. Cell cultures were fed every 2-3 days and characterized after 21-25 days by Alizarin Red S and Von Kossa staining.

2.7.1 Mineralization Assay: Alizarin Red S Stain

At the end of the third and fourth week of treatment, cells were washed three times with PBS and fixed in 10% formalin for 10 minutes at room temperature. Cells were rinsed twice with distilled water and incubated in 1% Alizarin Red solution (Spectrum) for 10 minutes, followed by a gentle wash with distilled water.

2.7.2 Mineralization Assay: Von Kossa Stain

At the end of both treatment weeks (week three and four), cells were washed three times with PBS and fixed in 10% formalin for 10 minutes at room temperature. Cells were rinsed twice with distilled water and incubated in 2% silver nitrate and exposed to UV light for 1 hour. The silver nitrate solution was then removed and cells were rinsed with distilled water three times, exposed to bright light for 15 minutes, and dehydrated in 100% ethanol.

2.8 Cell Proliferation Study

HGMSCs grown to 80-90% confluency were then trypsinized and plated into a 96 well plate at 10,000 cells/well. Cells were assigned into treatment groups as illustrated in Table 2.1. Cells were incubated at 37°C and 5% CO_2 and 95% O_2 for 24 hours, then further incubated for 1,3, or 5 days within their assigned culture media (CM, Bromelain or Curcumin). Cells incubated in CM alone were considered the control group. Cells were then analyzed for proliferation via the MTT assay (a colorimetric assay). The MTT assay was completed for each group at each day interval following manufacturer protocol. Each well of cells received 10µL of MTT reagent ((3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) and were incubated at 37°C for 4 hours. A negative control of 10µL MTT reagent was added to 100µL of complete medium alone. 50µL of dimethyl sulfoxide (DMSO) was then added to each well and mixed. Cells were again incubated at 37°C for 10 minutes. Absorbance was read at 540nm. A reduction in color from yellow to purple indicated viable cell metabolic activity (Figure 2.2).

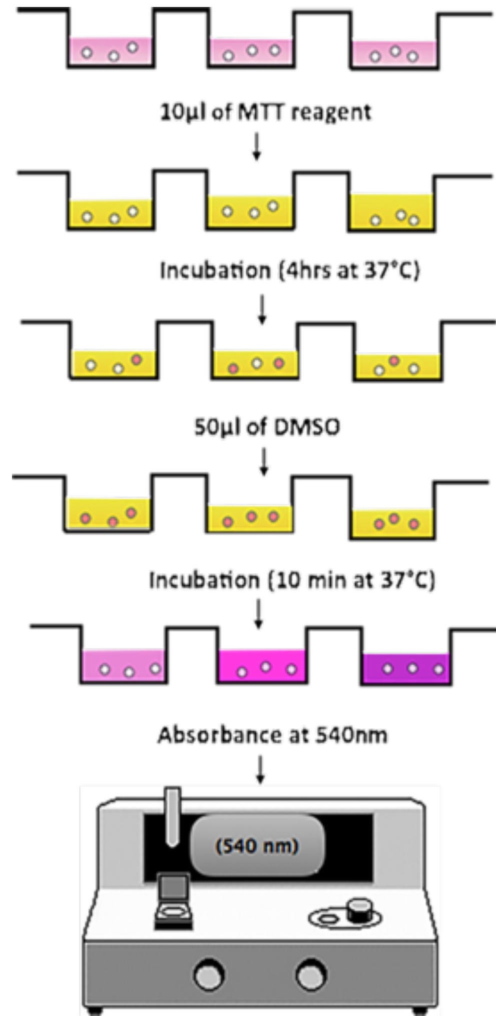


Figure 2.2 Depiction of the MTT Assay Procedure

2.9 Gene Expression Study

HGMSCs grown to 80-90% confluency were then further cultured in an assigned media, as found in Table 2.2, and were treated for 2 weeks at 37°C and 5% CO_2 and 95% O_2. Cells treated with complete medium supplemented with ascorbic acid and β-glycerophosphate was assigned as the control (OM) and cells treated with either dexamethasone (DEX), curcumin, or bromelain were designated as experimental groups. After two weeks of treatment, cells were harvested and RNA was extracted using the TRIzol method per manufacturer protocol. cDNA was then prepared via reverse

transcriptase following manufacturer guidelines. Gene expression analysis of selected osteogenic markers (Alkaline phosphatase, Collagen type I, Osteonectin, Osteopontin and Osteoprotegerin) was then measured by PCR and gel electrophoresis to determine cell differentiation and osteogenic differentiation (Figure 2.3).

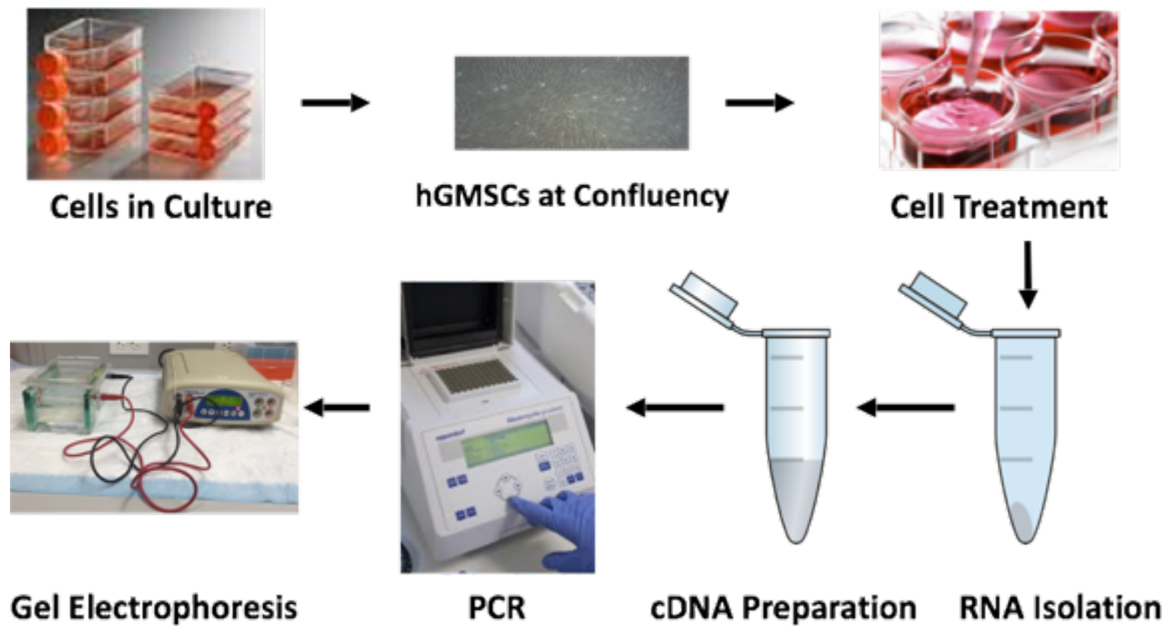


Figure 2.3 Depiction of Gene Expression Studies

2.10 Statistical Analysis

Data was analyzed usind the repeated measures of one way analysis of variance (ANOVA). A P-value ≤ 0.05 was selected for significance.

CHAPTER 3. RESULTS

3.1 Cell Morphology

Cells were seeded ($0.5x10^6$ cells cm^2) and cultured in a medium containing DMEM supplemented with 10% FBS and 100 U/ml penicillin/100µg/ml streptomycin at 37°C with 5% CO_2 and 95% O_2 (Figure 3.1A). Cells reached 80-90% confluency at one week after seeding (Figure 3.1D). Images depict a homogenous cell population of spindle shaped cells (Figure 3.1B, 3.1C, and 3.1D).

