# Effect of Curcumin on TNF-α-induced Cyclooxygenase-2 Expression in Bone Marrow Stromal Cells

# ผลของสารสกัดจากขมิ้นชันต่อการแสดงออกของจีนไซโคลออกซิจีเนสทูในเซลล์ไลน์จากไขกระดูกชั้นสตอร์มาลซึ่งถูกกระตุ้นด้วยทูเมอร์เนคโครซิสแฟคเตอร์แอลฟา

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**ABSTRACT**

The objective of this study was to investigate the effect of curcumin on TNF-α-induced expression of COX-2 in mouse ST2 bone marrow-derived stromal cells. ST2 cells were treated with TNF-α to determine the optimum concentration and treatment duration for inducing COX-2 expression. Then, ST2 cells were treated with TNF-α in the absence or presence of various concentration of curcumin and COX-2 expression was determined using reverse transcriptase polymerase chain reaction. TNF-α at concentrations of 1-50 ng/mL significantly increased COX-2 expression, with the maximum induction at 20 ng/mL. The time-course assay demonstrated that optimum durations for TNF-α treatment to induce COX-2 expression were 6 and 24 hours. Curcumin at 1 and 10 μM significantly decreased TNF-α-induced COX-2 expression in ST2 bone marrow stromal cells.

**บทคัดย่อ**

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของสารสกัดจากขมิ้นชัน (เคอร์คูมิน) ต่อการแสดงออกของจีนไซโคลออกซิจีเนสทูในเซลล์ไลน์จากไขกระดูกชั้นสตอร์มาลเอสทีทูซึ่งถูกกระตุ้นด้วยทูเมอร์เนคโครซิสแฟคเตอร์แอลฟา (ทีเอ็นเอฟแอลฟา) โดยทำการกระตุ้นเซลล์เอสทีทูด้วยทีเอ็นเอฟแอลฟาเพื่อหาความเข้มข้นและระยะเวลาที่เหมาะสมในการกระตุ้นการแสดงออกของไซโคลออกซิจีเนสทู จากนั้นจึงศึกษาผลของเคอร์คูมินที่ความเข้มข้นต่างๆ ต่อการแสดงออกของไซโคลออกซิจีเนสทูในเซลล์ที่ถูกกระตุ้นด้วยทีเอ็นเอฟแอลฟาโดยวิธีปฏิกิริยาลูกโซ่พอลิเมอเรสแบบย้อนกลับ พบว่าทีเอ็นเอฟแอลฟาที่ความเข้มข้น 1-50 นาโนกรัม/ลิตร กระตุ้นการแสดงออกของจีนไซโคลออกซิจีเนสทูได้อย่างมีนัยสำคัญ โดยที่ความเข้มข้น 20 นาโนกรัม/ลิตรกระตุ้นการแสดงออกของจีนไซโคลออกซิจีเนสทูได้สูงที่สุดที่ระยะเวลา 6 และ 24 ชั่วโมง และเคอร์คูมิน (1 และ 10 ไมโครโมลาร์) ลดการแสดงออกของจีนไซโคลออกซิจีเนสทูในเซลล์เอสทีทูซึ่งถูกกระตุ้นด้วยทีเอ็นเอฟแอลฟาอย่างมีนัยสำคัญ

**Key Words:** Curcumin, TNF-α, COX-2, ST2, Bone marrow stromal cells

**คำสำคัญ:** สารสกัดจากขมิ้นชัน ทูเมอร์เนคโครซิสแฟคเตอร์แอลฟา ไซโคลออกซิจีเนสทู เซลล์ไลน์จากไขกระดูกชั้นสตอร์มาลเอสทีทู

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**Introduction**

Orthodontic pain is a major concern of patients and parents both prior to and during orthodontic therapy. This concern is the main reason for declining treatment, lack of cooperation and discontinuing treatment (Krishnan, 2007). The application of orthodontic force causes an initial compression to the vasculature followed by cascades of inflammatory response. Cytokines and biochemical mediators such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α) and prostaglandins are among the major molecules released and these factors play an important role during orthodontic tooth movement (Masella and Meister, 2006).

