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Effect of Curcumin on the Expression of Wound Healing-related Genes
in Human Gingival Fibroblasts
ผลของสารสกัดจากขมิ้นชันต่อการแสดงออกของยีนที่เกี่ยวข้องกับการหายของแผล
ในเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์

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ABSTRACT

This study investigated the effect of curcumin on the expression of wound healing- related genes including TGF-β1 and 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 30 and 50 µM curcumin are cytotoxic. Curcumin dose dependently increased the TGF-β1 expression while 1 µM curcumin is the optimal concentration for inducing 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.

บทคัดย่อ

การศึกษาผลของเคอร์คูมินต่อการแสดงออกของยีนที่เกี่ยวข้องกับการหายของแผลในเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์ เช่น ทีจีเอฟเบต้าชนิดที่ 1 และ วีอีจีเอฟ ความเป็นพิษของเคอร์คูมินถูกทดสอบด้วยวิธีเอ็มทีที จากนั้นเซลล์จึงถูกกระตุ้นด้วยเคอร์คูมินที่ความเข้มข้นต่างๆเป็นเวลา 24 ชั่วโมงและทำการตรวจสอบการแสดงออกของยีนด้วยวิธีควอนทิเททีฟพีซีอาร์ พบว่าเคอร์คูมินที่ 0.1- 20 ไมโครโมลาร์ ไม่มีผลต่อการมีชีวิตของเซลล์ ส่วนความเข้มข้น 30 และ 50 ไมโครโมลาร์มีความเป็นพิษต่อเซลล์ เคอร์คูมินกระตุ้นการแสดงออกของทีจีเอฟเบต้าชนิดที่ 1 ตามความเข้มข้นที่มากขึ้นขณะที่ความเข้มข้น 1 ไมโครโมลาร์เป็นความเข้มข้นที่กระตุ้นการแสดงออกของวีอีจีเอฟ ได้ดีที่สุด อย่างไรก็ตามไม่พบความแตกต่างอย่างมีนัยสำคัญจากการกระตุ้นดังกล่าว โดยสรุป เคอร์คูมินน่าจะมีผลควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการหายของแผลในเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์ แต่จำเป็นต้องมีการศึกษาเพิ่มเติมต่อไป

Keywords: Curcumin, Gingival fibroblasts, Wound healing process
คำสำคัญ: สารสกัดจากขมิ้นชัน เซลล์ไฟโบรบลาสต์จากเหงือก กระบวนการหายของแผล

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Introduction

Ulceration in the oral cavity is the cause of pain and discomfort that could significantly affect normal food intake and quality of life. Although there are several recommended remedies to relieve the symptoms, none of them accelerates the healing process (Scully and Shotts, 2001). Wound healing is a complex process controlled by signals from several cell types including keratinocyte, immune cells, endothelial cell and fibroblasts (Pastar et al., 2014). The process of wound healing consists of four stages: 1) hemostasis, 2) inflammation, 3) proliferation, and 4) remodeling (Diegelmann and Evans, 2004). Gingival fibroblasts play an important role in the proliferative phase of oral wound healing by secreting several growth factors such as transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) (Aukhil, 2000). It has been demonstrated that TGF-β1 play a key role in wound healing process such as initiating the inflammation, forming the granulation tissues, and stimulating collagen synthesis and wound contraction (Montesano and Orci, 1988; Pakyari et al., 2013). On the other hand VEGF is important for re-epithelialization, granulation tissue and scar tissue formation during the wound healing (Barrientos et al., 2008). Therefore, these two biological molecules are crucial for the healing of wound (Barrientos et al., 2008).

Curcumin (diferuloylmethane) belongs to a family of chemicals known as curcuminoids, a major constituent in turmeric rhizome responsible for its yellow colour (Sharma et al., 2004). It has been used in India for thousands of years as a spice and medicinal herb (Chattopadhyay et al., 2004). Curcumin has anti-inflammatory (Liang et al., 2009), anti-oxidant (Meng et al., 2013), anti-bacterial (Tyagi et al., 2015) and anti-carcinogenic properties (Aggarwal et al., 2004).