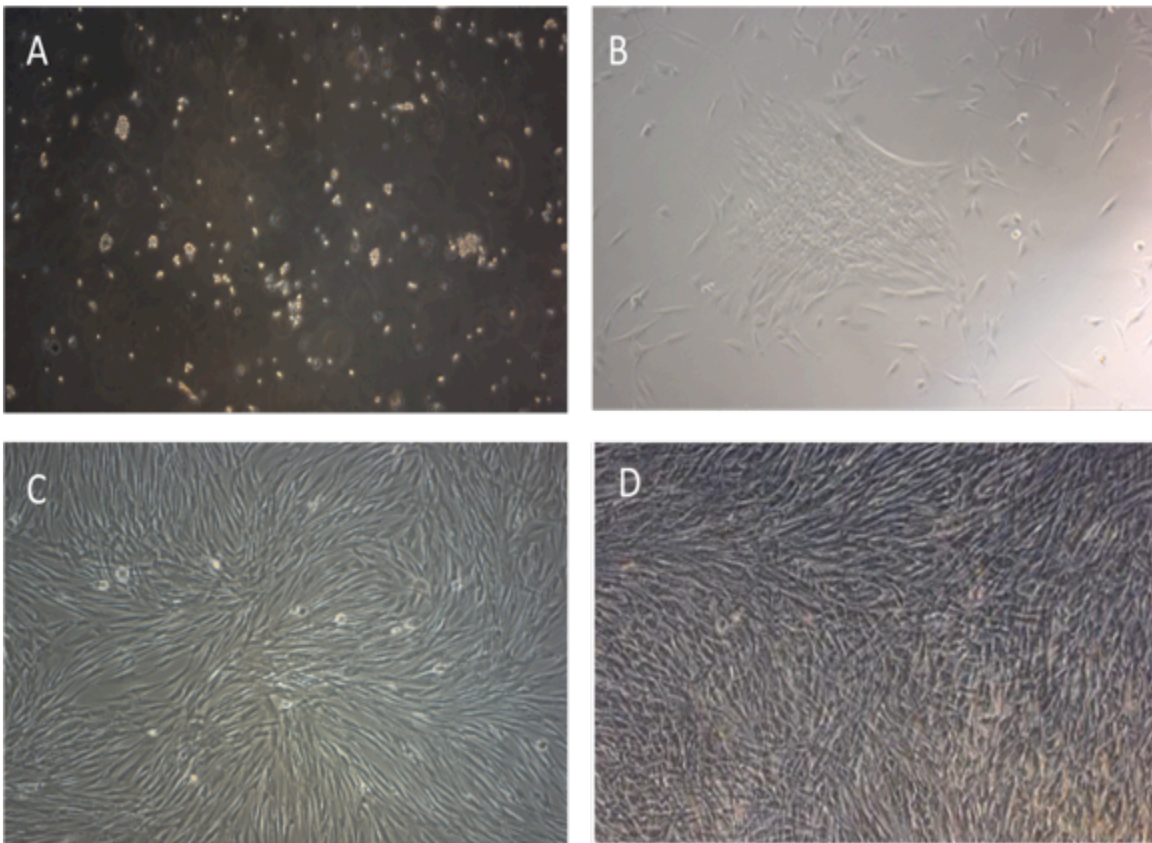


Figure 3.1 Morphologic Images of HGMSCs. Phase contrast micrographs at Day 0 (A), Day 1 (B), sub-confluence (C) and confluence (D).

3.2 Flow Cytometry Analysis

Cell surface markers were examined to confirm that they exhibit the characteristic features found in mesenchymal stem cells. Results revealed that the cells were positive for mesenchymal stem cell markers CD73, CD90, CD105 and were negative for hematopoietic stem cell markers CD34, CD14, CD45, and CD20 (Figure 3.2).

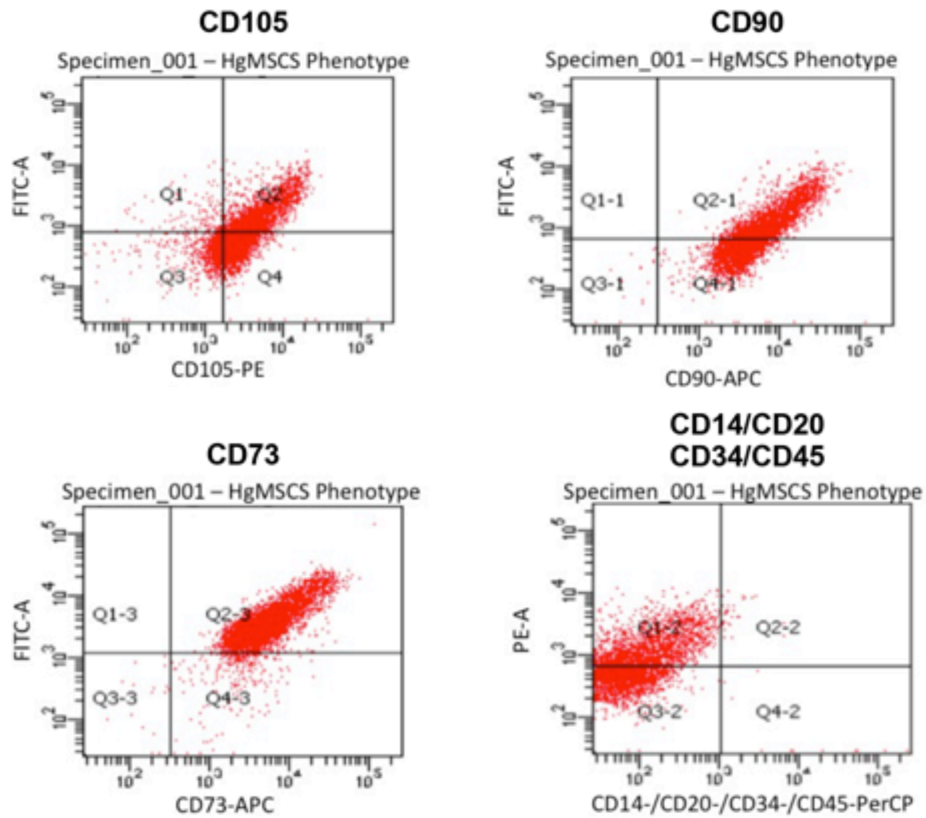


Figure 3.2 Flow Cytometric Analysis of HGMSCs.
Cells were more than 90% positive for the expression of cell surface markers related to those found in mesenchymal stem cells (CD105, CD90 and CD73) and negative for those found in hematopoietic stem cells (CD14, CD20, CD34 and CD45).

3.3 *In vitro* Mineralization

The osteogenic differentiation potential of human gingiva derived mesenchymal stem cells was determined by culturing cells in osteogenic induction medium for 4 weeks. At the end of week 4, they were examined for mineral nodule formation. Results revealed that cells induced with osteogenic supplements were positive for mineral aggregates and calcification by Alizarin Red S and Von Kossa stain (Figure 3.3B, 3.3D).

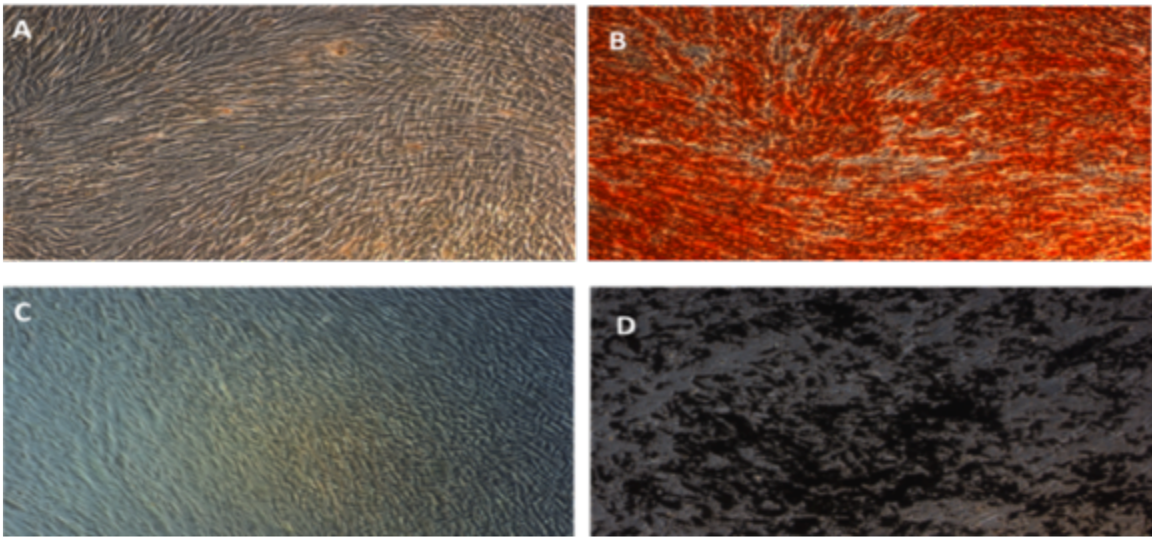


Figure 3.3 Osteogenic Differentiation Potential of Gingiva Derived Mesenchymal Stem Cells. Alizarin Red S stain negative to cells cultured in complete medium (A) and positive to cells cultured in osteogenic medium (B). Von Kossa stain negative to cells cultured in complete medium (C) and positive to cells cultured in osteogenic medium (D).

