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Simvastatin inhibits the expression of inflammatory cytokines and cell adhesion molecules induced by LPS in human dental pulp cells

JY Jung[1*], SM Woo[1*], WJ Kim[1,6], BN Lee[2], JE Nör[3], KS Min[4], CH Choi[5], JT Koh[6], KJ Lee[7], YC Hwang[2,6]

*Ji-Yeon Jung and Su-Mi Woo contributed equally to this study.

[1]Department of Physiology, School of Dentistry, Dental Science Research Institute, Chonnam National University, Gwangju, Korea, [2]Department of Conservative Dentistry, School of Dentistry, Dental Science Research Institute, Chonnam National University, Gwangju, Korea, [3] Angiogenesis Research Laboratory, Department of Cariology, Restorative Sciences, Endodontics, University of Michigan, School of Dentistry; Ann Arbor, MI, 48109-1078, USA, [4]Department of Conservative Dentistry, School of Dentistry, Chonbuk National University, Jeonju, Korea [5] Department of Preventive and Public Health Dentistry, School of Dentistry, Dental Science Research Institute, Chonnam National University, Gwangju, Korea, [6]Research Center for Biomineralization Disorders, Chonnam National University, Gwangju, Korea, [7]Department of Convergence Medicine, Asan Institute for Life Sciences, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Korea

Running title: Anti-inflammatory effect of simvastatin in pulp cells

Key Words: Simvastatin, lipopolysaccharide, inflammatory cytokines, cell adhesion molecules, NF-κB

Corresponding author:
Yun-Chan Hwang, DDS, PhD
Department of Conservative Dentistry, School of Dentistry, Chonnam National University. Yong-bong ro 77, Bukgu, Gwangju, Korea
Tel: +82-62-530-5831
Fax: +82-62-530-5629
E-mail: ychwang@chonnam.ac.kr

Abstract

Aim To investigate the effect of simvastatin on lipopolysaccharide (LPS) stimulated inflammatory cytokines, cell adhesion molecules, and Nuclear factor-κB (NF-κB) transcription factors in human dental pulp cells (HDPCs)

Methodology The effect of LPS and simvastatin on human dental pulp cell (HDPCs) viability was measured using a 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT) assay. Expression of inflammatory cytokines and cell adhesion molecules was evaluated by reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and Western blot analysis. NF-κB transcription factors were evaluated by Western blot analysis. Statistical analysis was performed with analysis of variance (ANOVA).

Results The viability of cells exposed to different concentrations of *E. coli* LPS, *P. gingivalis* LPS, and simvastatin was not significantly different compared with that of control cells ($P>0.05$). LPS significantly increased interleukin (IL)-1β ($P<0.05$) and IL-6 mRNA expression ($P<0.05$) and vascular cell adhesion molecule-1 (VCAM-1) ($P<0.05$) and intercellular adhesion molecule-1 (ICAM-1) protein expression ($P<0.05$) in HDPCs. Treatment with simvastatin significantly attenuated LPS-stimulated production of IL-1β, IL-6, VCAM-1, and ICAM-1 ($P<0.05$). Treatment with simvastatin decreased LPS-induced expression of p65 and phosphorylation of IκB and also significantly decreased the phosphorylation of p65 and IκB in the cytoplasm and the level of p65 in the nucleus ($P<0.05$).

Conclusions Simvastatin has a suppressing effect on LPS-induced inflammatory cytokine, cell adhesion molecules, and NF-κB transcription factors in HDPCs. Therefore, simvastatin might be a useful candidate as a pulp capping agent in vital pulp therapy.

Introduction

Caries penetration into the pulp results in bacterial invasion and causes pulp inflammation (Hilton 2009) as a result of the bacterial components and byproducts. Although this reaction represents a protective response, it can also be destructive. Unlike other body structures, the dental pulp is encased

in a rigid hard tissue with no collateral circulation. As a result, the pulp can become inflamed, eventually leading to pulp necrosis (Massey *et al*. 1993, Martin 2003). When the pulp becomes irreversibly inflamed or infected, root canal treatment is necessary, and such therapy can be challenging when the morphology of the canal is complex (Vertucci 2005).

