Morus alba Stem Extract Suppresses Matrix Metalloproteinases (MMP)-1, MMP-9, and Tissue Inhibitors of Metalloproteinase (TIMP)-1 Expression via Inhibition of IκBα Degradation Induced by *Porphyromonas gingivalis* LPS Signal in THP-1 Cells

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Abstract

Objectives The aim of this study is to evaluate the inhibitory effects of *M. alba* stem extract (MSE) on the expression of matrix metalloproteinases (MMP)-1, MMP-9, and tissue inhibitors of metalloproteinase (TIMP)-1 in *Porphyromonas gingivalis* lipopolysaccharide (LPS)-activated-acute monocytic leukemia cell line (THP-1).

Materials and Methods THP-1 cells were treated with noncytotoxic concentrations of MSE combined with 1 μg/mL of *P. gingivalis* LPS. The mRNA levels of MMP-1, MMP-9, and TIMP-1 were evaluated via quantitative real-time polymerase chain reaction. The secreted proteins in the culture media were detected by enzyme-linked immunosorbent assay. The degradation of inhibitor of kappa B-alpha (IκBα) protein was tracked by Western blotting.

Statistical Analysis Comparisons in experiments were analyzed with analysis of variance followed by Tukey honestly significant difference comparison test.

Results Twenty and 40 μg/mL of MSE significantly downregulated MMP-1 and MMP-9 genes and protein expression but upregulated the gene expression of TIMP-1 ($p < 0.05$). *P. gingivalis* LPS induced degradation of IκBα, while addition of MSE (20 and 40 μg/mL) increased IκBα cytosolic levels. MSE was able to suppress the *P. gingivalis* LPS-induced MMPs expression and also increased the gene expression of TIMP-1 via the inhibition of the cytoplasmic IκBα degradation in THP-1 cells.

Conclusions The present observations suggest that MSE exerted a positive effect on the regulatory mechanism between MMPs and TIMP, which is an important implication for the therapeutic potential of MSE in periodontitis.

Keywords
- matrix metalloproteinases
- lipopolysaccharide
- monocytes
- *M. alba* stem extract

Introduction

Periodontitis is initiated by products of bacteria in plaque biofilm such as lipopolysaccharide (LPS), which leads to periodontal connective tissue degradation and alveolar bone resorption. One of the important inflammatory mediators involved in periodontitis is matrix metalloproteinases (MMPs), which initiate extracellular matrix degradation of the periodontal tissues.[1] The activities of MMP are generally balanced by endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs), and any imbalance between MMP and TIMP levels plays an important role in the periodontal disease progression.[2]

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MMP-1 is produced by defense cells during inflammation to degrade types I and III collagens, which are the predominant types of interstitial collagens in periodontal extracellular matrix. Gingival crevicular fluid (GCF) is composed of various proteolytic enzymes that have been reported to be possible diagnostic markers in periodontitis.[3] MMP-1 levels in the GCF are increased in patients with chronic periodontitis, and MMP-1 levels decrease after phase I therapy. However, TIMP-1 levels were lower in periodontitis group when compared with a healthy group, and TIMP-1 levels increased after phase I therapy.[4]

MMP-9, a predominant form of gelatinase in periodontitis, is capable of degrading denatured interstitial collagens, gelatins, laminin, elastin, fibronectin, and collagens type IV and type VII. A high MMP-9 level was correlated with disease activity in GCF from chronic periodontitis patients,[5] and periodontal treatment has decreased the level of this enzyme.[6]

Nuclear factor kappa B (NFκB), which is a ubiquitously expressed family of transcription factors of various genes involved in inflammation, is known to play a key regulatory role in the expression of MMP genes in response to *Porphyromonas gingivalis* LPS signals.[7] In unstimulated cells, NFκB is sequestered in the cytoplasm in an inactive form by inhibitory proteins such as IκB.[8] Stimulation of cells with LPS leads to degradation of inhibitor of kappa B-alpha (IκBα) and IκBβ, resulting in the complex releases and rapid translocation of NFκB to the nucleus. Activation of NFκB mediates a coordinated regulation of target genes, followed by production of inflammatory mediators, including MMPs.