TNF-α is a prototype of cytokines that binds to transmembrane receptors and activates several cell signaling pathways. TNF-α plays an important role in acute and chronic inflammation. Produced by activated monocytes, macrophages, bone marrow stromal cells and osteoblasts, TNF-α acts as one of the key players in pain and inflammation (David and Schett, 2010). TNF-α has been shown to induce prostaglandin production in various cell types such as murine fibroblast L929 cells, human gingival fibroblasts, tracheal smooth muscle cells, and colonic myofibroblasts (Hai et al., 2011; Lin et al., 2004; Nakao et al., 2002; Pettus et al., 2003).

Prostaglandins are a family of arachidonic acid-derived lipid compounds that play a major role in the acute inflammatory response (Smith et al., 2000; Williams and Peck, 1977). Cyclooxygenase-1 (COX-1) enzymes, produce the baseline level of prostaglandins in most tissues while COX-2 contribute to the increased prostaglandins during inflammation (Smith et al., 2000). PGs, released after orthodontic force application, are associated with hyperalgesia of the periodontal ligament by making the periodontal ligament sensitive to other pain mediators such as histamine, bradykinin, and substance P (Ferreira et al., 1978; Polat and Karaman, 2005).

There is a great interest in identifying compounds that can be used in pain control and increasing attention is paid on the use of natural substances to reduce pain associated with orthodontic treatment. Curcumin, a polyphenolic phytochemical found in turmeric plant, has anti-inflammatory, anti-carcinogenic and antioxidant properties. Studies have reported the effect of curcumin on the production and activity of many inflammatory cytokines including TNF-α, IL-6, IL-1β, $PGE_2$ (Cho et al., 2007; Lee et al., 2012; Lev-Ari et al., 2006; Singh and Aggarwal, 1995). It has been reported that curcumin blocked $PGE_2$ synthesis via down-regulation of COX-2 in human colon carcinoma cell line, synovial fibroblasts of rheumatoid arthritis patients, and human lung epithelial A549 cells (Lev-Ari et al., 2006; Moriyuki et al., 2010; Park et al., 2007). In contrast, curcumin induced COX-2 expression but failed to increase $PGE_2$ synthesis in coronary artery endothelial cells, suggesting a cell-type specific effect of this compound (Tan et al., 2011).

Bone marrow stromal cells play a key role in the inflammatory process by direct interaction with granulocyte and macrophage-colony forming cells as well as by secreting various cytokines including $PGE_2$. Although curcumin has been shown to modulate COX-2 expression in several cell types *in vitro* (Hong et al., 2004; Lee et al., 2012; Lev-Ari et al., 2006; Moriyuki et al., 2010; Park et al., 2007; Tan et al., 2011), the effect of curcumin on COX-2 expression in bone marrow stromal cell remains

unknown. We hypothesize that curcumin might be able to inhibit COX-2 expression in bone marrow stromal cells thus reducing $PGE_2$ release and hyperalgesia in the alveolar bone and periodontal tissues.

**Objective of the study**

The purpose of this study was to investigate the effect of curcumin on the TNF-α induced gene expression of COX-2 in a mouse ST2 bone marrow stromal cell line.

**Methodology**

**Cell culture**

Mouse ST2 bone marrow-derived stromal cells were maintained in 60-mm dishes in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) containing 10% Fetal bovine serum (FBS, Gibco, USA), 2 mM L-glutamine (Gibco, USA), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, USA), and 5 µg/mL amphotericin B (Gibco, USA) at 37°C in a humidified atmosphere with 5% carbon dioxide. After reaching confluence, the cells were subcultured with 0.125% Trypsin-EDTA (Gibco, USA) at a 1:5 ratio. During subculture, the medium was changed every 2 days.