Vital pulp therapy, such as pulp capping, partial pulpotomy, or full pulpotomy is an alternative to pulpectomy and can be successful (Aguilar & Linsuwanont 2011). Partial pulpotomy was reported to be effective in treating permanent teeth with deep carious lesions (Mass & Zilberman 2011), and partial pulpotomy using ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK, USA) or Dycal (L.D. Caulk, Milford, DE, USA) led to favourable outcomes in patients with reversible pulpitis (Chailertvanitkul *et al.* 2014). Direct pulp capping is also of value in certain cases (Willershausen *et al.* 2011) with cariously exposed pulps as well as traumatic or mechanical pulp exposure (Matsuo *et al.* 1996). However, partial pulpotomy and full pulpotomy have more predictable results than direct pulp capping in permanent teeth with cariously exposed pulps (Aguilar & Linsuwanont 2011).

For vital pulp therapy to be successful when the pulp is inflamed, the material used to cap the pulp should have an anti-inflammatory effect and should also induce mineralisation to create a dentine bridge. Therefore, an ideal pulp capping agent should suppress inflammation and induce pulp tissue mineralisation during treatment for cariously exposed pulps (Komabayashi & Zhu 2010). Previously, ketoprofen was used to inhibit dental pulp inflammation in a laboratory model (Choi *et al*. 2013). Ketoprofen is a nonsteroidal anti-inflammatory agent that is used on oral lesion such as pharyngitis or inflammation of the mouth in orthodontic therapy (Choi *et al*. 2013). Ketoprofen has been reported to inhibit expression of inflammatory mediators in dental pulp cells stimulated with LPS (Choi *et al*. 2013). However, ketoprofen does not have a mineralisation-inducing effect on the dental pulp (Choi *et al*. 2013).

Simvastatin, an HMG-CoA reductase inhibitor, has been used to reduce the risk of cardiovascular disease. It has an excellent tolerability profile and is associated with a low risk of adverse effects (Pedersen & Tobert 2004, Robinson 2007). Many studies have shown that simvastatin exerts an anti-inflammatory effect and it has been shown to prevent the inflammatory process induced by lipopolysaccharide (LPS) (Hernandez-Romero *et al*. 2008). Atorvastatin, another HMG-CoA reductase inhibitor, has been shown to reduce inflammation through the inhibition of nuclear factor-kappa B (NF-κB) activity (Ortego *et al*. 1999). In addition, simvastatin was found to have a bone formation–promoting effect in animal studies (Mundy *et al*. 1999). When injected subcutaneously or administered orally, simvastatin stimulated bone formation in mice calvaria and increased the volume of cancellous bone in rats (Mundy *et al*. 1999). Simvastatin has also been reported to promote osteoblastic differentiation and mineralisation in MC-3T3-E1 cells (Maeda *et al.* 2001) and to promote odontoblastic differentiation in human dental pulp cells (HDPCs) (Min *et al.* 2010).

Therefore, although it is possible that simvastatin could suppress inflammation in the dental pulps no

study has assessed the anti-inflammatory effect of simvastatin in HDPCs. The purpose of this study was to investigate whether simvastatin suppresses expression of inflammatory cytokine and cell adhesion molecule in HDPCs. The null hypothesis tested was that there are no differences in the anti-inflammatory effect between control and simvastatin treated groups.

Material and Methods

Cell isolation and culture

HDPCs were isolated from intact, caries-free supernumerary teeth freshly extracted from healthy children 7 to 10 years of age. All procedures were conducted after obtaining informed consent. The study protocol was approved by the internal review board of the Chonnam National University Dental Hospital (CNUDH-2013-002). Immediately after extraction, the teeth were kept in phosphate buffered saline and were then split open. The pulp tissues were removed under sterile conditions, minced with a surgical knife, and placed in 60-mm culture dishes containing α-minimum essential medium (α-MEM) (Gibco Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 mg/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cell passage numbers from 3 to 4 were used for the following experiment.