3.4 Effects of Bromelain and Curcumin on Cell Proliferation

Cells were treated as described in the methodology section 2.8. On day 1, cells induced with bromelain showed increased proliferation at 1μg/ml concentration. At all other concentrations, cell proliferation was comparable to those untreated with the antioxidant. On day 3, bromelain treatment enhanced human gingival mesenchymal stem cell proliferation at all concentrations. On day 5, there was a gradual decrease in cell proliferation with increasing concentrations of bromelain. Bromelain did not inhibit HGMSCs survival at any concentration (Figure 3.4).

In the presence of curcumin, cells displayed higher proliferation rates at wide concentration ranges (0.5-5μM) with a significant decrease at 15μM on day 1. Curcumin exhibited a dose dependent proliferation on day 3. On day 5, curcumin inhibited cell growth at higher concentrations (10-15μM), while lower concentrations did not affect cell growth (Figure 3.5).

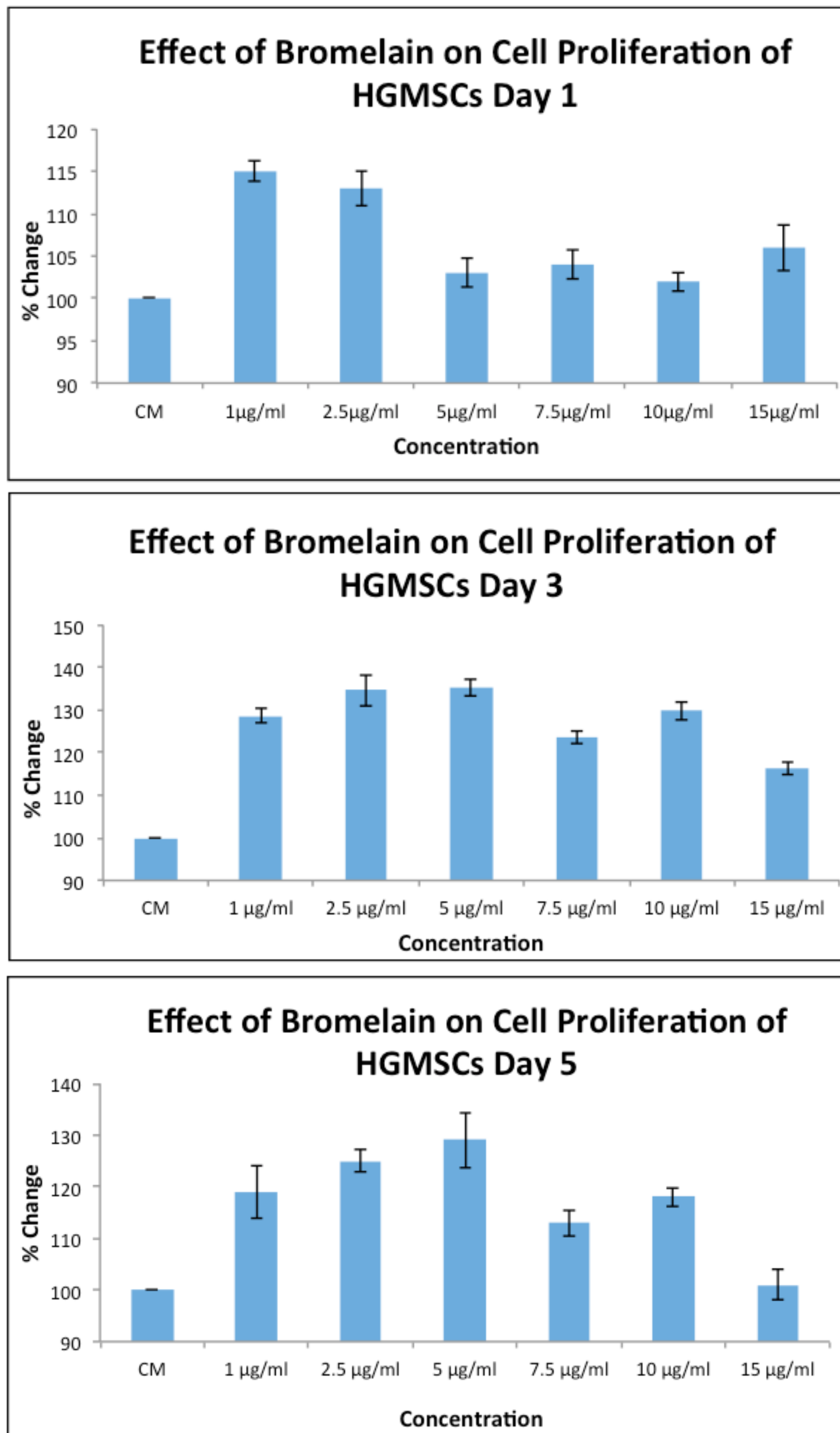
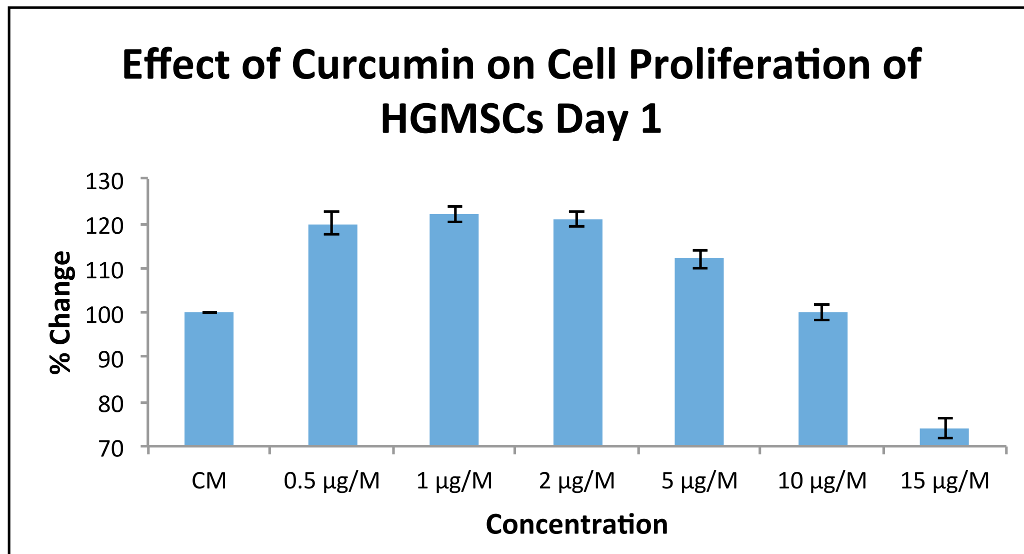
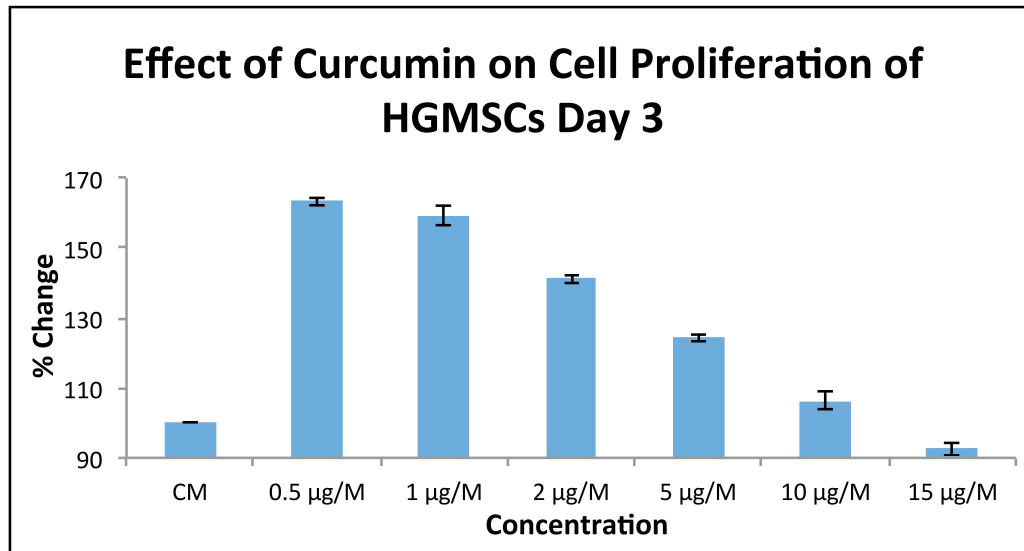