Different parts of the plant of *Morus alba* L. have been widely used as herbal medicine for the treatment of various systemic conditions in East Asia.[9] The ethanolic extract obtained from stems has the highest amount of its bioactive compound, polyphenol oxyresveratrol.[10] *Morus alba* stem extract (MSE) has a wide spectrum of biological properties including anti-inflammatory.[11,12] Recently, the use of natural compounds from plant sources such as alkaloids, flavonoids, and phenolic compounds have shown promising results regarding MMPs inhibition.[13] However, the effect of MSE on MMPs and TIMPs expression in THP-1 cells has so far not been investigated. The objective of the present study was to evaluate the in vitro effect of MSE on the expression of MMP-1, MMP-9, and TIMP-1 in THP-1 stimulated with *P. gingivalis* LPS. The degradation of IκBα protein involved in this process was also determined.

Materials and Methods

Plant Materials and Preparation of Plant Extract

M. alba stems were obtained from the Queen Sirikit Sericulture Center, Tak Province, Thailand. The fresh stems were chopped and dried. Then, the dried plant was extracted by maceration technique using 80% ethanol for two cycles at room temperature as previously described.[12] MSE was dissolved in 0.05% dimethyl sulfoxide and diluted with the culture medium to obtain the designed concentrations.

Cell Culture

This study was approved by the Naresuan University Institutional Review Board (COE No.339/2016). THP-1 cells were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom). The cells were cultured in RPMI-1640 medium (Hyclone, Cramlington, United Kingdom) containing 10% heat-inactivate fetal bovine serum (Hyclone), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) in 5% CO_2 at 37°C.

Determination of Cell Viability

THP-1 cells were seeded at a density of 5×10^4 cells per well in 24-well plates. After 24 hours, cells were treated with 0, 10, 20, 40, 80, and 100 µg/mL of MSE in serum-free medium for 24 hours. The cytotoxicity of MSE was evaluated by the CellTiter 96 Aqueous One Solution Cell Proliferation Kit (MTS assay; Promega, Wisconsin, United States) according to the manufacturer's protocol. These experiments were repeated three independent times in triplicate wells.

Reverse Transcription-Polymerase Chain Reaction (Real-Time PCR) Analysis

THP-1 cells (5×10^5 cells per well) were transferred to 6-well plates in serum-free medium. The cells were treated with 0, 10, 20, and 40 µg/mL of MSE for 1 hour, followed by 1 µg/mL of *P. gingivalis* LPS (InvivoGen, California, United States). The plates were maintained in an incubator at 37.8C with 5% CO_2 for 24 hours. After incubation, the cell culture was collected and centrifuged. The cell supernatant was collected for enzyme-linked immunosorbent assay (ELISA). Total RNA was then isolated by Nucleospin RNAII (Macherey-Nagel GmbH&-Co. KG) according to the manufacturer's instruction. cDNA is synthesized from 1 µg of total mRNA and mixed with LightCycler 480 DNA SYBR Green I Master (Roche Diagnostics, Mannheim, Germany). Two-step quantitative real-time PCR was performed in duplicate using the LightCycler 480 II real-time PCR System (Roche Diagnostics) as previously described.[12] The sequences of the PCR primers were used as follow: MMP-1, forward, TGGACCTGGAGGAAATCTTGC, and reverse, AGAGTCCAAGAGAAT GGCCGA; MMP-9, forward, GAGGTGGACCGGA TGTTCC, and reverse, AAC TCACGCGCCAGTAGAAG; TIMP-1, forward, ACTGCAGGA TGGACTCTTGCA, and reverse, TTTCAGAGCCTTGGAGGAGCT. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as a control. Data are analyzed with the LightCycler 480 software version 1.5.

Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

The levels of MMP-1 and MMP-9 protein were measured using the human MMP-1, and MMP-9 ELISA kits (Abcam, Massachusetts, United States) according to the manufacturer's instructions. The optical densities at a wavelength of 450 nm were measured. All measurements were performed in duplicate and were repeated at three different occasions.

Western Blot Analysis

THP-1 cells (5×10^6 cells/well) were preincubated in the absence or presence of 20 and 40 µg/mL of MSE prior to the

addition of 1 µg/mL of *P. gingivalis* LPS. After incubation for 30 minutes, the cells were collected on ice, washed twice with ice-cold phosphate-buffered saline (PBS), and suspended in 40 µL of the lysis buffer (Boehringer Mannheim, Mannheim, Germany). The cytoplasmic extracts were prepared as described previously.[14] The expressions of IκBα were assayed in line with Western blotting protocol. Briefly, the volume of extracted protein was determined to 80 µg in each well of 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. A rabbit anti-IκBα (44D4) polyclonal antibody (Cell Signaling Technology, Massachusetts, United States) was used as the primary antibody for the IκBα degradation analysis. Immunoreactive bands were detected and analyzed with Chemi-Doc MP Imaging System and Image Laboratory Software (Bio-Rad, California, United States). β-actin was used as the loading control.