Cell viability assay

Cell viability was measured using a 3-[4, 5-dimethylthiazolyl-2-2, 5 diphenyltetrazolium bromide (MTT) assay. For the cell viability experiments, a suspension of HDPCs at a concentration of 1×10^5 cells per well was seeded in 48-well plates containing α-MEM with 10% FBS. The concentrations of *Escherichia coli* (*E. coli*) LPS (Sigma-Aldrich, St. Louis, MO, USA) and of *Porphyromonas gingivalis* (*P. gingivalis*) LPS (Invivogen, San Diego, CA, USA) were set at 0.1, 1, 10, and 20 μg/mL. The concentrations of simvastatin were set at 0.1, 1, 5, and 10 μM. After the cells were incubated for 24 h, MTT was added to each well for the last 4 h of the experiment, which was then stopped by the addition of dimethyl sulfoxide (DMSO). Optical density was determined at a 570-nm wavelength on a multi-well plate reader. Background absorbance of medium in the absence of cells was subtracted.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA of dental pulp cells was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's instructions. Then, 2 μg of RNA was reverse-transcribed for the synthesis of first-strand complementary DNA (cDNA) (Gibco BRL, Rockville, MD, USA). The cDNA was amplified in a final volume of 20 μL containing 2.5 mmol/L of magnesium dichloride, 1.25 U of Ex Taq Polymerase (Bioneer, Daejeon, Korea), and 1 mmol/L of specific primers. Thermocycling conditions consisted of 94°C for 5 m and then 30 cycles at 94°C for 40 s, at 55°C for 40 s, and at 72°C for 90 s, followed by a final 10 m extension at 72°C. Primer

sequences for PCR are described in Table 1. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide and visualised with a UV Transilluminator/Polaroid camera BioImaging System (UVP, LLC, Upland, CA, USA). PCR results were quantified using ImageJ software, version 1.47 (National Institutes of Health, Bethesda, MD, USA). The band density of each gene was normalized with the density of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control.

Western blot analysis

Cell lysates (50 to 100 μg) were placed in a lysis buffer (30 mM of Tris-Cl [pH 7.5], 1% NP-40, 1 mM of EDTA, 150 mM of NaCl, 1 mM of phenylmethanesulfonyl fluoride [PMSF], and a protease inhibitor mixture containing 1 μg/mL of aprotinin and leupeptin), separated by 12% polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to standard procedures. The membrane was blocked in 5% non-fat dry milk and incubated with primary antibodies for vascular cell adhesion molecule-1 (VCAM-1) and for intercellular adhesion molecule-1 (ICAM-1) (Santa Cruz Biotechnology), p65 (Abcam, Cambridge, UK), and p-IκB and IκB (Cell Signaling, Danvers, MA, USA) for 1 h at room temperature. After incubation with the specific peroxidase-coupled secondary antibodies (Thermo Scientific, Rockford, IL, USA) for 1 h, the blotted bands were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Little Chalfort, UK).

Cells that had been incubated with 1 μg/mL of *E. coli* LPS or with 1 μg/mL of *P. gingivalis* LPS in the absence or presence of 5 μM of simvastatin for 24 h were harvested. The nuclear and cytoplasmic proteins were collected by the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, San Diego, CA, USA). Western blot assay was performed, and the nitrocellulose membrane was incubated overnight at 4°C with primary antibodies for p-p65 (Cell Signaling), p-65, p-IκB, and IκB. After incubation with the specific peroxidase-coupled secondary antibodies (Thermo Scientific) for 1 h, the blotted bands were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). Histone H1 and β-actin were used as the internal controls for the nuclear and cytoplasmic proteins, respectively.

Enzyme-linked immunosorbent assay (ELISA)

Cells were incubated with 1 μg/mL of *E. coli* LPS or with *P. gingivalis* LPS in the absence or presence of 5 μM of simvastatin for 24 h. The levels of IL-1β and IL-6 were determined by means of enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc, Minneapolis, MN, USA). In all cases, a standard curve was constructed from the standards provided by the manufacturer. Cytokine levels were normalised to the protein concentration in lysate.

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test, as compared with control. Differences for which *P*-values were less than 0.05 were considered statistically significant.