Curcumin has also been shown to have significant wound healing properties (Akbik et al., 2014). It is suggested that curcumin play a role in wound healing by stimulating the production of the growth factors therefore accelerating wound healing (Lopez-Jornet et al., 2011; Sidhu et al., 1999). Slow delivery of curcumin from collagen matrix has been shown to improve dermal wound healing in rats (Gopinath et al., 2004). Biochemical and histological analysis showed decreased wound area, enhanced fibroblast proliferation and higher level of antioxidant enzyme in rat wounds treated with curcumin incorporated collagen matrix compared with collagen matrix alone (Gopinath et al., 2004). Curcumin promoted collagen production and decreased matrix metalloproteinase-9 production in the superficial abrasion skin of rats (Bhagavathula et al., 2009). A biodegradable hydrogel system containing curcumin encapsulated in micelles enhanced cutaneous wound repair with increased tensile strength and epidermal thickness in incision model and enhanced wound closure in excision model (Gong et al., 2013). Curcumin improved the healing process of irradiated wounds by decreasing the duration of healing period while enhancing the rate of wound contraction and the synthesis of collagen, hexosamine, DNA and nitric oxide (Jagetia and Rajanikant, 2004).

Curcumin has also been shown to promote oral wound healing. Recent animal study, demonstrated faster wound healing of mucosal oral ulcer at upper labial gingiva in curcumin-treated group in comparison with the control group (Lim et al., 2016). In addition, clinical study reported that topical curcumin gel significantly reduced the size of minor aphthous ulcers in comparison with placebo. Moreover, curcumin gel decreased pain intensity in these patients based on perceived pain rating scale (Manifar et al., 2012). Although curcumin has been shown to promote oral wound

healing, the cellular response to curcumin treatment remains unclear. The purpose of this study is to investigate the effect of curcumin on the expression of TGF-β1 and VEGF in human gingival fibroblasts.

Objectives of the study

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

2. To study the effect of curcumin on the expression of TGF-β1 and VEGF in human gingival fibroblasts by qPCR.

Methodology

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 (HREC-DCU 2017-037).

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 Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) consisting of 10% Fetal bovine serum (FBS, Gibco, USA), 1% L-glutamine (Gibco, USA), and 1% Antibiotic-Antimycotic (Gibco, USA), under the humidified atmosphere with 37°C and 5% carbon dioxide. After reaching the confluence, the cells were subcultured with 0.125% trypsin (Gibco, USA). 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 assay

To study the toxicity of curcumin, 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 replaced with the serum-free-DMEM along with 0-50 µM of curcumin and then incubated for another 24 hours. Following incubation, cell viability was determined by the MTT assay. When the medium was removed, 100 µL of the MTT solution (Invitrogen, USA) 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 mixed 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 a starvation medium (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 (Geneaid, Taiwan). The concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm with a Thermo Scientific NanoDrop™ 2000 Spectrophotometer. Two μg of total RNA of each sample was reverse transcribed to single-strand cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, USA) following the manufacturer's instruction.

Real-time PCR assay

The amplification of the cDNA template was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA). 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 primer sequences used for PCR amplification were shown in Table 1.

Table 1 Primer sequences used for PCR

Gene	Primer sequence
TGF-β1	Forward: 5'-GGATACCAACTATTGCTTCAGCTCC- 3' Reverse:5'-AGGCTCCAAATGTAGGGGCAGGGCC- 3'
VEGF	Forward: 5'-AGACCCTGGTGGACATCTTC- 3' Reverse: 5'-TGCATTCACATTTGTTGTGC- 3'
GAPDH	Forward: 5'-TGAACGGGAAGCTCACTGG- 3' Reverse: 5'-TCCACCACCCTGTTGCTGTA- 3'

Statistical analysis

Each experiment was repeated at least 3 times with gingival fibroblasts from 3 different subjects. The data were reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using Kruskal-Wallis test followed by Mann-Whitney U test. The differences at $p < 0.05$ were considered as statistically significant.

Results

Effects of curcumin on the viability of human gingival fibroblasts

First, we examined the cytotoxicity of curcumin on human gingival fibroblasts. Figure 1 showed that the concentration of curcumin at up to 20 µM caused no change in cell viability whereas the higher concentrations of curcumin at 30 and 50 µM induced significant dose dependent cytotoxicity ($p < 0.05$). Thus, the concentration of curcumin at 0-20 µM was selected to be used in the subsequent experiments.
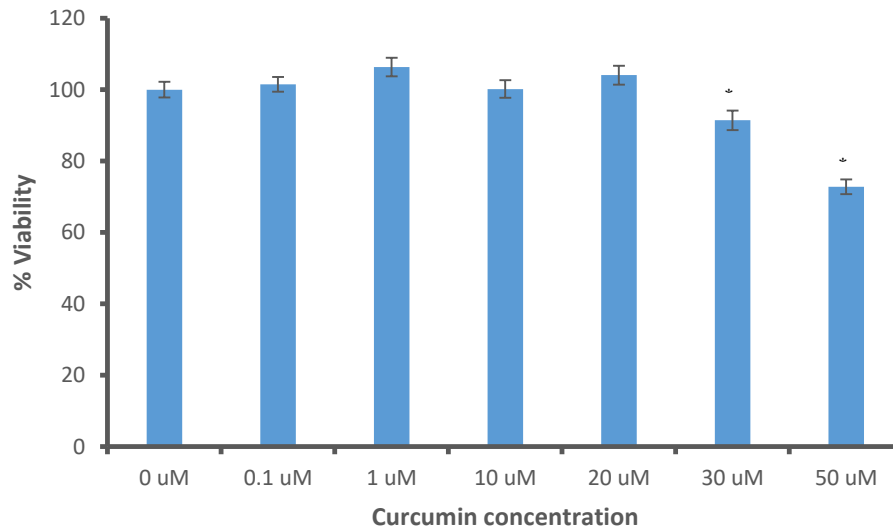

