ผลของสารสกัดจากขมิ้นชันต่อการแสดงออกของยีนที่เกี่ยวข้องกับการหายของแผล
ในเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์

นางสาวอัสปรียา รุจิระโชติวัฒน์

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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EFFECT OF CURCUMIN ON THE EXPRESSION OF WOUND HEALING-RELATED GENES IN HUMAN GINGIVAL FIBROBLASTS

Miss Auspreeya Rujirachotiwat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Pediatric Dentistry
Department of Pediatric Dentistry
Faculty of Dentistry
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อัสปรียา รุจิระโชติวัฒน์ : ผลของสารสกัดจากขมิ้นชันต่อการแสดงออกของยีนที่เกี่ยวข้องกับการหายของแผลในเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์ (EFFECT OF CURCUMIN ON THE EXPRESSION OF WOUND HEALING-RELATED GENES IN HUMAN GINGIVAL FIBROBLASTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ทพญ. ดร.สุภาพร สุทธมนัสวงษ์, 36 หน้า.

การศึกษาผลของสารสกัดจากขมิ้นชัน (เคอร์คูมิน) ต่อการแสดงออกของยีนที่เกี่ยวข้องกับการหายของแผลในเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์ซึ่งได้แก่ ยีนทรานสฟอร์มมิ่งโกรทแฟคเตอร์เบต้าชนิดที่ 1, ยีนทรานสฟอร์มมิ่งโกรทแฟคเตอร์เบต้ารีเซปเตอร์ชนิดที่ 1, ยีนทรานสฟอร์มมิ่งโกรทแฟคเตอร์เบต้ารีเซปเตอร์ชนิดที่ 2 และวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ ความเป็นพิษของเคอร์คูมินถูกทดสอบด้วยวิธีเอ็มทีที จากนั้นทำการกระตุ้นเซลล์ด้วยเคอร์คูมินที่ความเข้มข้นต่างๆเป็นระยะเวลา 24 ชั่วโมงและทำการตรวจสอบการแสดงออกของยีนด้วยวิธีควอนทิเทฟพีซีอาร์ ผลการศึกษาพบว่าเคอร์คูมินที่ความเข้มข้น 0.1-20 ไมโครโมลาร์ไม่มีผลต่อการมีชีวิตของเซลล์ขณะที่ความเข้มข้นที่สูงขึ้น (30 และ 50 ไมโครโมลาร์) มีความเป็นพิษต่อเซลล์ เคอร์คูมินที่ 0.1-20 ไมโครโมลาร์กระตุ้นการแสดงออกของยีนทรานสฟอร์มมิ่งโกรทแฟคเตอร์เบต้าชนิดที่ 1 ตามความเข้มข้นที่มากขึ้น ในขณะที่ความเข้มข้น 1 ไมโครโมลาร์เป็นความเข้มข้นที่เหมาะสมต่อการกระตุ้นการแสดงออกของยีนทรานสฟอร์มมิ่งโกรทแฟคเตอร์เบต้ารีเซปเตอร์ชนิดที่ 1, ยีนทรานสฟอร์มมิ่งโกรทแฟคเตอร์เบต้ารีเซปเตอร์ชนิดที่ 2 และวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ อย่างไรก็ตามการกระตุ้นแสดงออกของยีนทั้งหมดโดยเคอร์คูมินไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุม สรุปผลเคอร์คูมินน่าจะมีผลต่อการควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการหายของแผลในเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์ แต่จำเป็นต้องมีการศึกษาเพิ่มเติมต่อไป

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ภาควิชา ทันตกรรมสำหรับเด็ก ลายมือชื่อนิสิต ....
สาขาวิชา ทันตกรรมสำหรับเด็ก ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
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AUSPREEYA RUJIRACHOTIWAT: EFFECT OF CURCUMIN ON THE EXPRESSION OF WOUND HEALING-RELATED GENES IN HUMAN GINGIVAL FIBROBLASTS. ADVISOR: SUPAPORN SUTTAMANATWONG, D.D.S., Ph.D., 36 pp.

This study investigated the effect of curcumin on the expression of wound healing-related genes including transforming growth factor beta 1 (TGF-β1), transforming growth factor beta receptor type I (TGFβR I), transforming growth factor beta receptor type II (TGFβR II) and vascular endothelial growth factor (VEGF) in human gingival fibroblasts. The cytotoxicity of curcumin was determined by MTT assay. Then, cells were treated with non-cytotoxic concentrations of curcumin for 24 hours and the level of gene expression was determined by quantitative polymerase chain reaction (qPCR). Curcumin at 0.1-20 μM caused no significant change in cell viability while higher concentrations of curcumin (30 and 50 μM) are cytotoxic. Curcumin dose dependently increased the TGF-β1 expression while 1 μM curcumin is the optimal concentration for inducing TGFβR I, TGFβR II, and VEGF expression. However, no statistically significant difference was found in any of these inductions. In conclusion, curcumin may regulate the expression of genes involved in wound healing in human gingival fibroblasts but further investigation is needed.

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Department:	Pediatric Dentistry	Student's Signature ....
Field of Study:	Pediatric Dentistry	Advisor's Signature ....
Academic Year:	2017	

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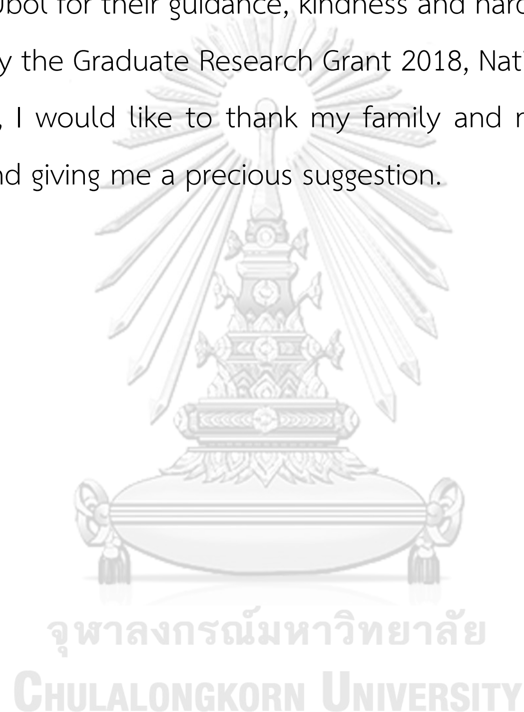
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Chapter 1
Introduction

Background and rationale

Oral ulceration can cause pain and discomfort which significantly affects daily food intake. Among all of the remedies for oral ulceration, none of them accelerates the healing process [1]. Wound healing is regulated by signals from various cell types including platelets, immune cells, and fibroblasts. Wound healing comprises 4 phases: hemostasis, inflammation, proliferation, and remodeling [2]. In the proliferative phase, gingival fibroblasts work by secreting several growth factors such as transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) [3]. These biological molecules are crucial for the healing of the wound [4].