Results

Effects of LPS and simvastatin on viability of HDPCs

The effect of *E. coli* LPS, *P. gingivalis* LPS, and simvastatin on cell viability of HDPCs is shown in Figure 1. The viability of cells exposed to different concentrations of *E. coli* LPS, *P. gingivalis* LPS, and simvastatin was not significantly different from that of the controls (P> 0.05).

Effects of LPS on expression of inflammatory cytokines and cell adhesion molecules

To investigate whether *E. coli* LPS and *P. gingivalis* LPS induced an inflammatory reaction in HDPCs, the expression of interleukin (IL)-1β and IL-6 was assessed in LPS-treated HDPCs. As shown in Figure 2a and 2b, *E. coli* LPS and *P. gingivalis* LPS significantly increased both IL-1β and IL-6 mRNA expression and IL-1β and IL-6 protein secretion. In addition, the expression of cell adhesion molecules, such as VCAM-1 and ICAM-1, was upregulated after the HDPCs were treated with both types of LPS (Fig. 2c). Densitometry showed that there was a significant increment in IL-1β (P<0.05) and IL-6 mRNA expression (P<0.05) and in VCAM-1 (P<0.05) and ICAM-1 expression (P<0.05) after treatment with LPS, beginning at concentrations of 0.1 μg/mL and higher (Fig. 2).

Effects of simvastatin on LPS-upregulated inflammatory cytokines and cell adhesion molecules

To determine the effect of simvastatin on LPS-induced pulpal inflammation, the expression and secretion of cytokines such as IL-1β and IL-6 and the expression of cell adhesion molecules such as VCAM-1 and ICAM-1 were examined in HDPCs stimulated with *E. coli* LPS or *P. gingivalis* LPS in the presence of simvastatin for 24 h. Treatment with simvastatin significantly attenuated the LPS-stimulated expression of IL-1β (P<0.05) and IL-6 (P<0.05) in HDPCs (Fig. 3a). The inhibitory effect of simvastatin on the secretion of cytokines into the supernatant was measured by ELISA. Detection of the secreted forms of IL-1β and IL-6 confirmed that simvastatin blocked the increments in IL-1β (P<0.05) and IL-6 proteins (P<0.05) in the supernatant of LPS-stimulated HDPCs (Fig. 3b). The effect of simvastatin on LPS-stimulated IL-1β and IL-6 protein secretion corresponded to its effect on mRNA expression. In addition, upregulated VCAM-1 and ICAM-1 production in HDPCs stimulated with LPS was inhibited by simvastatin treatment (Fig. 3c) (P<0.05 compared with the *E. coli* LPS–treated group; P<0.05 compared with the *P. gingivalis* LPS–treated group).

Effects of simvastatin on LPS-stimulated NF-κB pathway

To determine the mechanism involved in the response to simvastatin, the effect of simvastatin on LPS-stimulated NF-κB pathway was examined. Simvastatin treatment significantly decreased LPS-induced p65 expression and phosphorylation of IκB in the whole extract (Fig. 4a) ($P<0.05$), and it also significantly decreased LPS-increased phosphorylation of p65 and IκB in the cytoplasm and p65 level in the nucleus (Fig. 4b) ($P<0.05$) ($P<0.05$ compared with the *E. coli* LPS–treated group; $P<0.05$ compared with the *P. gingivalis* LPS–treated group).

Discussion

Caries lesions are associated with bacterial penetration of the pulp which evoke pulp inflammation, and eventually pulp necrosis and periapical pathosis (Hilton 2009). LPS, an endotoxin of gram-negative bacteria, mediates activity of the offending organism and induces an immune response in the host cells. LPS is also involved in the development of dental pulp inflammation and pulpitis. If the pulp can recover from the inflammatory state, the inflammation is classified as reversible pulpitis. Vital pulp therapy aims to treat reversible pulpitis and recover to a normal pulp state (Ward 2002). For the success of vital pulp therapy, factors such as age, size of exposure, state of pulp, extra-pulpal blood clot, and choice of capping material may affect to the outcomes (Ward 2002). Calcium hydroxide has been used as a vital pulp therapy for some time (Foreman & Barnes 1990). MTA has been used in the direct pulp capping and pulpotomy and has been associated with good results (Chailertvanitkul *et al.* 2014). Anti-inflammatory agents such as corticosteroid have also been tried as pulp capping agents (Obersztyn *et al.* 1968).