Figure 3.4 Effect of Bromelain Treatment on the Proliferation of HGMSCs. Cells were incubated with or without various concentrations of bromelain for 1,3, or 5 days. The CM group was treated as the control.





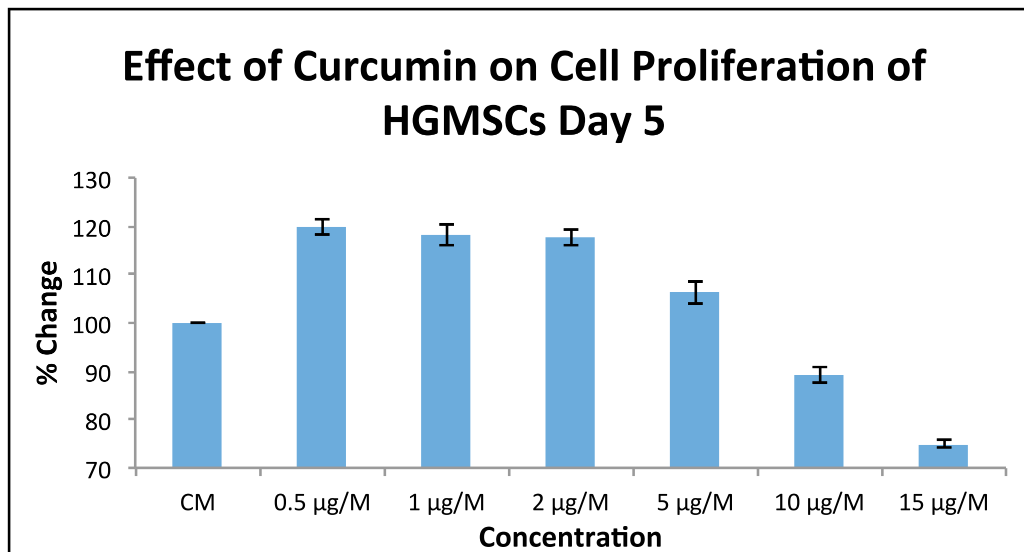
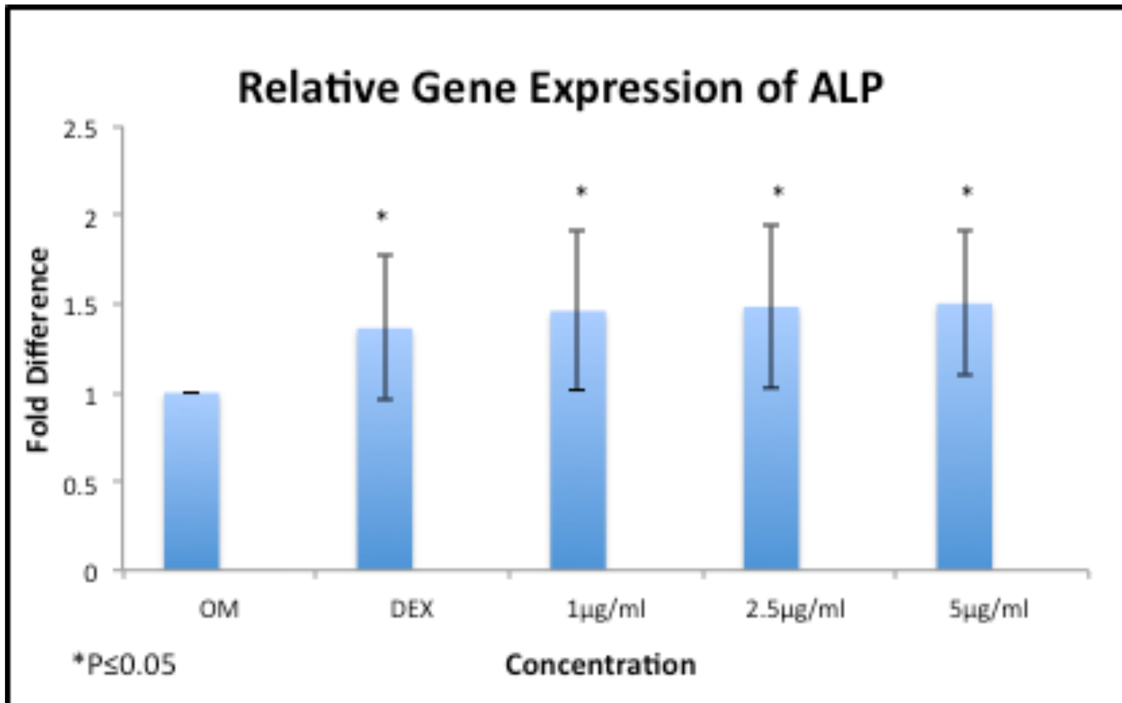


Figure 3.5 Effect of Curcumin Treatment on the Proliferation of HGMSCs. Cells were incubated with or without various concentrations of curcumin for 1, 3, or 5 days. The CM group was treated as the control.

3.5 Effects of Bromelain and Curcumin on Gene Expression

ALP, OPG, ONN and Collagen Type I gene expression was examined by PCR after HGMSCs were cultured in OM, DEX or different concentrations of bromelain or curcumin (as noted in Table 2.2) for two weeks (Figures 3.6, 3.7, 3.8, and 3.9). After 14 days of treatment, ALP ($p<0.01$), OPG ($p=0.04$), and ONN ($p<0.01$) were significantly up-regulated by bromelain and curcumin treatment in a dose-dependent manner. While DEX treatment induced the up-regulation of genes ALP, OPG, ONN after 14 days of treatment, collagen type I was comparable with the control (OM). As illustrated in Figure 3.6, the bromelain-treated groups exhibited significantly greater expression of ALP ($p<0.001$) and OPG ($p<0.05$) than cells treated with OM. While the bromelain treated group showed enhanced expression of ALP and OPG, the increase was not significantly higher than the DEX treated groups. Both OPG and ONN significantly increased at 5µg/ml (Figure 3.6 and 3.7). As depicted in Figure 3.8, ALP was significantly expressed with lower concentrations of curcumin. ONN expressed at the highest concentration of 10µM (Figure 3.9). Both bromelain and curcumin did not effect the expression of collagen type I in HGMSCs.