**Cell viability assay**

To study the toxicity of curcumin, ST2 cells were plated at $5 \times 10^3$ cells/well in 96-well flat-bottomed tissue culture plates for 24 hours. The medium was then replaced with DMEM containing 1% FBS and 0–50 µM of curcumin (Sigma, USA). After 24 hours, cell viability was determined by the MTT assay. The medium was removed, and 0.7 mg/mL of MTT reagent in 1% FBS DMEM was added into each well and incubated for 3 hours until Formazan crystal formation was visible under the microscope. 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 was measured using a plate reader at a wavelength of 570 nm. Cell survival was calculated as follows:

Percent survival = (mean experimental absorbance/mean control absorbance) × 100

**TNF treatment**

To determine the dosage and time course of TNF-α that induced COX-2 expression, ST2 cells were seeded at $5\times10^5$ cells per plate in 60-mm plates in 10% FBS DMEM. The next day, the cells were washed and switched to a starvation medium (serum-free-DMEM) for 24 hours. After that the cells were stimulated with 1, 10, 20 and 50 ng/mL TNF-α for 24 hours and 20 ng/mL of TNF-α for 3, 6, 9, 24 hours.

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

Total RNA was extracted using a total RNA Mini kit (Geneaid, USA). The RNA concentration 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 were reverse transcribed to single-strand cDNA using the SuPrimeScript RT Premix (2X) (GeNet Bio, USA) following the manufacturer's instruction. Polymerase chain reaction (PCR) was performed using glyceraldehye-3-phosphate dehydrogenase (GAPDH) as internal control. The primer sequences used for PCR amplification were as followed; COX-2 Forward: 5'-TTCGGGAGCACAACAGAGTG-3', Reverse: 5'-TAACCGCTCAGGTGTTGCAC-3';

GAPDH Forward: 5'-TGAACGGGAAGCTCACTGG-3', Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'

2 µL of cDNA was amplified by PCR using a thermal cycler. Conditions were as follows: denaturation for 30 sec at 94°C, annealing for 30 sec at 63°C for GAPDH and 60°C for COX-2 and extension for 30 sec at 72°C. After amplification, the PCR products were separated on a 1.2 % agarose gel with ethidium bromide and imaged by a digital imaging system (Syngene, G:Box). The lowest dose and time of TNF-α that most effectively induced COX-2 expression was used in subsequent experiments.

**Curcumin treatment**

To study the effect of curcumin on TNF-α-induced gene expression of COX-2, ST2 cells were seeded at $6\times10^5$ cells per plate in 60-mm plates in DMEM with 10% FBS. The next day, the cells were washed and switched to a starvation medium (serum-free-DMEM) for 24 hours. After that, 1, 10, 20 µM of curcumin were added 30 min prior to TNF-α treatment. After 6 hours, total RNA was extracted using the same kit as above and real-time PCR was performed on the LightCycler® 480 system, version 1.5 (Roche, Germany) . The primer sequences used for real-time PCR were the same as semi-quantitative PCR.

**Real-time PCR assay**

The amplification of the cDNA template was performed using LightCycler® 480 SYBR Green I Master kit (Roche, Germany). The mixture contained 10 µL of 2x LightCycler® 480 SYBR Green I Master, 10 µM of each primer, 5 µL of DNA extract. Nuclease-free water was added to a final volume of 20 µL. The PCR program setting was at 95°C for 5 min followed by 45 cycles of amplification phase, consisting of denaturation for 30 sec at 95°C, annealing for 30 sec at 56°C for GAPDH and 52°C for COX-2, and extension for 30 sec at 72°C.

**Statistical analysis**

All experiments were performed in triplicate, and each experiment was repeated at least 3 times. Data were reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using either ANOVA followed by Tukey test or Kruskal-Wallis H followed by Mann-Whitney U test. Differences at $p < 0.05$ were considered as statistically significant.

## Results

**Effect of curcumin on the viability of ST2 bone marrow-derived stromal cells**

We first examined the cytotoxic effect of curcumin on ST2 cells. The data is shown normalized to control (Figure 1). Concentrations of curcumin up to 10 µM significantly increased cell viability ($p < 0.05$), whereas curcumin 20 µM caused no change in cell viability. Higher concentrations of curcumin (30 and 50 µM) induced a significant dose dependent cytotoxicity ($p < 0.05$).