Simvastatin has been reported to have certain pharmacological effects in various tissues and cell types, including a wide range of anti-inflammatory effects. In one study, simvastatin reduced IL-1β, IL-6, and cyclooxygenase (COX) 2 expression in human umbilical vein endothelial cells (HUVECs) (Inoue *et al.* 2000), and in another report, treatment with simvastatin reduced serum levels of IL-6 and TNF-α in patients with hypercholesterolemia (Musial *et al.* 2001). In an experimental model of temporomandibular joint inflammation, simvastatin injected into the joint was effective in reducing subsynovial inflammation (George *et al.* 2013). Nevertheless, no studies have been carried out to assess the potential anti-inflammatory effect of simvastatin in human pulp cells. In the present study, simvastatin effectively decreased the expression of IL-1β, IL-6, and VCAM-1 and ICAM-1 induced by LPS in HDPCs. The suppressive effect of simvastatin on inflammatory cytokines is similar to that seen in other cell types, as shown in a previous study (Inoue *et al.* 2000).

IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) are well known as key pro-inflammatory mediators in the pathogenesis of inflammatory conditions (Greenhill *et al.* 2011, Tang *et al.* 2015), and LPS induces the expression of many inflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, in HDPCs (Coli *et al.* 2004, Nakanishi *et al.* 2010, Choi *et al.* 2013, Kim *et al.* 2015).

ICAM-1 and VCAM-1, which belong to the immunoglobulin superfamily of cell adhesion molecules,

are thought to regulate the attachment and migration of leukocytes during the inflammatory process (Blake & Ridker 2001). It has been reported that these molecules are expressed in inflamed human dental pulps and actually enhance pulp inflammation (Sawa *et al.* 1998). Because bacterial components such as LPS can induce ICAM-1 and VCAM-1 expression in dental pulp cells (Lee *et al.* 2008, Nakanishi *et al.* 2010), IL-1β, IL-6, and ICAM-1 and VCAM-1 were used as markers of inflammation induction. LPS increased the expression of these inflammatory cytokines in the HDPCs. This finding is in accordance with results reported by others (Coli *et al.* 2004, Lee *et al.* 2008, Choi *et al.* 2013).

NF-κB is a transcription factor that binds to the enhancer element of the immunoglobulin kappa light chain of activated B cells (Hoesel & Schmid 2013). In addition, the NF-κB pathway is known to be a key mediator of genes involved in controlling cellular proliferation and apoptosis. Therefore, NF-κB has an important role in regulating cellular proliferation, and activation of NF-κB can reduce apoptosis (Hoesel & Schmid 2013).

NF-κB transcription factor also plays an essential role in the regulation of inflammation and the immune response. Inflammatory processes are associated with activation of the NF-κB signaling pathway (Hoesel & Schmid 2013), so inhibition of this pathway could have a potential role in reducing inflammation (Yamamoto & Gaynor 2001). Several transduction cascades mediate stimulation of the NF-κB pathway. Activation of IκB kinase causes phosphorylation of IκB, leading to its ubiquitination and degradation. Degradation of IκB results in the translocation of NF-κB from the cytoplasm to the nucleus where it induces the expression of specific cellular genes (Yamamoto & Gaynor 2001).

To demonstrate the mechanism of simvastatin that inhibits inflammatory cytokine expression, the present study examined the effect of simvastatin on the NF-κB pathway. Simvastatin decreased the phosphorylation of IκB and p65 in the nucleus, which had been increased by LPS. Based on these results, the suppressive effect of simvastatin on LPS-induced inflammatory cytokines in HDPCs seems to be related to inhibition of the NF-κB pathway. This finding is consistent with a previous study of epidermal burn injury in mice showing that simvastatin reduced inflammatory cytokine production and apoptosis via downregulation of the TNF-α/NF-κB pathway (Zhao *et al.* 2015).