Figure 1 Cytotoxicity of curcumin on human gingival fibroblasts. Cells were plated at 5×10^3 cells per well in 96- well plates, then treated with varying concentrations of curcumin or DMSO for 24 hours. The cell viability was measured by MTT assay. The data are the mean ± SEM. *indicates compared to the controlled group; $p < 0.05$.

Effects of curcumin treatment on the gene expression

The expression of TGF-β1 and VEGF was determined by treating human gingival fibroblasts in the presence of various curcumin concentrations (0-20 µM) for 24 hours. The results showed that curcumin at the concentrations of 0.1, 1, 10, and 20 µM dose dependently increased TGF-β1 expression. Treatment with 1 and 10 µM curcumin increased VEGF expression. However, the difference between the curcumin induced gene expression was not statistically significant when compared with untreated control ($p > 0.05$) (Figure 2)
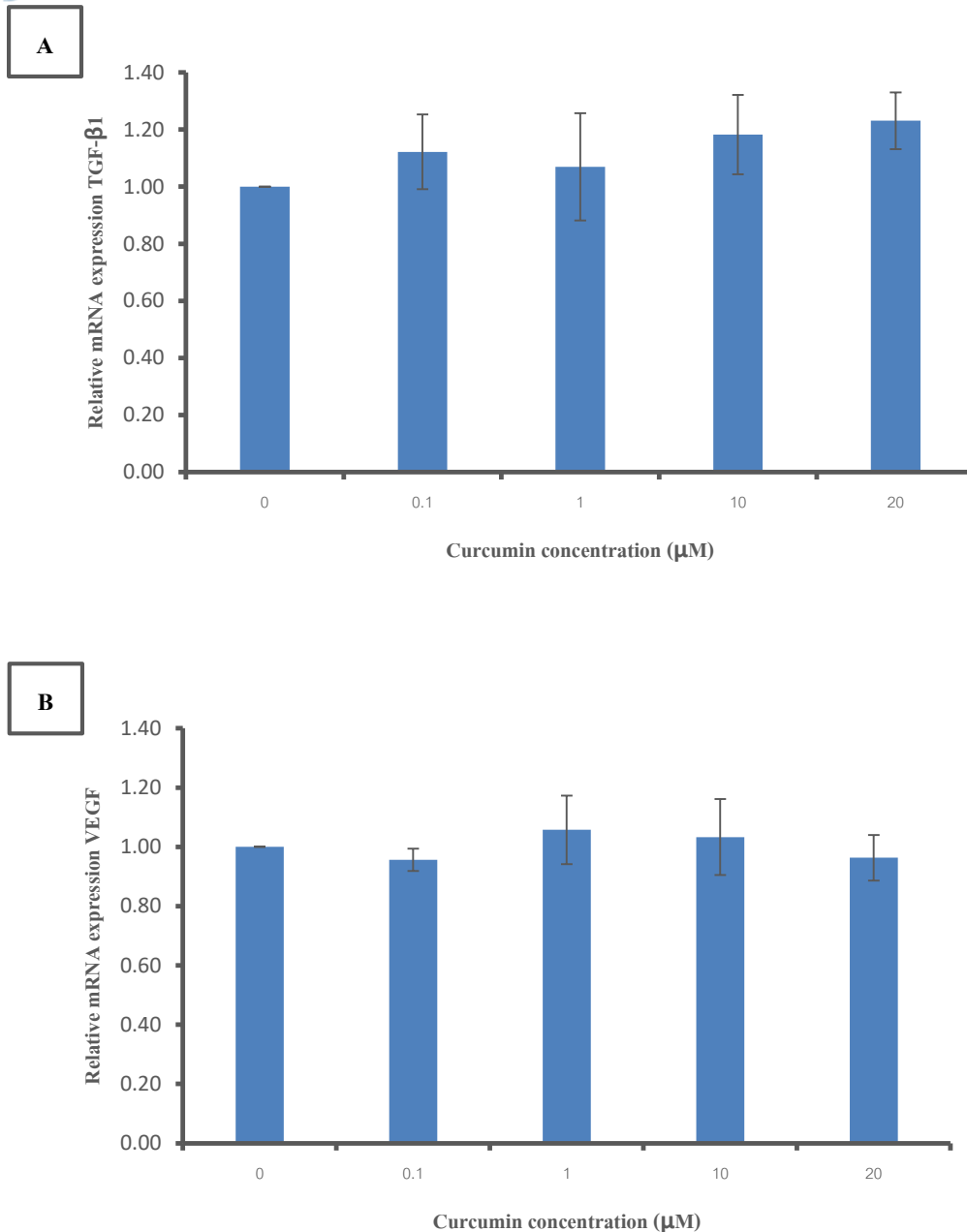
Figure 2 The expression of TGF-β1 (A) and VEGF (B) in human gingival fibroblasts in response to curcumin. Cells were plated at 6×10^5 cells per plate in tissue culture dishes, then treated with varying concentrations of curcumin or DMSO for 24 hours. The level of genes expression was determined with the real-time PCR. The data are the mean ± SEM.