Statistical Analysis

Statistical analyses were performed using the SPSS software package version 17.0. All data were expressed as mean ± standard deviation. Comparisons in experiments were analyzed with analysis of variance followed by Tukey honestly significant difference comparison test. Statistical significance was considered when $p < 0.05$.

Results

Cytotoxic Effect of MSE on THP-1 Cells

The cytotoxic activity of MSE for the concentration range of 10 to 100 µg/mL was evaluated on THP-1 cells. The results showed decreased cell viability in a dose-dependent manner. MSE at concentrations between 10 and 40 µg/mL was not toxic to THP-1 cells as the cell viability was not significantly altered compared with the nontreated control. However, significant loss of cell viability was observed at the concentrations of 80 and 100 µg/mL, indicating cell death (►**Fig. 1**).

MSE Inhibits *P. gingivalis* LPS-Induced MMP-1, MMP-9, and TIMP-1 mRNA Expression

Expression of MMP-1, MMP-9, and TIMP-1 mRNA was assessed after treatment of *P. gingivalis* LPS-stimulated THP-1 cells with nontoxic concentrations of MSE. MMP-1 and MMP-9 mRNA expression increased significantly when stimulated with 1 µg/mL *P. gingivalis* compared with control, untreated cells ($p < 0.05$). The exposure to 10 µg/mL MSE extract showed no significant difference of both MMP and TIMP-1 mRNA expression compared with *P. gingivalis* LPS-treated cells. MSEs 20 and 40 µg/mL were able to significantly inhibit mRNA expression of MMP-1 and MMP-9 induced by *P. gingivalis* LPS

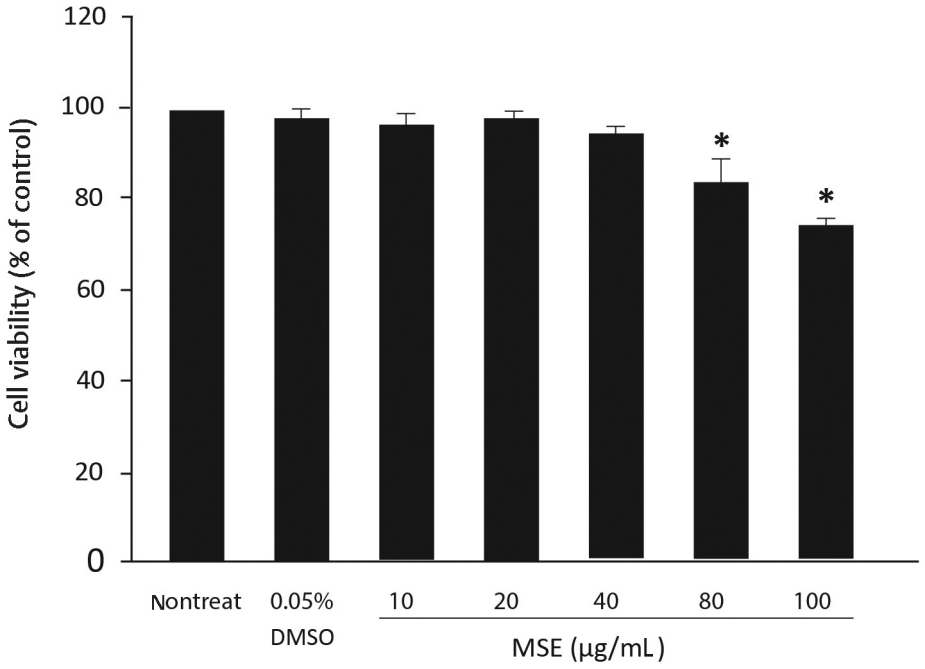

Fig. 1 Effect of *Morus alba* stem extract (MSE) on cell viability in THP-1 cells. Cells were treated with various concentrations of MSE or 0.05% dimethyl sulfoxide (DMSO) and cell viability was measured by MTS assay at 24 hours after exposure. The data are expressed as the percentage of viable cells compared with the nontreated cells (control). The values (mean ± standard deviation [SD]) are the average of three independent experiments performed in triplicate; * indicates significant difference from the control ($p < 0.05$).