Asl Aminabadi *et al.* (2013) assessed the use of simvastatin versus calcium hydroxide as a pulp capping agent, but the anti-inflammatory effect of simvastatin was controversial. It is possible that their result was related to the experimental design in that they studied the pulp in its normal state. Under such normal conditions, implantation materials can cause an inflammatory response (Jegat *et al.* 2007). However, in the present study, LPS activated the inflammatory cytokines, which were reduced by treatment with simvastatin. Moreover, in the study by Asl Aminabadi *et al.* (2013) sodium carboxymethyl cellulose was used as a carrier of simvastatin, and cellulose derivatives can be degraded by bacteria (Reese *et al.* 1950). For the clinical application of simvastatin in inflammatory

conditions, a non-degradable carrier, such as bioglass, would have been more appropriate.

Conclusion

Simvastatin had an anti-inflammatory effect on LPS-induced inflammation in HDPCs. Therefore, simvastatin might be a useful candidate as a pulp capping agent in vital pulp therapy.

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Conflict of Interest statement

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Figure legends

Figure 1 Effects of *E. coli* LPS, *P. gingivalis* LPS, and simvastatin on cell viability of HDPCs. The MTT assay was used to assess the viability of HDPCs for 24 h after treatment with different concentrations of *E. coli* LPS, *P. gingivalis* LPS, and simvastatin. Values are expressed as means ± SD of three replicates of one representative experiment.

Figure 2 Effects of LPS on the expression of IL-1β, IL-6, VCAM-1, and ICAM-1 in HDPCs. The cells were treated with 0.1, 1, and 10 μg/mL of *E. coli* LPS and of *P. gingivalis* LPS for 24 h. (a) The mRNA levels of IL-1β and IL-6 were determined by RT-PCR. (b) The protein levels of IL-1β and IL-6 were determined with the use of ELISA kits. (c) The protein levels of VCAM-1 and ICAM-1 were determined by means of Western blot analysis. Values are expressed as means ± SD of three replicates of one representative experiment. * $P<0.05$, vs. control. The data shown are representative of three independent experiments with triplicates for each experiment.

Figure 3 Effects of simvastatin on the expression of IL-1β and IL-6 and VCAM-1 and ICAM-1 expression induced by *E. coli* LPS and *P. gingivalis* LPS in HDPCs. Cells were cultured with or without 1 μg/mL of *E. coli* LPS or of *P. gingivalis* LPS for 24 h with the concentrations of simvastatin as indicated. (a) The mRNA levels of IL-1β and IL-6 were determined by RT-PCR. (b) The protein levels of IL-1β and IL-6 were determined by using ELISA kits. (c) The protein levels of VCAM-1 and ICAM-1 were determined by means of Western blot analysis. Values are expressed as means ± SD of three replicates of one representative experiment. The data shown are representative of three independent experiments with triplicates for each experiment. # $P<0.05$, vs. the *E. coli* LPS–treated group; + $P<0.05$, vs. the *P. gingivalis* LPS–treated group.

Figure 4 Effects of simvastatin on the NF-κB pathway in HDPCs. Cells were cultured with or without 1 μg/mL *E. coli* LPS or *P. gingivalis* LPS for 24 h with the concentrations of simvastatin as indicated. (a) The protein expression of p65, phospho-IκB, and IκB was determined by means of Western blot analysis. (b) Protein levels of phospho-p65 and phospho-IκB in the cytoplasm and p65 in the nucleus of HDPCs were assessed. Values are expressed as means ± SD of three replicates of one representative experiment. The data shown are representative of three independent experiments with triplicates for each experiment. # $P<0.05$, vs. the *E. coli* LPS–treated group; + $P<0.05$, vs. the *P. gingivalis* LPS–treated group.