Herbal medicine has long been common in eastern cultures despite the western advance medical and pharmaceutical products. Due to the alarm of side effects from the western medicine, the popularity of herbal medicine is even more strengthened and supported by its effectiveness. Curcumin (diferuloylmethane), a yellow substance from turmeric rhizome, is a member of curcuminoid family [5]. In India, curcumin has been widely used for a long time due to its anti-inflammatory, anti-oxidant, anti-bacterial and anti-carcinogenic properties [6].

Curcumin also demonstrates a significant wound healing property by enhancing fibroblast proliferation and increasing the level of antioxidant enzyme in dermal wound of rats [7, 8]. Another study reported that curcumin enhanced collagen production and decreased matrix metalloproteinase-9 production in rat wounds [9]. Curcumin promotes healing of cutaneous wound in both incision and excision models [10]. In

the irradiated wounds, curcumin decreased the healing period by accelerating wound closure and increasing the synthesis of collagen, hexosamine, DNA and nitric oxide [11].

Curcumin has also been reported to promote oral wound healing. An animal study showed that curcumin accelerated the wound healing of mucosal oral ulcer at upper labial gingiva of rabbits [12]. Clinically, topical curcumin gel significantly reduced the size of minor aphthous ulcers in comparison with placebo and pain intensity among these patients [13]. However, the cellular response to curcumin treatment during oral wound healing remains unclear. This study aim to investigate how curcumin affect the expression of genes involved in wound healing in human gingival fibroblasts.

Research question

What is the effect of curcumin on the expression of wound healing-related genes in human gingival fibroblasts?

Research Objectives

1. To examine the effect of curcumin on human gingival fibroblast viability by MTT assay.

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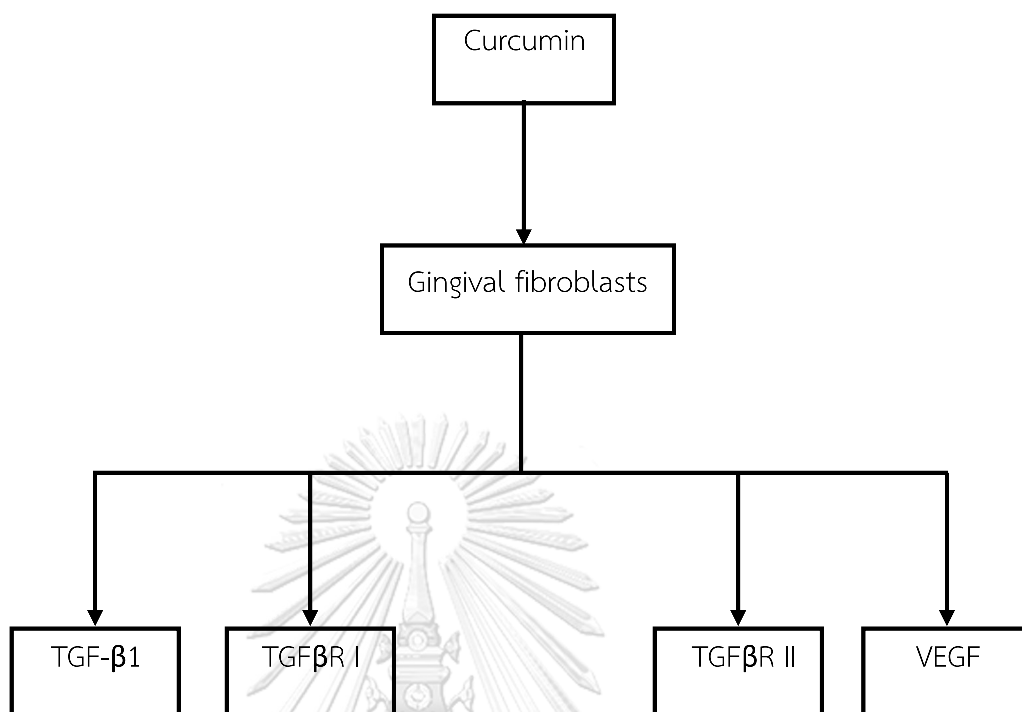
2. To determine the effect of curcumin on the expression of TGF-β1, TGFβR I, TGFβR II, and VEGF in human gingival fibroblasts by qPCR.

Hypothesis

Curcumin can alter the expression of wound healing-related genes in human gingival fibroblasts.

Conceptual framework



Research design

Laboratory experimental research

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Keywords

Curcumin, TGF-β1, TGFβR I, TGFβR II, VEGF, gingival fibroblasts

Expected Results

Curcumin can regulate the expression of wound healing-related genes in human gingival fibroblasts.

Expected benefits

This research may provide more understandings of the mechanisms by which curcumin stimulates wound healing. Moreover, curcumin can be further developed as a topical agent to promote oral wound healing.

Chapter 2
Review of literature

Oral Ulceration and Remedy

Oral ulceration is characterized as the complete loss of epithelium with different levels of losing the underlying connective tissue [14]. The common causes are the physical, chemical, thermal or trauma [15-19]. The simplest injuries may develop from daily activities such as accidental biting during mastication or from hard textures of food. Another group of injuries may cause from sharp edges of broken teeth, restorations, and appliances (e.g. orthodontic and prosthodontics appliance) especially ill-fitting dentures, which may cause chronic ulcers. The other group of injuries may be iatrogenic from rotary instrument, saliva ejector, dental material (e.g. sodium hypochlorite) and medicament (e.g. aspirin) during dental treatment. Moreover, one of the most common ulcers is the wound after dental extraction.

Oral ulcers can cause significant pain and discomfort due to the inflammatory process of mucosal lesions as a part of tissue injury response. The inflammation releases a series of inflammatory mediators such as histamine, bradykinins, serotonin, and prostaglandins. These mediators are reported as the factors of pain and hyperalgesia [20-23]. Normally, ulcers can naturally be resolved within a few weeks. However, it takes longer to heal both cutaneous and oral ulcers in patients with systemic conditions such as diabetes mellitus (DM) due to their defective healing process [2, 24]. These patients may benefit from some remedies to relieve the pain and discomfort such as benzydamine hydrochloride mouthwash or spray [1].