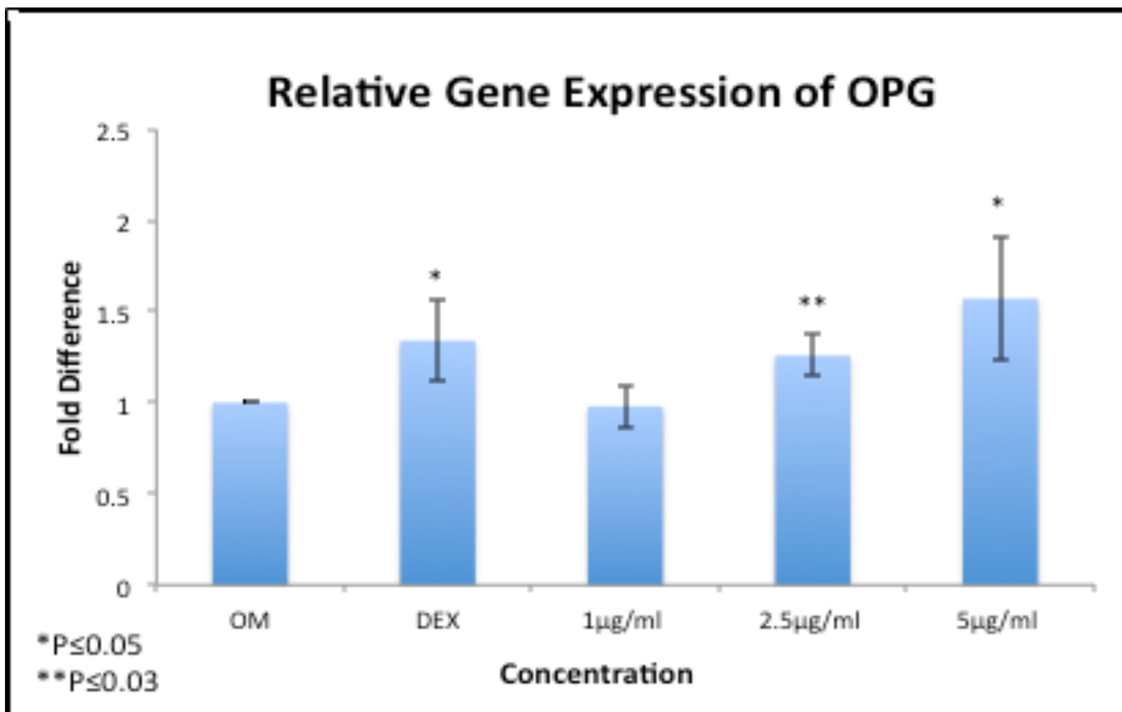
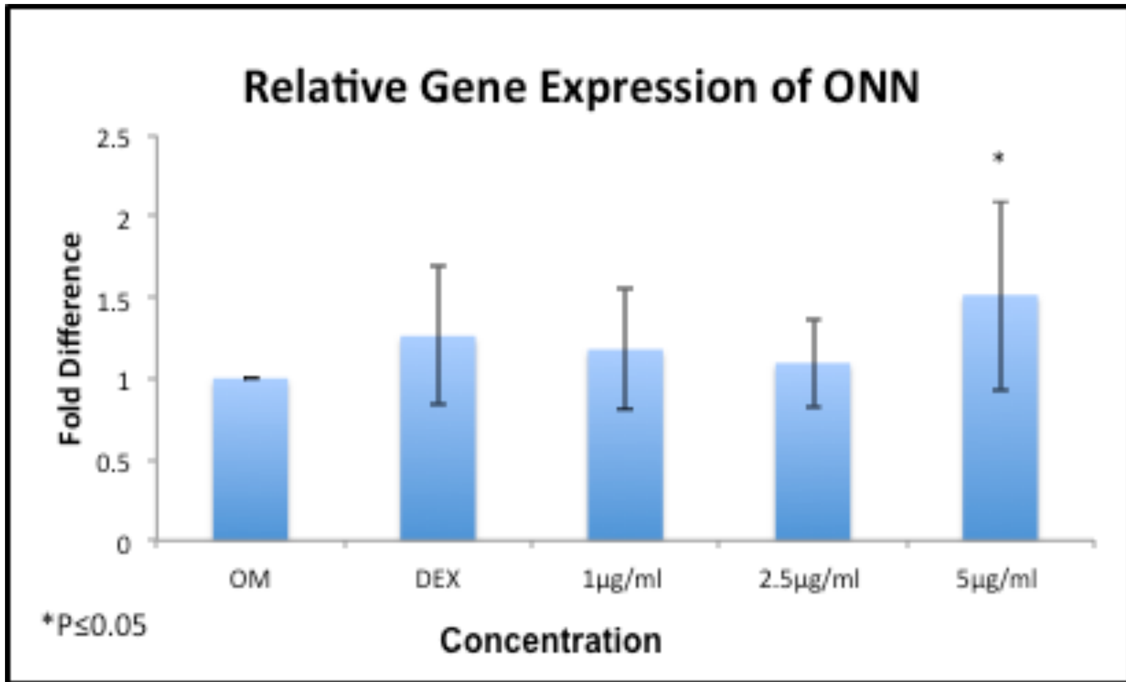


Figure 3.6 The Expression of ALP and OPG in HGMSCs Cultured in OM, DEX, and Bromelain.



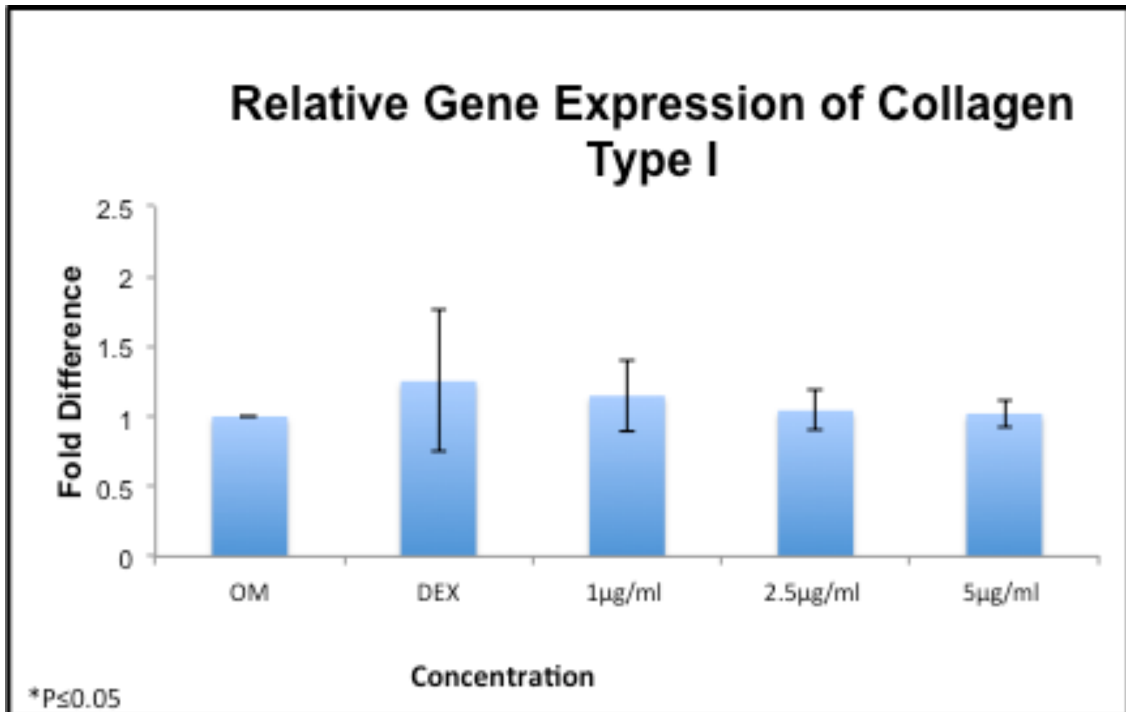
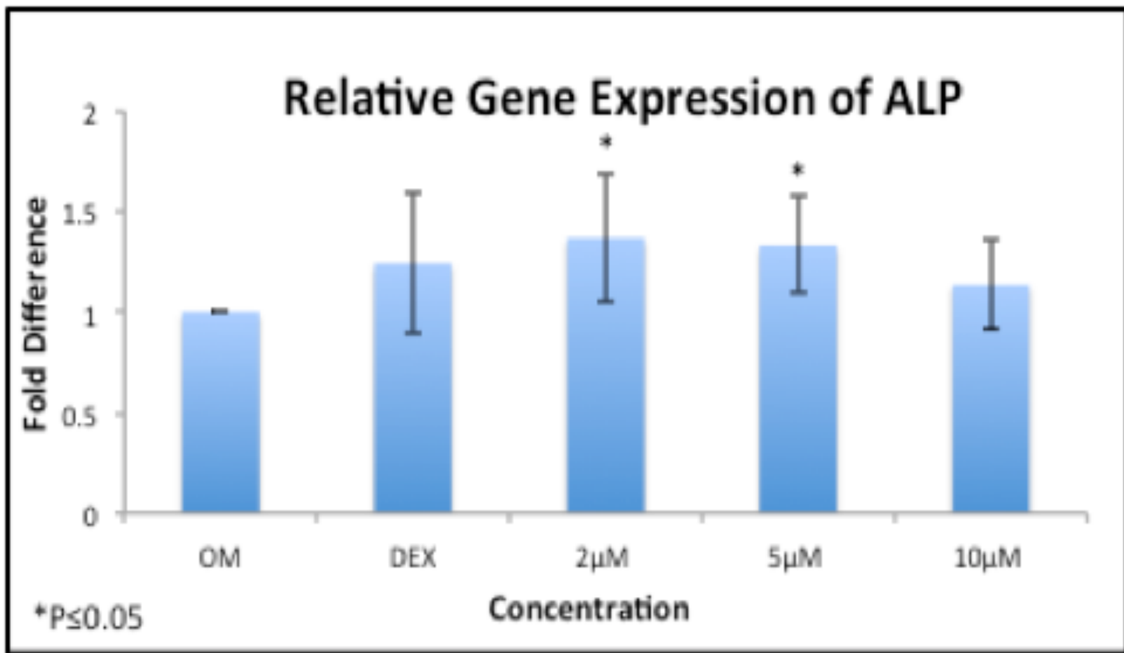