**Effect of TNF-α treatment on COX-2 expression**

The optimum concentration of TNF-α concentration to induce COX-2 expression, and the time-course of COX-2 expression were determined using semi-quantitative PCR. TNF-α at concentrations of 1, 10, 20, and 50 ng/ml significantly increased COX-2 expression compared to control in a dose dependent manner ($p < 0.05$) (Figure 2a). However,

the highest increase was seen at 20 ng/mL (2.6 fold compared to control group), which was used in subsequent experiments. The time-course experiment showed that TNF-α at 20 ng/mL induced COX-2 expression in a time-dependent manner with maximum induction seen at 6 and 24 hours (Figure 2b).

**Effect of curcumin on TNF-α-induced COX-2 expression**

COX-2 expression level was examined in ST2 cells pretreated with curcumin 30 min prior to TNF-α stimulation using real-time PCR. The results showed that curcumin at concentrations of 1 and 10 μM significantly decreased TNF-α-induced COX-2 expression from 4.3 fold to 3.2 fold compared to TNF-α-treated ST2 cells alone ($p < 0.05$) (Figure 3).

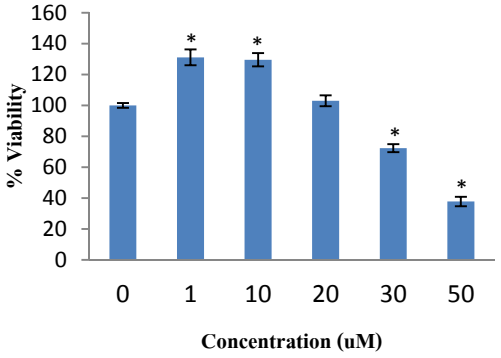


**Figure 1** Cytotoxicity test. ST2 cells were incubated with a range of concentrations of curcumin for 24 h. Cell viability was detected using MTT cytotoxicity assay. Data are mean ± SEM. * indicates $p < 0.05$ vs. control.

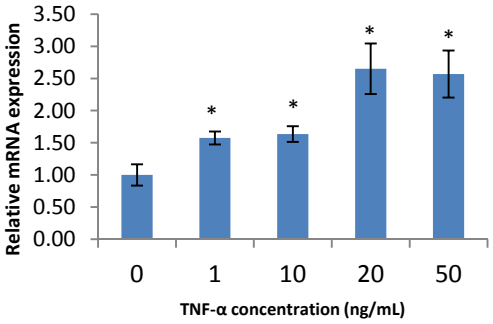


**Figure 2a** COX-2 expression in TNF-α-treated ST2 cells. Cells were incubated with various concentrations of TNF-α. Total RNA was prepared at 6 h after TNF-α treatment. COX-2 expression was determined by RT-PCR. Data are mean ± SEM. *indicates $p < 0.05$ vs. control.

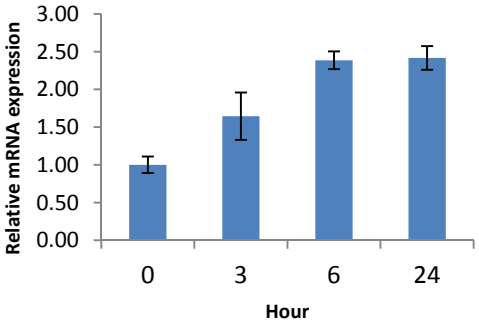


**Figure 2b** COX-2 expression in TNF-α-treated ST2 cells. Cells were incubated with TNF-α at 20 ng/mL. Total RNA was isolated over 24h after TNF-α treatment. COX-2 expression was determined by RT-PCR. Data are mean ± SEM.