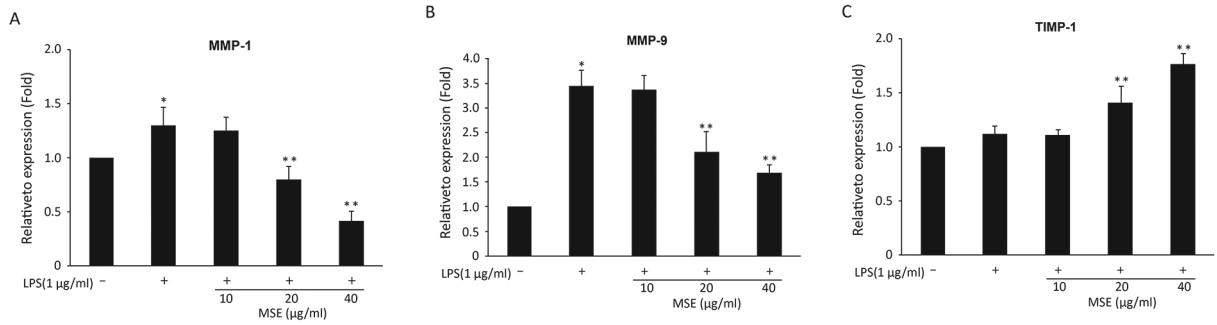

Fig. 2 Modulation of *P. gingivalis* lipopolysaccharide (LPS)-induced matrix metalloproteinase-1 (MMP-1) (**A**), MMP-9 (**B**), and tissue inhibitors of metalloproteinase-1 (TIMP-1) messenger ribonucleic acid (mRNA) expression by *Morus alba* stem extract (MSE). THP-1 cells were incubated with 20 and 40 µg/mL MSE or 0.05% dimethyl sulfoxide (DMSO) for 1 hour, followed by 1 µg/mL *P. gingivalis* LPS stimulation. After 24 hours, cells were extracted for real-time polymerase chain reaction (PCR). Levels of MMP-1, MMP-9, and TIMP-1 mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The values (mean ± standard deviation) are the average of three independent experiments performed in triplicate; * indicates significant difference from the nontreated cells ($p < 0.05$); ** indicates significant difference from *P. gingivalis* LPS treatment alone.