Discussion and Conclusions

In this study, we investigated the effect of curcumin on the expression of genes involved in wound healing in human gingival fibroblasts including TGF-β1 and VEGF. Our data revealed that curcumin slightly increased TGF-β1 and VEGF mRNA expression but the induction was not considered statistically significant.Both TGF-β1 and VEGF play important role in wound healingstudies the increasing rate of wound healing by the application of exogenous TGF-β1 (Mustoe et al., 1987; Quaglino et al., 1991; Quaglino et al., 1990). VEGF is a strong positive regulator of

angiogenesis to stimulate endothelial cell functions needed for the new blood vessel formation (Leung et al., 1989). The VEGF-A deficient mice showed the delay of the wound closure because of the reduction of the vessel density (Rossiter et al., 2004). It has been reported that curcumin enhanced blood vessel formation and promoted wound healing by increasing the expression of VEGF and TGF-β1 in granulation tissues of diabetic rats (Kant et al., 2015; Sidhu et al., 1999). Another study showed that curcumin enhanced cutaneous wound healing in excision wound model in rats by promoting wound contraction and increasing the level of TGF-β1 significantly as compared with control group (Prasad et al., 2017). Sharma et al., has also reported that curcumin promoted the healing of indomethacin-induced gastric ulceration by increasing of collagenization and angiogenesis via up-regulation the expression of matrix metalloproteinase (MMP)-2, TGF-β and VEGF at protein and mRNA levels (Sharma et al., 2012). In addition, TGF-β1 was enhanced by curcumin treatment in both unimpaired and dexamethasone-impaired wounds in animal model (Mani et al., 2002).

In contrast, our data revealed that curcumin treatment of gingival fibroblasts culture only slightly increased TGF-β1 and VEGF mRNA expression. Similar results were obtained in another study that curcumin has no effect on the fibroblast migration after infliction of localized mechanical damage to the cultures (Topman et al., 2013). These data suggested that since the *in vivo* wound healing process involves several cell types migrating into the wound and then interacting with each other cells, our *in vitro* cell culture of single cell type may have some limitations. Regulation of gene expression in human gingival fibroblasts during the complex wound healing process may require other signals or cellular interaction that we have not included in our *in vitro* assay. In addition, it is interesting to study the effect of curcumin on gene expression in a "wounded" gingival fibroblast culture to mimic the injury to these cells *in vivo*.

Although the results of this study revealed only a slight increase in mRNA expression of TGF-β1 and VEGF when stimulated with curcumin, the effect of curcumin on the protein level of these cytokines are unknown. It is possible that curcumin may regulate these genes at a different time point or at the post-transcriptional level. Curcumin may also affect some other genes involved in wound healing such as collagen type I, epidermal growth factor or keratinocyte growth factor. Moreover, the effect of curcumin on acceleration of wound healing *in vivo* may arise from stimulation of other cell types such as macrophage or endothelial cells.

In summary, this study reported that curcumin did not significantly alter the mRNA expression of TGF-β1 and VEGF in human gingival fibroblasts at 24 hours after treatment. However, further investigations such as an *in vitro* wound healing model that cause damage to the gingival fibroblasts as well as the effect of curcumin on other wound healing-related genes are required to understand the mechanism by which curcumin promote wound healing.

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