Wound Healing Process

The process of wound healing involves four overlapping but distinct phases: hemostasis, inflammation, proliferation, and remodeling [2] (Figure 1). The normal response starts immediately after tissue injury. First, hemostasis is achieved through the platelet aggregation and the formation of blood clots to prevent further blood loss and becoming a provisional matrix for infiltrating cells [25, 26]. In addition to releasing the clotting factors, the platelets also secrete important growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β). These mediators trigger the wound healing process by attracting neutrophil, macrophage, endothelial cells, smooth muscle cell and fibroblast into the wound [27]. Second, inflammation is the phase when the neutrophils migrate into the wound area to phagocytose damaged tissues and bacteria [28, 29]. Mast cell release cytokines resulting in inflammation at the wound [30]. Toward the end of the inflammatory phase, the macrophages continue phagocytosis and secrete PDGF and TGF-β to stimulate fibroblast migration and proliferation [2]. Then, fibroblasts migrate into the cleaned area and proliferate. New blood vessels are formed by the process called angiogenesis to restore blood supply to the new tissues [31]. The fibroblasts also synthesize and deposit collagen and new extracellular matrix which result in granulation tissue formation [25]. Re-epithelialization occurs within hours after the injury [26]. The final phase of wound healing involves remodelling of collagen and formation of scar tissue [32].

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Although both oral and cutaneous wounds heal through the same 4 phases, they are clinically different [33]. Oral wounds heal more rapidly with less scar formation than cutaneous wounds. The difference in healing could be from the phenotypic

difference between oral and cutaneous fibroblasts [34]. Gingival fibroblasts (GFs) plays important roles in the proliferative phase of oral wound healing [3]. GFs are the connective tissue cell responsible for essential collagen and extracellular matrix deposition for the processes of wound healing and repair [35]. For an effective healing process to occur, various growth factors are required including TGF-β, VEGF, EGF, FGF and Col.

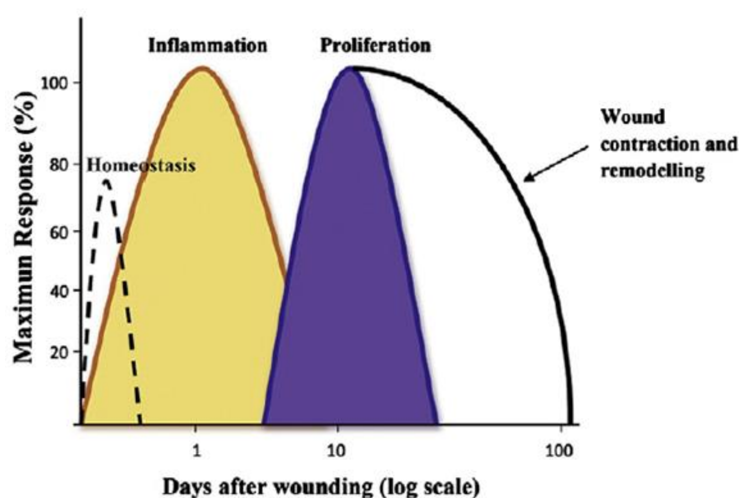

Figure 1 : Four phases of wound healing process

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Transforming growth factor beta (TGF-β)

TGF-β plays an important part to control many cellular functions [36]. The 3 isoforms of TGF-β (TGF-β1, -β2, -β3) share 60-80% of the structural similarity but differ in encoded genes. All TGF-β isoforms are secreted as inactive forms that require activation before binding to their specific receptors—receptor type I (TGFβRI) and receptor type II (TGFβRII) [37]. This cytokine is produced by activated macrophages, keratinocytes, platelets, and fibroblasts [38-41]. TGF-β1 is an important player during healing of the wound by initiating the inflammation, forming the granulation tissue, and stimulating collagen synthesis and wound contraction [42-44]. The expression of TGF-β1 was remarkably less in non-healing wounds, commonly found in diabetic foot

ulcers and chronic venous leg ulcers compared to normal healing ulcers [45, 46]. Moreover, many studies reported the increasing rate of healing after treating the wounds with exogenous TGF-β1 [47-49]. Similar to TGF-β1, TGF-β2 recruits immune cells and fibroblasts into the wound site leading to angiogenesis, granulation tissue formation and collagen deposition [4]. In contrast, TGF-β3 has TGF-β1 antagonistic effect in scar formation. When compared with dermal wounds, the TGF-β3 to TGF-β1 ratio is significantly higher in oral wounds, suggesting that TGF-β3 promotes healing with minimal or no scar formation [50-52]. Furthermore, injection of exogenous TGF-β3 decreased collagen type I synthesis and scar formation [42, 52].

Vascular endothelial growth factor (VEGF)

The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) [53]. VEGF is produced by endothelial cells, fibroblasts, platelets, neutrophils, and macrophages [54-58]. VEGF binds to the receptor Flt-1 (fms-like tyrosine kinase or VEGFR-1) and KDR (kinase-insert domain containing receptor or VEGFR-2) [59]. VEGF was originally recognized as a vascular permeability factor that helps recruiting inflammatory cells. Up-regulation of VEGF is observed during wound healing [60]. The presence of VEGF is important for inflammation, re-epithelialization, granulation tissue formation, and scar tissue formation during the wound healing. In addition, VEGF stimulates endothelial cells to form new blood vessels. The VEGF-A deficient mice showed the delay of the wound closure because of the reduction of the vessel density [61]. Human and mouse saliva contains high concentrations of VEGF which are primarily secreted from parotid gland and submandibular gland, respectively [62, 63]. Removal of murine submandibular gland resulted in reduction of salivary VEGF level and vessel densities in palatal wound. The decreased salivary VEGF level was correlated with

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impaired oral wound healing. Moreover, oral supplement of VEGF in drinking water promoted neovascularization and re-epithelialization in the impaired wounds [64]. Therefore, salivary VEGF is implicated in the superior healing capacity of oral mucosa.

Curcumin

Curcumin (diferuloylmethane) belongs to a family of chemicals known as curcuminoids, a major constituent in turmeric rhizome responsible for its yellow colour [5] (Figure 2 and 3).