Figure 3.7 The Expression of ONN and Collagen Type I in HGMSCs Cultured in OM, DEX, and Bromelain.



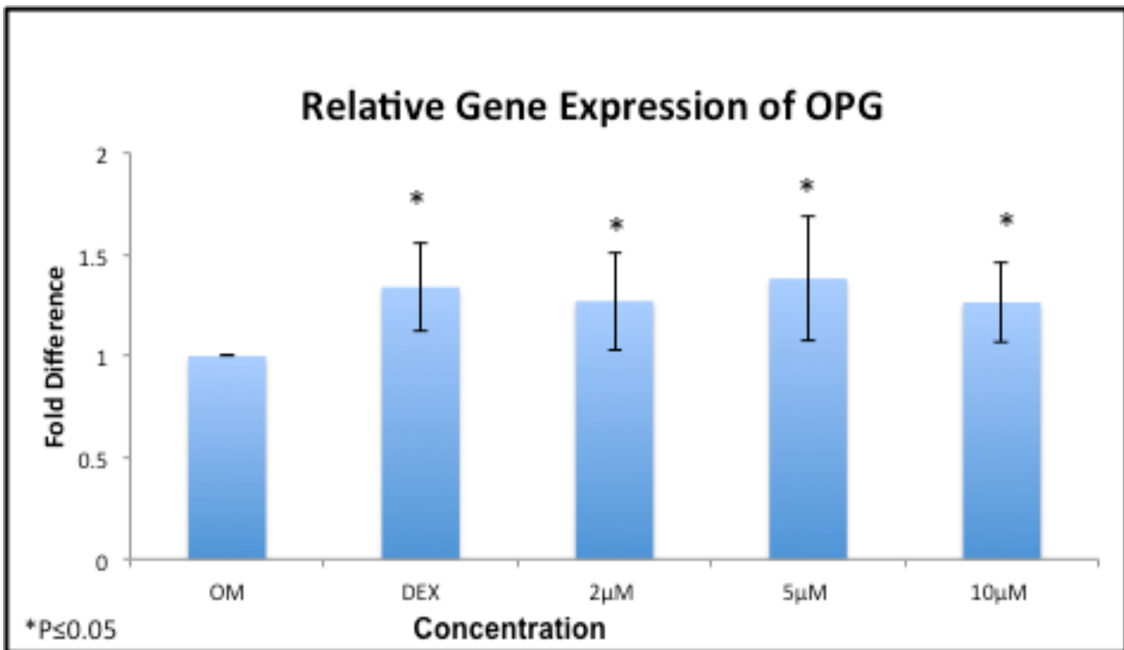
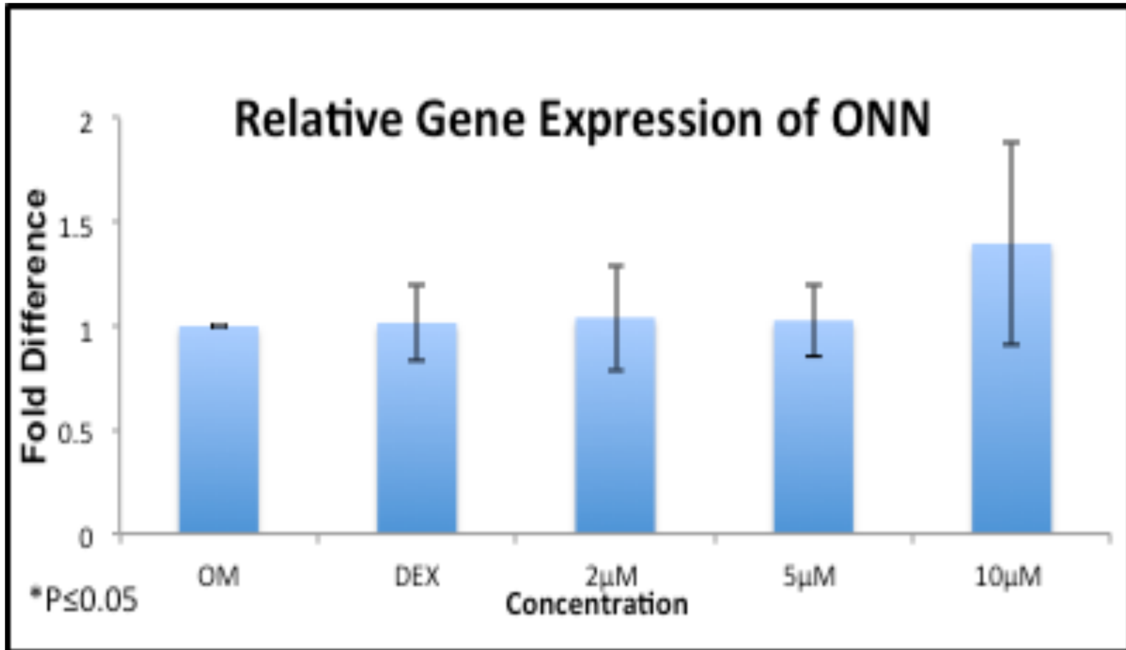


Figure 3.8 The Expression of ALP and OPG in HGMSCs Cultured in OM, DEX, and Curcumin.