Table 1 Primer sequences used for RT-PCR

Genes	GeneBank number	Sequences (5′-3′)	Length of product
IL-1β	NM_000576.2	F: GATACAAACTGATGAAGCTCGTCA R: GAGATAGTGTTTTCCACATCCTGA	172 bp
IL-6	NM_000600.3	F: GAACAAGCCAGAGCTGTCCA R: TGAGGTGCCCATGCTACATT	230 bp
GAPDH	NM_001256799	F: AGTCACGGATTT GGTCGT R: ACAAGCTTCCCGTTCTCAG	185 bp

Figure 1

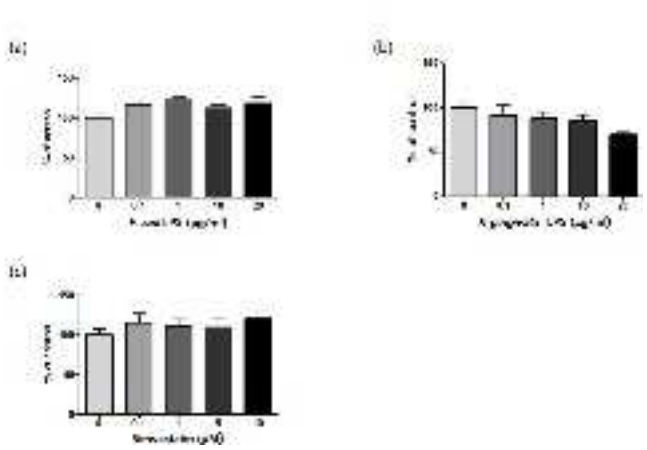