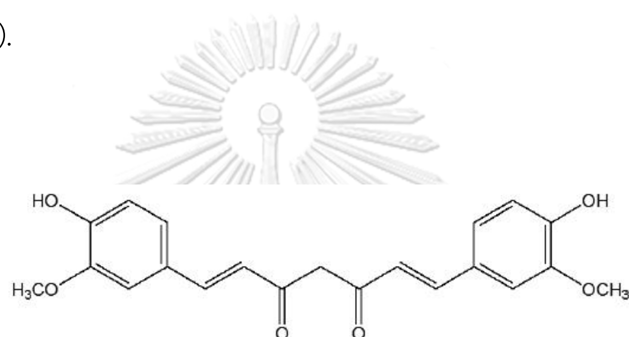

Figure 2 : Chemical structures of curcuminoids

Figure 3 : Turmeric rhizome

The accumulating data demonstrated that curcumin is considered as a potent wound healing agent because of its strong anti-oxidant [65], anti-inflammatory [66], and anti-infective activities [67]. Previous studies showed that curcumin promote healing in different types of wounds. Application of curcumin on CO_2 laser-induce skin

wounds promoted re-epithelialization [68]. Previous study also reported faster healing of wounds in the curcumin-treated diabetic mice when compared with the control group. Histological study revealed greater re-epithelialization, migration of numerous cells, neovascularization in the dermis, and deposition of collagen. Moreover, immunohistochemistry showed an increase of TGF-β1 known to accelerate wound healing suggesting that curcumin might regulate the TGF-β1 activity [43, 69]. Another study showed that curcumin treatment promoted wound healing possibly by affecting the levels of several enzymes involved in reactive oxygen species production [70]. Furthermore, curcumin significantly protected human keratinocytes and fibroblasts from H_2O_2-induced oxidative damage respectively [71]. Topical application of curcumin to irradiated skin can stimulated wound healing and reduced the expression of cyclooxygenase-2 (COX-2) and nuclear factor-kappa B (NF-kB) [72]. Curcumin also enhanced blood vessel formation and promoted wound healing by increasing the expression of VEGF and TGF-β1 in granulation tissues of diabetic rats [73]. For oral wound healing, an animal study demonstrated faster wound healing of mucosal oral ulcer at upper labial gingiva in curcumin-treated group when compared with the control group [12]. Curcumin – ghee formulation and hyaluronic acid is therapeutically effective for periodontal wound healing in dogs. Histologic analysis found substantial decrease in number of the inflammatory cells and acceleration of wound healing process [74]. Clinical study showed that turmeric gel can be a good adjunctive treatment to routine periodontal prophylaxis and deep cleaning [75]. The authors reported significant improvement of all clinical periodontal parameters in comparison with control group [75]. Moreover, curcumin gel has been shown to significantly reduce the severity and the size of lesion of mucositis in patients undergoing head and neck

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cancer radiotherapy [76, 77]. Similarly, curcumin mouthwash expedited wound healing compared with 0.2% chlorhexidine mouthwash in radio-chemotherapy-induced oral mucositis patients [78]. These data has established the benefits of curcumin in acceleration of wound healing.

Chapter 3
Materials and methods

Materials

1. Dulbecco’s modified Eagle’s medium (DMEM); Sigma, USA
2. Fetal bovine serum (FBS); Gibco, USA
3. L-glutamine; Gibco, USA
4. Antibiotic-antimycotic solution; Gibco, USA
5. Phosphate buffered saline (PBS); Gibco, USA
6. 0.25% Trypsin-EDTA; Gibco, USA
7. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT); Invitrogen, USA
8. Curcumin; Sigma, USA
9. Primers for TGF-β1; Wardmedic, Thailand
10. Primers for TGFβR I; Wardmedic, Thailand
11. Primers for TGFβR II; Wardmedic, Thailand
12. Primers for VEGF; Wardmedic, Thailand
13. Total RNA MiNi kit; Geneaid, USA
14. DNase I, RNase-free; Thermo scientific, USA
15. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT); Promaga, USA
16. dNTP Mix, Thermo scientific, USA
17. Ribolock RNase inhibitor, Thermo scientific, USA
18. Tag DNA polymerase; Thermo scientific, USA
19. iTaq™ Universal SYBR® Green Supermix; Bio-Rad, USA

20. ELISA kit; R&D Systems, USA
21. Nuclease free water
22. Dimethylsulfoxide (DMSO)
23. Distilled water
24. Agarose
25. Ethidium bromide
26. Absolute ethanol
27. 70% ethanol
28. Microcentrifuge tubes
29. PCR tubes
30. Disposable pipette tips (10, 200, and 1000 microlitre)
31. 60- and 100-mm tissue culture dishes
32. 24- and 96-wells tissue culture polystyrene plates
33. Disposable latex gloves

Methods

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Cell culture

Human gingival fibroblasts were prepared from healthy gingival tissue explants from patients who were undergoing a minor oral surgery such as tooth extraction or surgical removal of third molars for orthodontic reasons. The complete consent forms were obtained from the subjects. The study protocol was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University.

The gingival tissue was removed from the cervical third of extracted tooth with scalpel, and then washed twice with PBS. The collected gingival tissue was cut into small pieces and placed in tissue culture dishes (60-mm dishes) with the DMEM

supplement consisting of 10% FBS, 1% L-glutamine, and 1% Antibiotic-Antimycotic, under the humidified atmosphere with 37°C and 5% carbon dioxide. Once confluent, the cells were subcultured with 0.125% trypsin. During the subculturing, the medium was renewed every 2 days. The cells from the third to the fifth passage were used in the experiments.

Cell viability assays

To determine curcumin toxicity, human gingival fibroblasts were plated at 5×10^3 cells per well in 96-well flat-bottomed tissue culture plates in DMEM with 10% FBS for 24 hours. Next, the medium was changed to the serum-free-DMEM containing 0-50 μM of curcumin and then incubated for another 24 hours. Following incubation, cell viability was measured by the MTT assay. After removing the culture media, 100 μL of the MTT solution was added into each well and incubated for 90 minutes until the formazan crystal formation was visible under the microscope. At the end of the incubation period, the MTT solution was removed, and 100 μL of DMSO was added to the well and swirled gently to solubilize the formed formazan crystals. Absorbance of the dye was measured using a plate reader (EZ Read 400; Biochrom) at a wavelength of 570 nm. Cell survival was calculated as follows:

Percentage of survival = (mean experimental absorbance/mean control absorbance) × 100

Curcumin treatment

To study the effect of curcumin on gene expression, human gingival fibroblasts were plated at 6×10^5 cells per plate in tissue culture dishes (60-mm dishes) in DMEM with 10% FBS. On the following day, the cells were washed and switched to serum-

free DMEM for 24 hours. After that, the cells were treated with 0, 0.1, 1, 10, 20 μM of curcumin for 24 hours.

RNA extraction and Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Total RNA Mini Kit. The concentration of the RNA is was determined by measuring the absorbance at 260 and 280 nm with a Thermo Scientific NanoDrop™ 2000 Spectrophotometer. Total RNA (2 μg) was reverse transcribed by Moloney Murine Leukemia Virus Reverse Transcriptase according to the manufacturer's instruction.