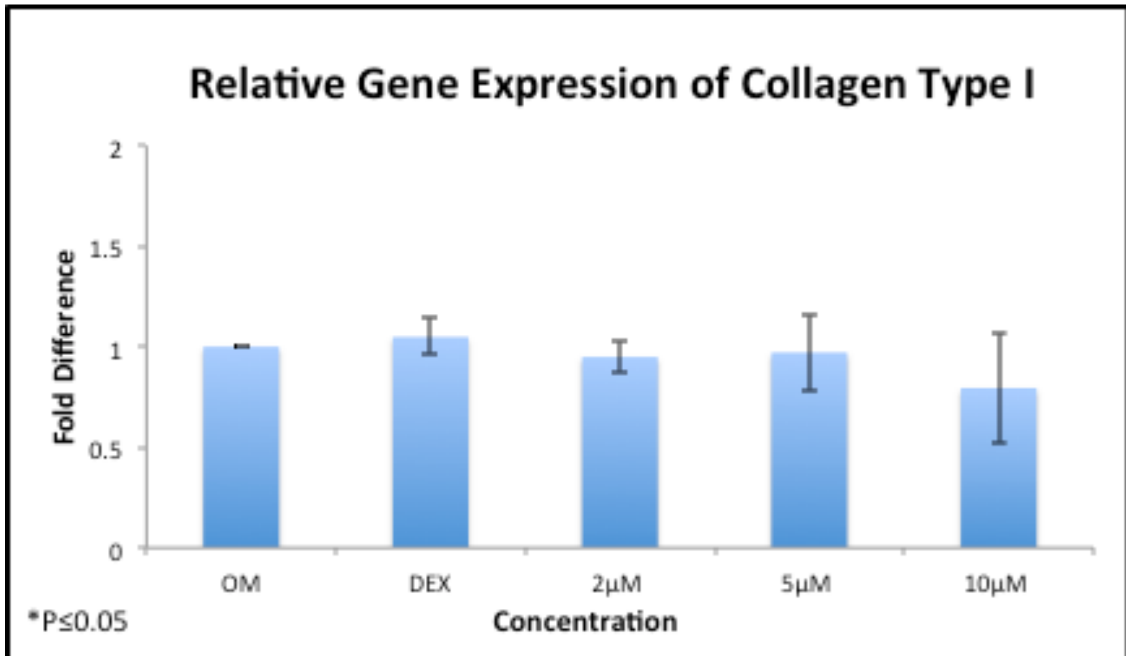


Figure 3.9 The Expression of ONN and Collagen Type I in HGMSCs Cultured in OM, DEX, and Curcumin.

3.6 Osteogenic Marker Genes

Osteogenic marker genes were investigated via RT-PCR to confirm the osteogenic differentiation of human gingival mesenchymal stem cells when grown in media treated with dexamethasone, curcumin or bromelain. Results demonstrated that curcumin and bromelain induced osteogenic differentiation in a dose dependent manner. At 2.5μg the peak up-regulation could be seen for genes Col Type I, ALP, and OPG for bromelain treated cells (Figure 3.10). In curcumin, the maximum enhancement has been observed at 2μM for all genes (ALP, Col Type I, OPN, and ONN) as depicted in Figure 3.11.

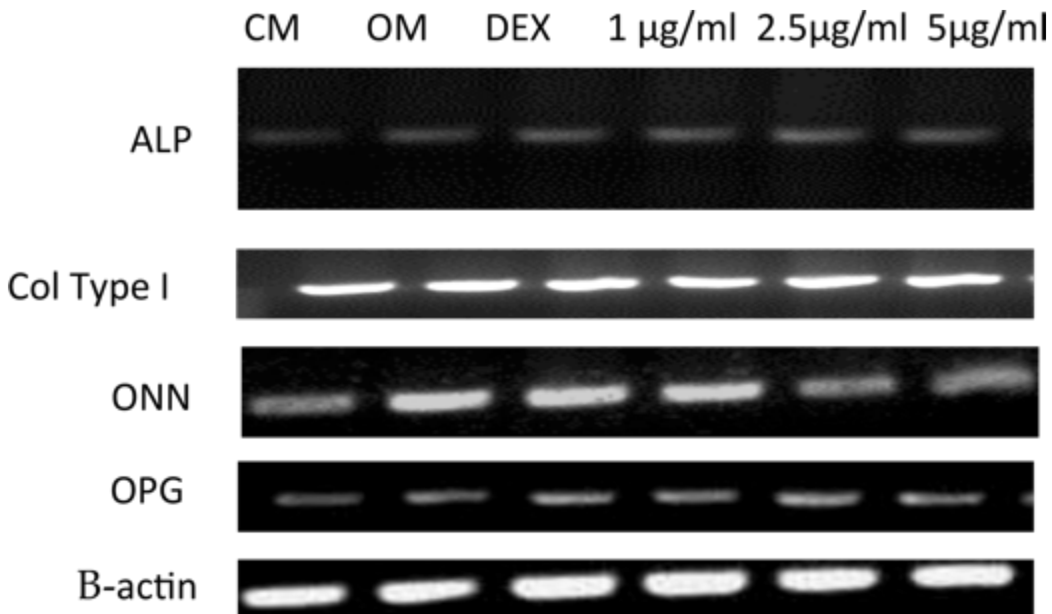


Figure 3.10 Identification of Osteogenic Marker Genes in HGMSCs Treated with Bromelain. Osteogenic differentiation of HGMSCs was determined by investing the osteogenic marker genes ALP, Col Type I, ONN, and OPG by RT-PCR. B-actin was used as an endogenous control. CM and OM were considered control media. At 2.5μg, the peak up-regulation could be seen for Collagen Type I, ALP, and OPG.

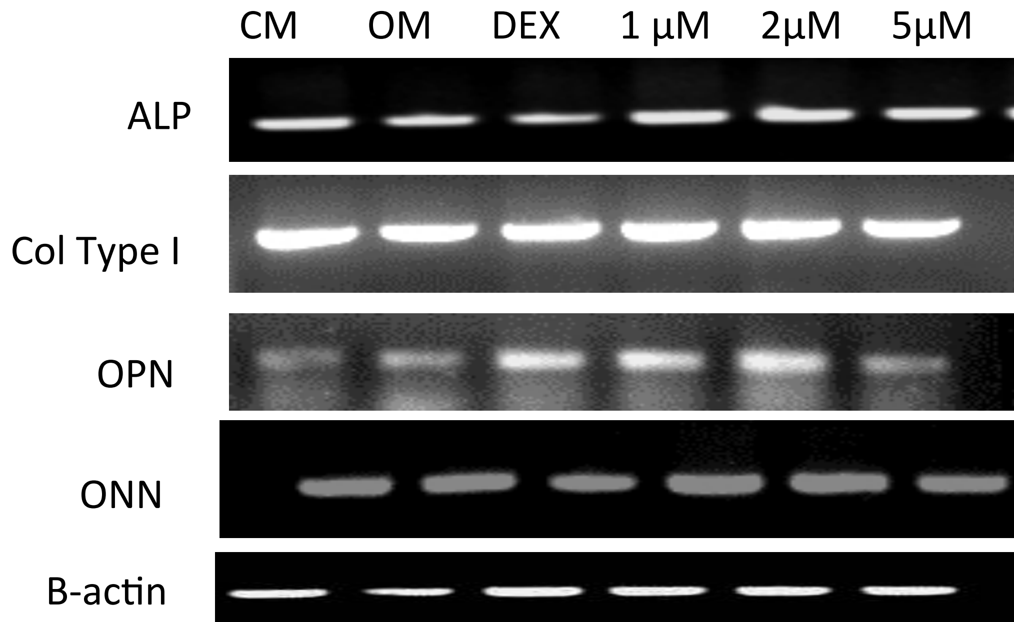


Figure 3.11 Identification of Osteogenic Marker Genes in HGMSCs Treated with Curcumin. Osteogenic differentiation of HGMSCs was determined by investing the osteogenic marker genes ALP, Col Type I, OPN, and ONN by RT-PCR. B-actin was used as an endogenous control. CM and OM were considered control media. At 2µM, the peak up-regulation could be seen for Collagen Type I, ALP, and OPG.

CHAPTER 4. DISCUSSION

Due to their highly proliferative capacity and multilineage differentiation potential, mesenchymal stem cells have become an attractive candidate for clinical application.[9,25] Most cell based therapies were conducted using mesenchymal stem cells derived from bone marrow, however, their limitation of availability and difficulty of harvesting large volumes have led to the investigation of other tissue sources.[13,23] In 2010, Zhang et al. isolated mesenchymal stem cells from human gingiva and characterized them as having the ability to differentiate into multi-lineages. Tomar et al. has demonstrated that mesenchymal stem cells derived from human gingiva are far more superior to those derived from bone marrow.[13] A study conducted by Xu et al. has demonstrated that 90% of the mesenchymal stem cells isolated from gingiva are derived from cranial neural crest cells and exhibit the same multipotency.[26] Based on these findings, HGMSCs may be an ideal cell source for repairing craniofacial defects.