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Figure 2

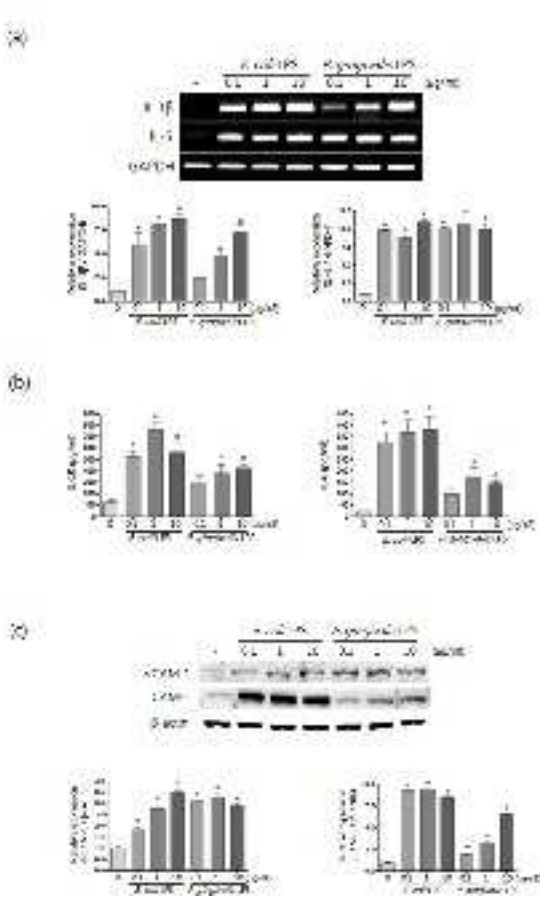

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Figure 3

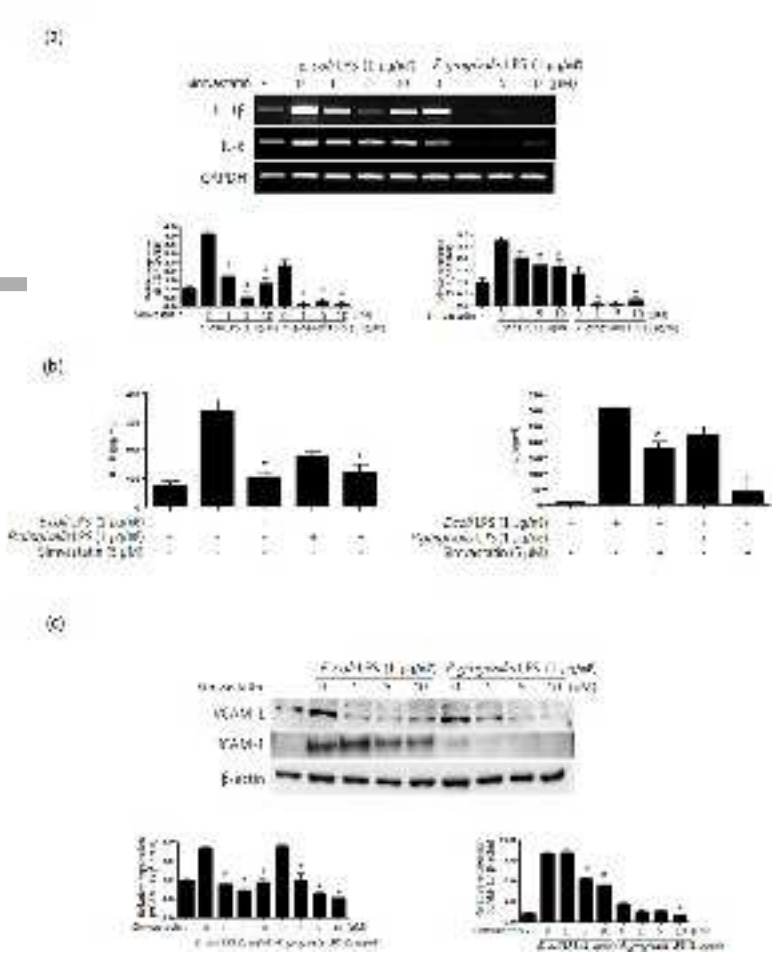

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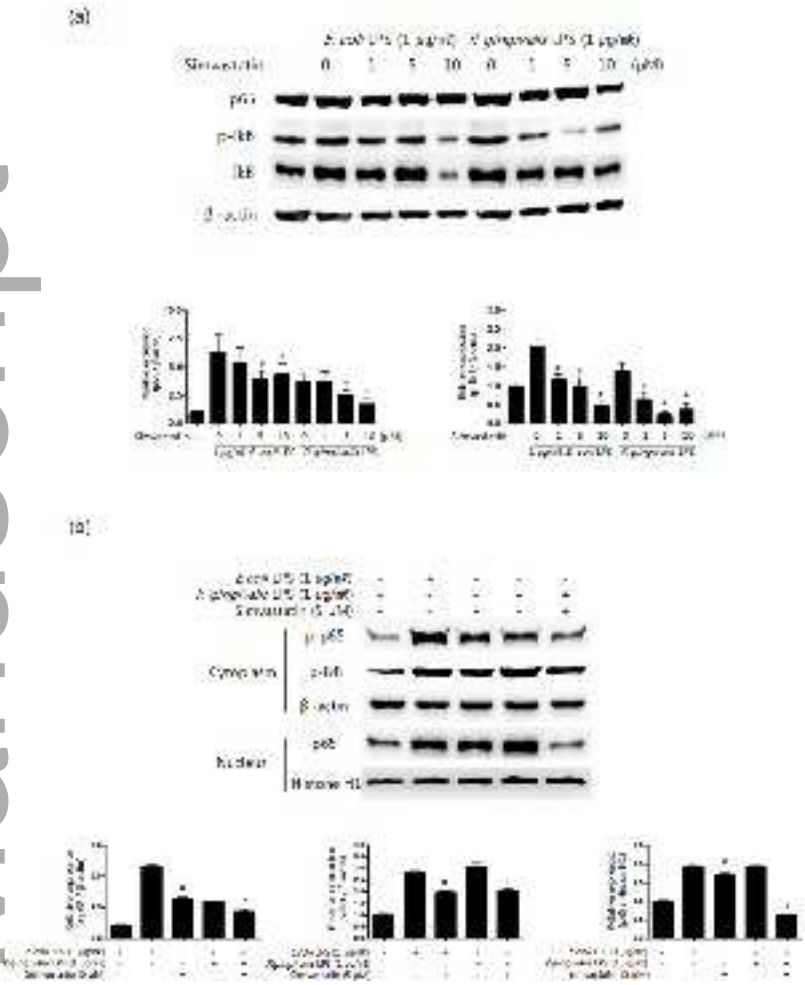
Figure 4



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