Real-time PCR assay

The cDNA template was amplified by iTaq™ Universal SYBR® Green Supermix. The mixture contains 5 μL of using iTaq™ Universal SYBR® Green Supermix (2x), 0.25 μL of each primer, 2.5 μL of DNA template. Nuclease-free water was added to a final volume of 10 μL. The PCR program setting was at 95°C for 5 min followed by 45 cycles for the amplification phase; each consists of denaturation for 30 sec at 95°C, annealing for 30 sec at 56°C for GAPDH and 50°C for other genes, and extension for 30 sec at 72°C. The sequences of PCR primers are shown in Table 1.

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Table 1: Primer sequences used for PCR

Gene	Primer sequences	Product size
TGF-β1	Forward: 5′-GGATACCAACTATTGCTTCAGCTCC-3′ Reverse: 5′-AGGCTCCAAATGTAGGGGCAGGGCC-3′	156
TGFβR I	Forward: 5′-GGTCTTGCCCATCTTCACAT-3′ Reverse: 5′-TCTGTGGCTGAATCATGTCT-3′	155
TGFβR II	Forward: 5′-GTCTACTCCATGGCTCTGGT-3′ Reverse: 5′-ATCTGGATGCCCTGGTGGTT-3′	197
VEGF	Forward: 5′-AGACCCTGGTGGACATCTTC-3′ Reverse: 5′-TGCATTCACATTTGTTGT GC-3′	225
GAPDH	Forward: 5′-TGAACGGGAAGCTCACTGG-3′ Reverse: 5′-TCCACCACCCTGTTGCTGTA-3′	307

Statistical analyses

Each experiment was repeated at least 3 times using gingival fibroblasts from 3 different subjects. The data were reported as mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA test followed by post-hoc or Kruskal-Wallis test followed by Mann-Whitney U test. The differences at $p < 0.05$ was considered as statistically significant.

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Chapter 4
Results

Effects of curcumin on the viability of human gingival fibroblasts

Our results demonstrated that curcumin at 0.1-20 μM caused no significant change in cell viability, whereas 30 and 50 μM curcumin caused a significant cytotoxicity ($p < 0.05$) (Figure 4). Therefore, curcumin at 0.1-20 μM was used in the subsequent experiments.