The *in vitro* differentiation of mesenchymal stem cells depends upon the conditions in which they are cultured. Typical *in vitro* osteogenic differentiation media contains ascorbic acid, dexamethasone, and β-glycerophosphate.[9,10] A constant presence of dexamethasone in culture medium is necessary to achieve complete differentiation of mesenchymal stem cells into osteoblasts. Although long-term exposure of dexamethasone enhances *in vitro* differentiation of MSCs (bone formation), its effects *in vivo* are contradictory and can lead to bone degeneration and loss.[9] Antioxidants play a vital role in stem cell proliferation and differentiation, therefore, may provide an alternative to dexamethasone in osteogenic media [14,15] The present study was focused on

investigating the effects of curcumin and bromelain on osteogenic differentiation of human gingiva derived mesenchymal stem cells (HGMSCs) and to compare their differentiation potential to dexamethasone.

In this study, the addition of bromelain to the culture medium enhanced cell proliferation at lower concentrations (1µg/ml-5µg/ml). Cells exposed to bromelain for 24 hours showed a high proliferation rate at 1µg/ml. On day 3, an increase in proliferation was observed with all concentrations. There was a gradual decrease in higher concentrations of bromelain on day 5. Overall, results demonstrated that bromelain did not inhibit the survival of HGMSCs at any concentration. Amini et al., Dave et al., and Grabowska et al., reported that bromelain inhibited cell proliferation in a concentration dependent manner. In all studies, cell viability was significantly reduced when exposed to higher concentrations of bromelain.[21,27,28] Amini et al., exposed experimental cells to bromelain concentrations ranging from 5µg/ml-600µg/ml. Their results showed a concentration dependent cell inhibitory effect with half maximal inhibitory values ranging from 29µg/ml-142µg/ml.[28] Our pilot studies revealed that concentrations over 15µg/ml were causing apoptosis in HGMSCs exposed to bromelain for 72 hours. Based on these results, further experiments were conducted with a concentration range of 1µg/ml-15µg/ml.

In the presence of curcumin, our data exhibited higher proliferation rates of HGMSCs at wide concentration ranges (0.5µg/M-5µg/M). Cells displayed higher proliferation rates at lower concentrations (0.5µg/M-5µg/M) with a significant decrease at 15µg/M on

day 1. Curcumin exhibited a dose dependent proliferation on day 3. A similar effect was found in the study conducted by Moran et al., who showed that the treatment of human osteoblast–like cells with curcumin inhibited their proliferation in a dose dependent manner.[29] On day 5, curcumin inhibited cell growth at higher concentrations (10µg/M-15µg/M). Overall, the proliferation of HGMSCs was significantly higher when the cells were exposed to curcumin at lower concentrations (0.5µg/M-5µg/M), while higher concentrations 10µg/M and 15µg/M caused cytotoxicity after prolonged period of exposure. Kim et al. revealed similar findings, which found that lower concentrations of curcumin can induce the proliferation of mouse multi-potent neural progenitor cells, while high doses of curcumin are cytotoxic.[30]

Our study demonstrated that the treatment of curcumin and bromelain significantly up-regulated the osteogenic gene expression of ALP, OPG, ONN and Collagen Type I in HGMSCs. These findings are supported by the study conducted by Son et al., where curcumin increased the expression of genes such as RunX2, ALP, and OC (osteocalcin), which consequently induced the differentiation of mouse embryonic stem cells into osteoblasts.[31] With curcumin treatment, gene expression patterns were not concentration dependent, suggesting that curcumin is an effective inducer of osteoblast differentiation at all concentrations in this study. However, cells were never exposed to a concentration more than 10ug/ml due to the fact that it caused apoptosis at higher concentrations, as evident in previous studies. The bromelain treated groups exhibited significantly greater expression of ALP than cells treated with DEX. To our knowledge, this is the first evaluation of bromelain as an inducer of osteoblast differentiation. Since a similar

osteogenic induction was also seen in the DEX treated groups, the results from our the study suggests that curcumin or bromelain can be used as substitute of DEX.

In conclusion**,** curcumin and bromelain treatment may help establish an environment that facilitates osteodifferentiation of mesenchymal stem cells *in vivo*. This strategy may support the regeneration of bone in individuals with orofacial clefts.

APPENDICES

MTT ASSAY: Bromelain

Day I

	Mean	SD
CM	100	0
1µg/ml	115	0.0116
2.5µg/ml	113	0.0205
5µg/ml	103	0.0165
7.5µg/ml	104	0.017
10µg/ml	102	0.0114
15µg/ml	106	0.0276

Day III

	Mean	SD
CM	100	0
1µg/ml	128.58	0.02
2.5µg/ml	134.69	0.04
5µg/ml	135.26	0.02
7.5µg/ml	123.53	0.01
10µg/ml	129.73	0.02
15µg/ml	116.26	0.01

Day V

	Mean	SD
CM	100	0
1µg/ml	119	0.05
2.5µg/ml	125	0.02
5µg/ml	129	0.05
7.5µg/ml	113	0.03
10µg/ml	118	0.02
15µg/ml	101	0.03

MTT ASSAY: Curcumin

Day I

	Mean	SD
CM	100	0
0.5 µg/M	120	0.024
1 µg/M	122	0.017
2 µg/M	121	0.017
5 µg/M	112	0.020
10 µg/M	100	0.017
15 µg/M	74	0.022

Day III

	Mean	SD
CM	100	0
0.5 µg/M	163	0.012
1 µg/M	159	0.027
2 µg/M	141	0.014
5 µg/M	124	0.011
10 µg/M	106	0.025
15 µg/M	93	0.019

Day V

	Mean	SD
CM	100	0
0.5 µg/M	119.64	0.017
1 µg/M	118.15	0.023
2 µg/M	117.55	0.016
5 µg/M	106.21	0.022
10 µg/M	88.96	0.016
15 µg/M	75.02	0.008

Gene Expression: Bromelain

ALP

	Mean	SD
OM	1	0
DEX	1.363629	0.404275
1µg/ml	1.460883	0.445922
2.5µg/ml	1.484082	0.458073
5µg/ml	1.502686	0.409826

OPG

	Mean	SD
OM	1	0
DEX	1.339321	0.22053
1µg/ml	0.979491	0.116551
2.5µg/ml	1.258179	0.116411
5µg/ml	1.570101	0.336333

Collagen Type I

	Mean	SD
OM	1	0
DEX	1.251893	0.505175
1µg/ml	1.147669	0.253731
2.5µg/ml	1.042571	0.141393
5µg/ml	1.020194	0.0962

ONN

	Mean	SD
OM	1	0
DEX	1.261195	0.428771
1µg/ml	1.178231	0.369885
2.5µg/ml	1.094126	0.274837
5µg/ml	1.513552	0.587276

Gene Expression: Curcumin

ALP

	Mean	SD
OM	1	0
DEX	1.2415	0.3498
2µg/M	1.368	0.3165
5µg/M	1.3309	0.238
10µg/M	1.1355	0.2198

OPG

	Mean	SD
OM	1	0
DEX	1.339321	0.22053
2µg/M	1.270759	0.241164
5µg/M	1.380983	0.30444
10µg/M	1.264004	0.195137

Collagen Type I

	Mean	SD
OM	1	0
DEX	1.0484	0.0913
2µg/M	0.9491	0.0802
5µg/M	0.9713	0.1902
10µg/M	0.7946	0.2767

ONN

	Mean	SD
OM	1	0
DEX	1.01557	0.181343
2µg/M	1.041345	0.254943
5µg/M	1.027247	0.172606
10µg/M	1.397643	0.487244

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