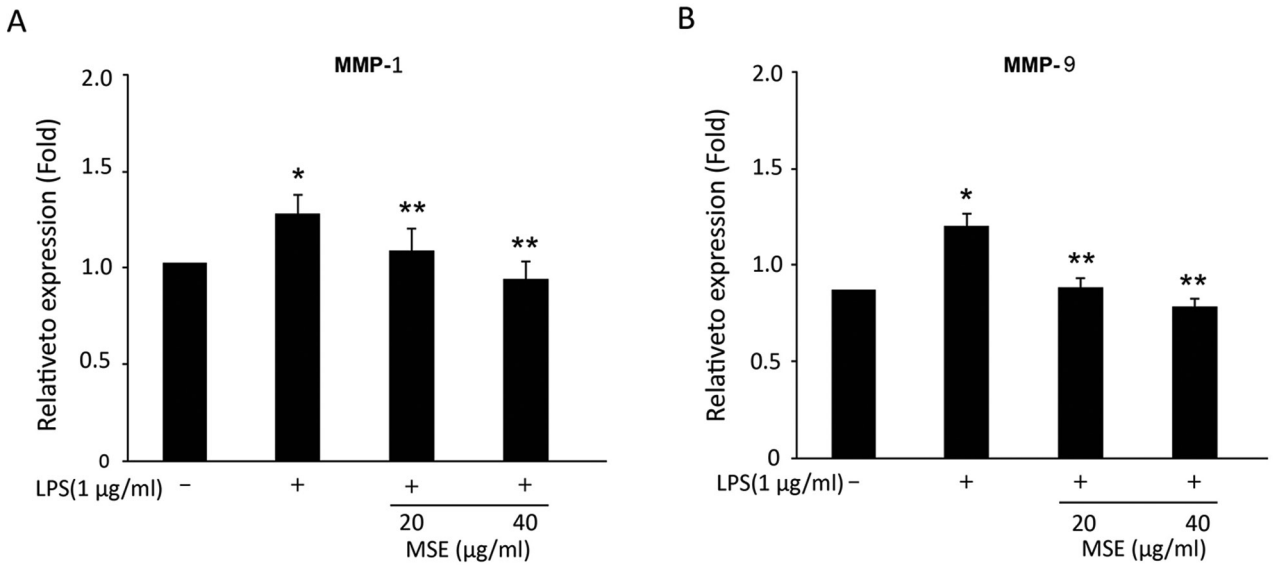

Fig. 3 Modulation of *P. gingivalis* lipopolysaccharide (LPS) induced matrix metalloproteinase-1 (MMP-1) (**A**) and MMP-9 (**B**) protein expression by *Morus alba* stem extract (MSE). THP-1 cells were incubated with 20 and 40 µg/mL MSE or 0.05% dimethyl sulfoxide (DMSO) for 1 hour, followed by 1 µg/mL *P. gingivalis* LPS stimulation. The culture supernatants were collected 24 hours later. The amount of MMP-1 and MMP-9 protein expression was measured by enzyme-linked immunosorbent assay (ELISA). The values (mean ± standard deviation [SD]) are the average of three independent experiments performed in triplicate; * indicates significant difference from the nontreated cells ($p < 0.05$); ** indicates significant difference from *P. gingivalis* LPS treatment alone.

($p < 0.05$). However, 20 and 40 µg/mL MSE significantly stimulated mRNA expression of TIMP-1 compared with *P. gingivalis* LPS-treated cells ($p < 0.05$) as shown in ►**Fig. 2**.

MSE Inhibits *P. gingivalis* LPS-Induced MMP-1 and MMP-9 Production

To further confirm the inhibitory effect of the MSE on MMP-1 and MMP-9 secretion, ELISA was used to detect the levels of these mediators in supernatants. MSE suppressed *P. gingivalis* LPS-induced MMP-1 and MMP-9 production in a concentration-dependent manner. MMP-1 and MMP-9 protein expression of *P. gingivalis* LPS treated THP-1 cells was significantly decreased ($p < 0.05$) by the both MSE testing concentrations (►**Fig. 3**).

MSE Inhibits *P. gingivalis* LPS-Induced IκBα Degradation

To address the inhibition role of MSE on the nuclear translocation of NFκB via modulation of IκBα degradation, the levels of IκBα were examined in the cytoplasmic extracts. Initially, we investigated time-varying effects. As shown in ►**Fig. 4A**, *P. gingivalis* LPS stimulation induced gradually degradation of IκBα at 15 and 30 minutes. The IκBα immunoreactivity reappeared at 60 minutes, presumably due to a feedback mechanism that regulated its own synthesis. Protein expression levels of IκBα were significantly lower at 30 and 60 minutes after *P. gingivalis* LPS stimulation ($p < 0.05$) compared with nontreated cells (►**Fig. 4B**). Preincubation with MSE at a concentration of 20 and 40 µg/mL significantly inhibited IκBα degradation stimulated by *P. gingivalis* LPS (►**Fig. 4C**, **D**).