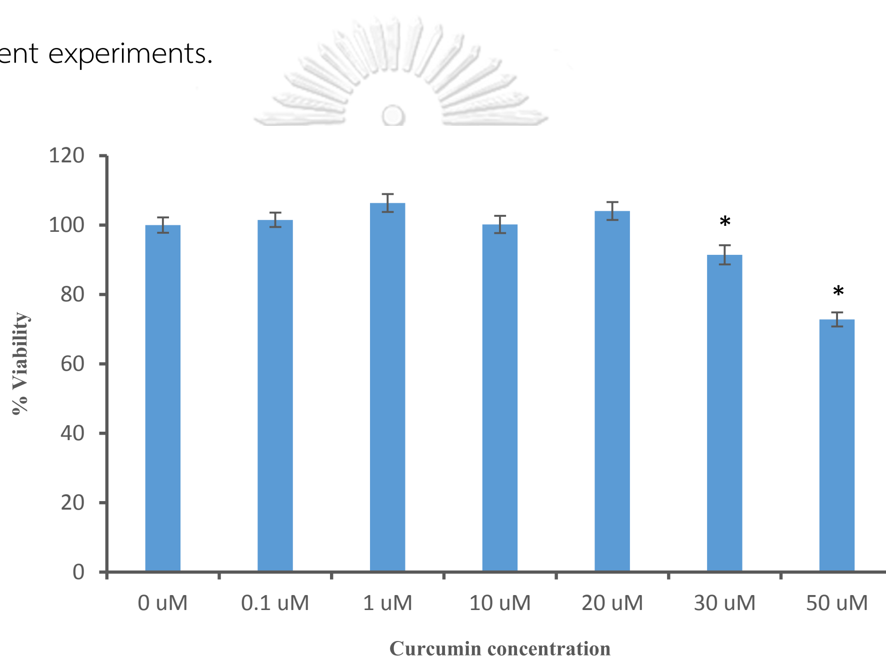

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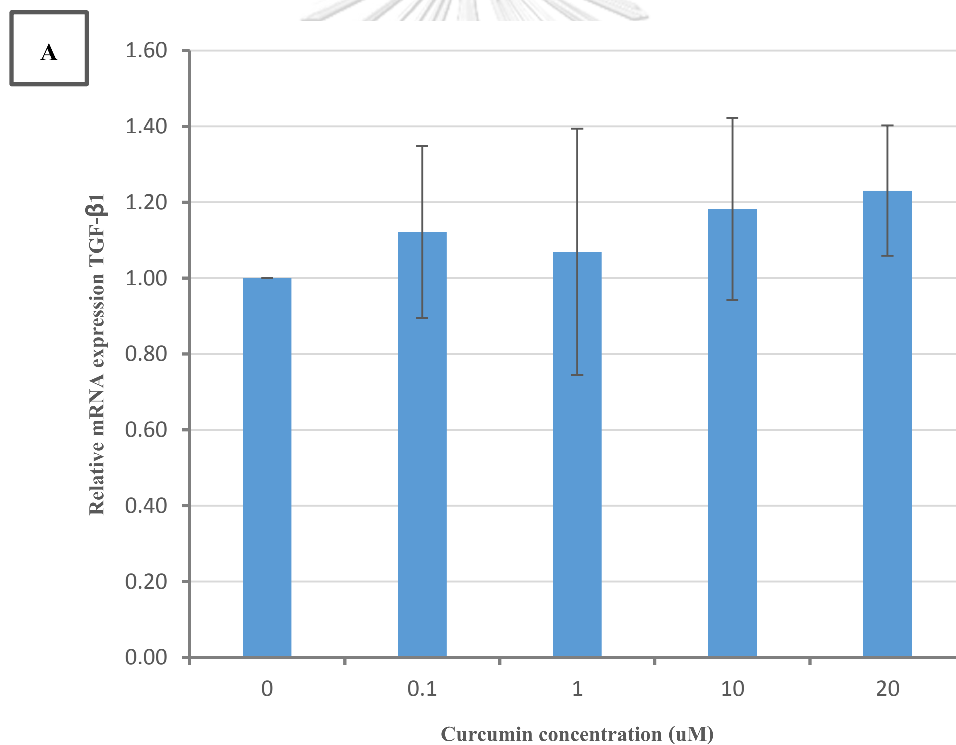
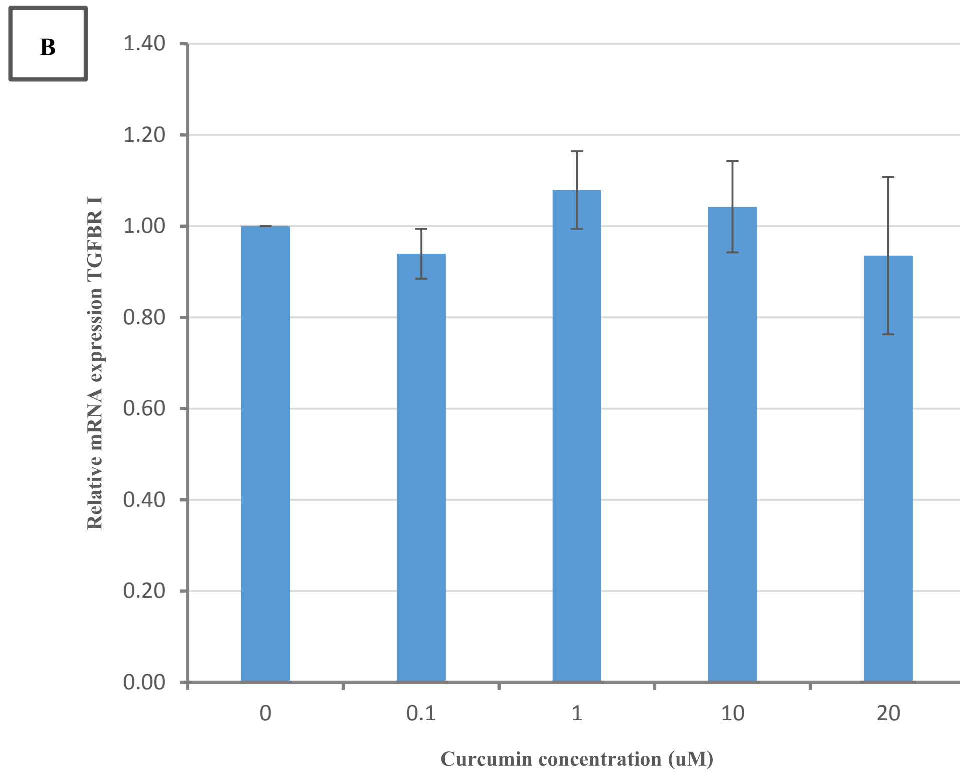
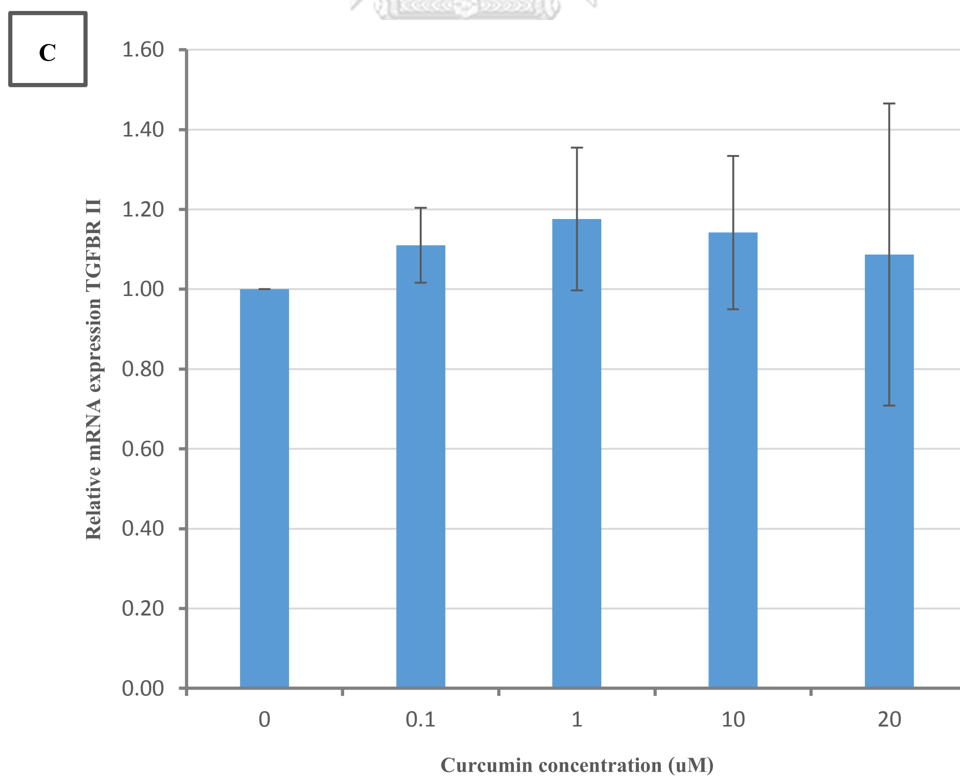
Figure 4 : Cytotoxicity test. Human gingival fibroblasts were plated at 5×10^3 cells per well in 96-well plates, then treated with varying concentrations of curcumin or DMSO for 24 hours. The cell viability was measured by MTT assay. The data showed percentage of cell viability compared to untreated group at 24 hours. *indicates compared to the untreated group; $p < 0.05$.

Effects of curcumin treatment on wound healing-related gene expression

The expression of TGF-β1, TGFβR I, TGFβR II and VEGF was determined after treating human gingival fibroblasts with various curcumin concentrations (0-20 μM) for 24 hours. The data showed that curcumin at the concentrations of 0.1-20 μM slightly increased TGF-β1 expression in a dose-dependent manner. On the other hand, only curcumin at 1 and 10 μM mostly increased TGFβR I, TGFβR II and VEGF expression. However, none of these induction showed statistical significant difference from untreated group (Figure 5).




B
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative mRNA expression TGFBR I
0
0.1
1
10
20
Curcumin concentration (uM)


C
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative mRNA expression TGFBR II
0
0.1
1
10
20
Curcumin concentration (uM)