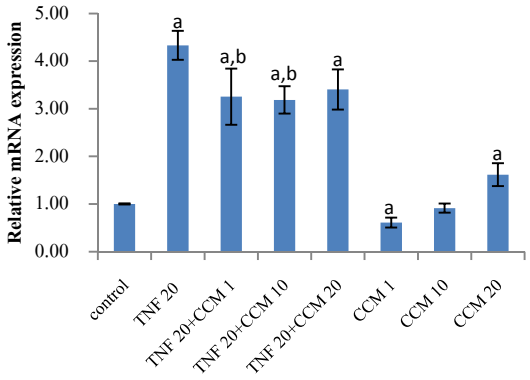


**Figure 3** Effect of curcumin (CCM) on COX-2 expression of TNF-α-treated ST2 cells. Cells were incubated with various concentrations of curcumin 30 min prior to 20 ng/mL TNF-α treatment. Total RNA was prepared at 6 h after TNF-α treatment. COX-2 expression was determined by real-time PCR. Data are mean±SEM. [a] indicates $p < 0.05$ vs. control, [b] indicates $p < 0.05$ vs. TNF-α 20 ng/mL.

**Discussion and Conclusions**

In the present study, we investigated the effect of TNF-α on COX-2 expression in ST2 bone marrow-derived stromal cells, and if the natural compound curcumin could affect COX-2 gene expression. We found that TNF-α significantly induced COX-2 expression in a concentration- and time-dependent manner in ST2 cells. Furthermore, curcumin significantly decreased TNF-α-induced COX-2 expression.

It has been demonstrated that the level of TNF-α in human gingival sulcus increased after application of orthodontic force (Karacay et al., 2007; Lowney et al., 1995). TNF-α has also been shown to play an important role in the regulation of many gene expressions involved in the inflammatory process (Bartlett et al., 1999; Ghosh et al., 1998; Subbaramaiah et al., 2000). TNF-α was found to involve in nuclear factor kappa B (NF-κB) activation (Fujisawa et al., 1996) and regulation of various genes that plays important role in immune and inflammatory responses including COX-2 (Ghosh et al., 1998). Several studies found that TNF-α increased COX-2 expression in many cell types such as synovial fibroblasts from human TMJ, human tracheal smooth muscle cells, and human gingival fibroblasts (Ke et al., 2007; Lin et al., 2004; Nakao et al., 2002). These studies showed that TNF-α-induced COX-2 expression is correlated to activation of transcription factor, in particular NF-κB. In contrast, TNF-α failed to markedly induce COX-2 expression in rat microglia cells (Bauer et al., 1997). These results suggest that COX-2 induction might be cell type-specific.

Curcumin has been shown to have anti-inflammatory, anti-carcinogenic and antioxidant properties. (Bharti et al., 2004; Duvoix et al., 2005; Hie et al., 2009; Notoya et al., 2006; Punithavathi et al., 2000; von Metzler et al., 2009). Previous studies showed that curcumin down-regulated COX-2 expression and blocked $PGE_2$ synthesis in several cell types including murine macrophage cells, human colon cancer cells, human lung epithelial cells, and human synovial fibroblasts (Hong et al., 2004; Koeberle et al., 2009; Lev-Ari et al., 2006; Moriyuki et al., 2010; Park et al., 2007). In this study, we found that curcumin decreased TNF-α-induced COX-2 expression in ST2 bone marrow stromal cells. In human colon epithelial cells, curcumin was found to inhibit the induction of COX-2 expression by TNF-α

in a dose dependent manner (Plummer et al., 1999), which is similar to our result. In contrast, other study demonstrated that curcumin increased expression of COX-1 and COX-2 but failed to increase $PGI_2$ or $PGE_2$ production in human coronary artery endothelial cells (Tan et al., 2011). Plummer et al. (1999) investigated the ability of curcumin to block NF-ΚB activation and the results suggested that curcumin can inhibit activation and nuclear translocation of NF-ΚB. The inhibitory effect of curcumin on TNF-mediated NF-kappa B (NF-ΚB) activation was also found in human myeloid cells (Singh and Aggarwal, 1995). The mechanism of action of curcumin in decreasing TNF-α-induced COX-2 expression in ST2 bone marrow stromal cells remains unknown. Further investigation is needed to elucidate the effect of curcumin on TNF-α-induced NF-ΚB activation and $PGE_2$ synthesis.

In summary, the results of this study indicate that curcumin (1-10 μM) can decrease the effect of TNF-α on COX-2 expression in ST2 cells. However, *in vivo* studies are required to justify the use of curcumin as an alternative for pain control.

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