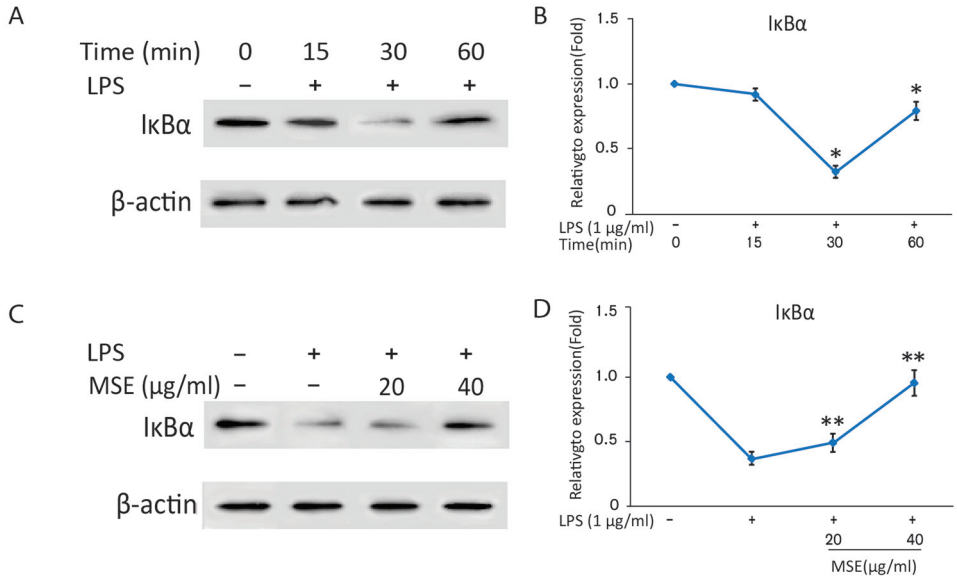

Fig. 4 Inhibitory effect of *Morus alba* stem extract (MSE) on the degradation of IκBα. (**A**) THP-1 cells were treated with *P. gingivalis* lipopolysaccharide (LPS) for the indicated times. (**C**) The cells were preincubated in the absence or presence of 20 or 40 μg/mL of MSE for 1 hour, followed *P. gingivalis* LPS stimulation. The cytoplasmic extracts were prepared 30 minutes later and were then assayed by Western blot analysis for inhibitor of kappa B-alpha (IκBα) and β-actin. (**B** and **D**) Quantitative analysis (mean ± standard deviation [SD]) of IκBα protein expression. *indicates significant difference from the nontreated cells ($p < 0.05$); ** indicates significant difference from *P. gingivalis* LPS treatment alone.

Discussion

In this study, we evaluated the inhibitory effect of MSE on MMPs production in human monocytes involved in inflammatory processes. Our results are the first to demonstrate that 20 and 40 μg/mL MSE significantly increased the mRNA and protein levels of MMP-1 and MMP-9. In addition, nontoxic concentration of MSE was able to enhance the gene expression of TIMP-1. To date, there is no published report on the effectiveness of MSE in MMPs and TIMPs expression, which make direct comparison difficult. Nonetheless, our findings of significantly decreased levels of MMP-1 and MMP-9 are in accordance with similar results in other studies on the different phenolic compounds.[15,16] Kou et al found that a dietary flavonoid, kaempferol inhibits *P. gingivalis*-induced mRNA expression of MMP-1 in human gingival cells.[16] Recent studies have shown that caffeic acid phenethyl ester (CAPE), an active component of propolis, inhibits the production of MMP-1 and -9, while enhances the gene expression of TIMP-1 in LPS-activated human monocytes.[16]

TIMP-1 is considered to be the most important endogenous inhibitor of extracellular matrix degradation mechanism because it suppresses the activity of all MMPs, except for membrane-bound MMP.[17] Vilela et al demonstrated that the TIMP-1 gene expression level was significantly elevated by treatment with high concentration (60 mM) of CAPE.[16] In the present study, TIMP-1 expression was slightly increased after the exposure to the *P. gingivalis* LPS, then the level increased significantly after treatment with nontoxic concentration of MSE. Studies have shown that the addition of TIMP-1 significantly reduced the activity of MMP-1[18] and MMP-9.[16,19] Thus, the inhibition effect of MSE on MMP1 and MMP-9 activities was probably the result of reduced gene expression with consequent modifications of MMP1 and MMP-9 production and the activity of TIMP-1 that inhibited their activity.

NFκB is pivotal for inflammatory mediators upregulation resulting in inflammation and destruction of periodontal tissues.[20] Treatment with *P. gingivalis* LPS results in phosphorylation and subsequent loss of cytoplasmic IκBα, which is an important event for NFκB activation. Oxyresveratrol has been previously reported to exert anti-inflammatory activity through inhibition of NFκB activation in LPS-activated murine macrophages.[11] The present study shown that MSE might regulate inflammatory reactions in human monocytes by inhibiting *P. gingivalis* LPS-inducible IκBα degradation and possibly suppress translocation of NFκB into nucleus. In addition to MMPs, NFκB regulates the production of various key proteins involved in triggering inflammation, including nitric oxide synthase, cyclooxygenase-2, and proinflammatory cytokines. The inhibitory effects of MSE on NFκB activation suggest that it may exert a wide range of anti-inflammatory effects in human monocytes. However, since the THP-1 cells are malignant transformed cells, further study using untransformed cells and other signaling molecules are required to directly link to the physiological roles of MSE.

Conclusion

MSE regulates the expression of MMP1, MMP-9, and TIMP-1 in *P. gingivalis* LPS-stimulated THP-1 cells by preventing the degradation of IκB, consequently blocking the activation of NFκB. These findings suggested that MSE may prevent

periodontal tissue destruction from bacterial challenge by changing the balance of the production between MMPs and TIMPs.

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Conflict of Interest
None declared.

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