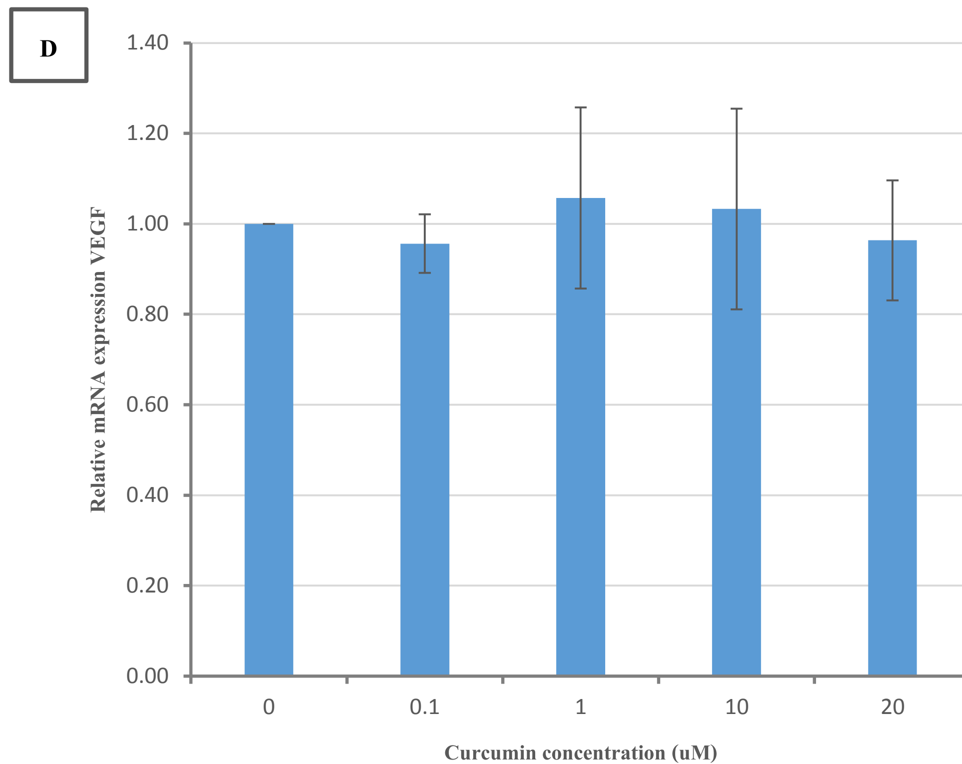


Figure 5 : The expression of TGF-β1 (A), TGFβR I (B), TGFβR II (C) and VEGF (D) in human gingival fibroblasts in response to curcumin. Cells were plated at 6×10^5 cells per plate in tissue culture dishes, then treated with varying concentrations of curcumin or DMSO for 24 hours. The level of genes expression was determined with the real-time PCR. The data are the mean ± SD. Kruskal-Wallis H analysis showed no statistical significance.

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Chapter 5
Discussion

Previous studies demonstrated that curcumin enhanced wound healing in several animal models [9, 11, 12]. However, the mechanism by which curcumin promotes healing of wounds remains unclear. This study investigated the effect of curcumin on the wound healing-related gene expression in human gingival fibroblasts. First, we determined the non-cytotoxic concentration of curcumin on human gingival fibroblasts. We found that curcumin at 0.1 - 20 μM did not significantly affect cell viability while 30 and 50 μM is cytotoxic. Chen et al. reported a similar finding that curcumin at 1-10 μM is not toxic to human gingival fibroblasts [79]. However, curcumin cytotoxicity was demonstrated at the concentration of 20 μM for renal fibroblasts [80]. These data suggest that the non-cytotoxic concentration of curcumin is different for each cell types and must be taken into consideration for the clinical application.

There are many growth factors that are secreted in response to injury and during wound healing. TGF signaling plays an important role for wound healing including scar formation and tissue regeneration [81]. TGF-β signals through TGFβR I/II that stimulate small mothers against decapentaplegic (Smad) protein in the cytoplasm to regulate the transcription of target genes [81]. Previous study showed that topical application of exogenous TGF-β1 expedited the rate of wound healing [47-49]. Mani et al. reported an increased expression of TGF-β1, mainly in macrophages, and TGFβR II in curcumin treated wounds, at both the protein and mRNA level [41]. Curcumin also increased TGFβR I expression in dexamethasone-impaired wounds [41]. Moreover, curcumin promoted the healing of indomethacin-induced gastric ulceration by enhancing collagenization and angiogenesis via induction of matrix metalloproteinase

(MMP)-2, TGF-β and VEGF at protein and mRNA levels [82]. Another study demonstrated that topical application of curcumin ointment promoted cutaneous wound healing in excisional model in rats by increasing the level of TGF-β1 as compare with control [83]. Sidhu et al. reported faster closure of curcumin-treated wound characterized by enhanced granular tissue formation through the increased expression of TGF-β in both normal and diabetic mice [69, 84].

VEGF is responsible for stimulating angiogenesis during wound healing [85]. VEGF acts predominantly through two high-affinity receptor tyrosine kinases including VEGFR-1 and VEGFR-2 [59]. Wilgus et al. demonstrated the importance of VEGF signaling by treating the wounds with anti-VEGFR-1 or inhibitors of VEGF signaling which resulted in delayed wound healing [86]. Sharma et al. showed that curcumin hastened the healing of indomethacin-induced ulcer by angiogenesis and collagenization via accelerating the expression of VEGF and TGF-β1 at earlier time points in wound healing process [82]. Furthermore, curcumin promoted neovascularization and accelerated wound healing in diabetic rats by increasing VEGF and TGF-β1 mRNA expressions [73].

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Our results showed no significant difference in TGF-β1, TGFβR I, TGFβR II and VEGF gene expression between curcumin treatment and control group. This probably due to some limitation of this *in vitro* model. It is well known that wound healing process requires various growth factors or various signal interactions from several cell types. Topman et al. showed that curcumin had no effect on fibroblast migration in the scratch test[31]. These results suggested that fibroblast migration is dependent on different factors which is not possible to mimic in an *in vitro* assay [31]. Mani et al. found that curcumin increases TGF-β1 expression in the wound, mainly in macrophages [41]. Therefore, fibroblasts may not be the main target cell of curcumin. Previous study

demonstrated that curcumin stimulated expression and secretion of interleukin-1 (IL-1) in macrophage [87]. Another study reported that IL-1 which mainly produced by macrophage induced VEGF expression in synovial fibroblasts [88]. Curcumin increased transcription and secretion of anti-inflammatory factors such as TGF-β in activated macrophage which stimulated collagen type I synthesis in fibroblasts [89, 90]. Therefore , curcumin may have an indirect effect on fibroblasts by acting on other cell types such as macrophages or lymphocytes which in turn regulate gene expression in fibroblasts [91] Furthermore, normal fibroblasts and wounded fibroblasts are phenotypically different [92]. Wounded fibroblasts undergo several phenotypical changes including decreased proliferation potential, increased collagen synthesis and matrix contraction [93, 94]. Moreover, wounded fibroblasts spontaneously synthesize nitric oxide while normal fibroblasts required stimulation [92]. Therefore, an *in vitro* wound healing model that simulating the wound in human gingival fibroblast culture is another way to study the effect of curcumin on wound healing.

In summary, our results showed that curcumin did not significantly alter the mRNA expression of TGF-β1, TGFβR I, TGFβR II and VEGF in human gingival fibroblasts
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at 24 hours after treatment. However, further studies are required to investigate the mechanism of curcumin to promote wound healing.

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APPENDIX

Statistical analysis

Test of normality (% cell viability)

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
%viability	.030	336	.200*	.992	336	.080

a. Lilliefors Significance Correction

*. This is a lower bound of the true significance.

Statistical analysis of % cell viability by one-way ANOVA test

ANOVA

%viability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37944.614	6	6324.102	22.703	.000
Within Groups	91644.430	329	278.554		
Total	129589.044	335			

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Statistical analysis of % cell viability by LSD post-hoc test (multiple comparisons)

Multiple Comparisons

%viability
LSD

(I) concentration of CCM	(J) concentration of CCM	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 uM	0.1 uM	-1.49313	3.40682	.661	-8.1950	5.2088
	1 uM	-6.31937	3.40682	.065	-13.0213	.3825
	10 uM	-.19042	3.40682	.955	-6.8923	6.5115
	20 uM	-4.05021	3.40682	.235	-10.7521	2.6517
	30 uM	8.56625*	3.40682	.012	1.8643	15.2682
	50 uM	27.17937*	3.40682	.000	20.4775	33.8813
0.1 uM	0 uM	1.49313	3.40682	.661	-5.2088	8.1950
	1 uM	-4.82625	3.40682	.158	-11.5282	1.8757
	10 uM	1.30271	3.40682	.702	-5.3992	8.0046
	20 uM	-2.55708	3.40682	.453	-9.2590	4.1448
	30 uM	10.05938*	3.40682	.003	3.3575	16.7613
	50 uM	28.67250*	3.40682	.000	21.9706	35.3744
1 uM	0 uM	6.31937	3.40682	.065	-.3825	13.0213
	0.1 uM	4.82625	3.40682	.158	-1.8757	11.5282
	10 uM	6.12896	3.40682	.073	-.5729	12.8309
	20 uM	2.26917	3.40682	.506	-4.4327	8.9711
	30 uM	14.88563*	3.40682	.000	8.1837	21.5875
	50 uM	33.49875*	3.40682	.000	26.7968	40.2007
10 uM	0 uM	.19042	3.40682	.955	-6.5115	6.8923
	0.1 uM	-1.30271	3.40682	.702	-8.0046	5.3992
	1 uM	-6.12896	3.40682	.073	-12.8309	.5729
	20 uM	-3.85979	3.40682	.258	-10.5617	2.8421
	30 uM	8.75667*	3.40682	.011	2.0548	15.4586
	50 uM	27.36979*	3.40682	.000	20.6679	34.0717
20 uM	0 uM	4.05021	3.40682	.235	-2.6517	10.7521
	0.1 uM	2.55708	3.40682	.453	-4.1448	9.2590
	1 uM	-2.26917	3.40682	.506	-8.9711	4.4327
	10 uM	3.85979	3.40682	.258	-2.8421	10.5617
	30 uM	12.61646*	3.40682	.000	5.9146	19.3184
	50 uM	31.22958*	3.40682	.000	24.5277	37.9315
30 uM	0 uM	-8.56625*	3.40682	.012	-15.2682	-1.8643
	0.1 uM	-10.05938*	3.40682	.003	-16.7613	-3.3575
	1 uM	-14.88563*	3.40682	.000	-21.5875	-8.1837
	10 uM	-8.75667*	3.40682	.011	-15.4586	-2.0548
	20 uM	-12.61646*	3.40682	.000	-19.3184	-5.9146
	50 uM	18.61312*	3.40682	.000	11.9112	25.3150
50 uM	0 uM	-27.17937*	3.40682	.000	-33.8813	-20.4775
	0.1 uM	-28.67250*	3.40682	.000	-35.3744	-21.9706
	1 uM	-33.49875*	3.40682	.000	-40.2007	-26.7968
	10 uM	-27.36979*	3.40682	.000	-34.0717	-20.6679
	20 uM	-31.22958*	3.40682	.000	-37.9315	-24.5277
	30 uM	-18.61312*	3.40682	.000	-25.3150	-11.9112

*. The mean difference is significant at the 0.05 level.

Statistical analysis of TGF-β1 gene expression by Kruskal-Wallis Test

Kruskal-Wallis Test

Ranks

	concentration of CCM	N	Mean Rank
TGFb1 fold change	0 uM	3	3.00
	0.1 uM	3	8.33
	1 uM	3	8.67
	10 uM	3	9.67
	20 uM	3	10.33
	Total	15	

Test Statistics[a,b]

	TGFb1 fold change
Chi-Square	5.103
df	4
Asymp. Sig.	.277

a. Kruskal Wallis Test

b. Grouping Variable: concentration of CCM

Statistical analysis of TGFβR I gene expression by Kruskal-Wallis Test

Kruskal-Wallis Test

Ranks

	concentration of CCM	N	Mean Rank
TGFbr1 fold change	0 uM	3	8.50
	0.1 uM	3	4.50
	1 uM	3	11.00
	10 uM	3	10.17
	20 uM	3	5.83
	Total	15	

Test Statistics[a,b]

	TGFbr1 fold change
Chi-Square	4.735
df	4
Asymp. Sig.	.316

a. Kruskal Wallis Test

b. Grouping Variable: concentration of CCM

Statistical analysis of TGFβR II gene expression by Kruskal-Wallis Test

Kruskal-Wallis Test

Ranks

	concentration of CCM	N	Mean Rank
TGFbr2 fold change	0 uM	3	4.50
	0.1 uM	3	10.33
	1 uM	3	9.67
	10 uM	3	9.50
	20 uM	3	6.00
	Total	15	

Test Statistics[a,b]

	TGFbr2 fold change
Chi-Square	4.089
df	4
Asymp. Sig.	.394

a. Kruskal Wallis Test

b. Grouping Variable: concentration of CCM

Statistical analysis of VEGF gene expression by Kruskal-Wallis Test

Kruskal-Wallis Test

Ranks

	concentration of CCM	N	Mean Rank
VEGF fold change	0 uM	3	8.00
	0.1 uM	3	6.50
	1 uM	3	9.50
	10 uM	3	9.00
	20 uM	3	7.00
	Total	15	

Test Statistics[a,b]

	VEGF fold change
Chi-Square	.984
df	4
Asymp. Sig.	.912

a. Kruskal Wallis Test

b. Grouping Variable: concentration of CCM

VITA

Miss Auspreeya Rujirachotiwat graduated with First class honors of D.D.S. (Doctor of Dental Surgery) from Faculty of Dentistry, Chulalongkorn University in 2012, and had worked at Banphue hospital in Udon Thani province for 3 years. She started her Master degree program and residency training program in Pediatric Dentistry at Graduate School, Chulalongkorn University in 2015.