



Dexmedetomidine and LPS co-treatment attenuates inflammatory response on WISH cells via inhibition of p38/NF- κ B signaling pathway

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Background: Inflammatory dental diseases that occur during pregnancy can cause preterm labor and/or intrauterine growth restriction. Therefore, proactive treatment of dental diseases is necessary during pregnancy. Dexmedetomidine (DEX) is a widely used sedative in the dental field, but research on the effect of DEX on pregnancy is currently insufficient. In this study, we investigated the effects of co-treatment with DEX and lipopolysaccharide (LPS) on inflammatory responses in human amnion-derived WISH cells.

Methods: Human amnion-derived WISH cells were treated with 0.001, 0.01, 0.1, and 1 μ g/mL DEX with 1 μ g/mL LPS for 24 h. Cytotoxicity of WISH cells was evaluated by 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay. The protein expression of cyclooxygenase-2 (COX-2), prostaglandin E₂ (PGE₂), p38, and nuclear factor kappa B (NF- κ B) was examined by western blot analysis. The mRNA expression of pro-inflammatory cytokines such as interleukin (IL)-1 β and tumor necrosis factor (TNF)- α was analyzed by real-time quantitative polymerase chain reaction.

Results: Co-treatment with DEX and LPS showed no cytotoxicity in the WISH cells. The mRNA expression of IL-1 β and TNF- α decreased after co-treatment with DEX and LPS. DEX and LPS co-treatment decreased the protein expression of COX-2, PGE₂, phospho-p38, and phospho-NF- κ B in WISH cells.

Conclusion: Co-treatment with DEX and LPS suppressed the expression of COX-2 and PGE₂, as well as pro-inflammatory cytokines such as IL-1 β and TNF- α in WISH cells. In addition, the anti-inflammatory effect of DEX and LPS co-treatment was mediated by the inhibition of p38/NF- κ B activation.

Keywords: Cyclooxygenase 2 Inhibitors; Dexmedetomidine; Lipopolysaccharides; Prostaglandin E₂; WISH Cells.



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INTRODUCTION

Various physical and hormonal changes occur in pregnant women, which can cause dental diseases such as gingivitis, benign gingival lesions, tooth erosion, periodontitis, and dental caries. Morning sickness in the first trimester of pregnancy increases dental caries as the

pH in the mouth becomes more acidic [1]. In particular, progesterone has an affinity for the fundamental constituent of the connective tissue that makes up the gingiva and increases the osmotic pressure of capillaries distributed in the gingiva, which makes the gingiva more vulnerable to inflammation [2]. In addition, physical fatigue and emotional sensitivity in pregnant women can easily lead to neglect of oral hygiene, which also leads

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to an increased risk of dental disease [3]. Some studies have reported that maternal periapical infections and untreated dental caries can cause preterm birth and/or intrauterine growth restriction (IUGR) [4,5]. Therefore, it is necessary to actively treat dental diseases in pregnant women.

Preterm labor is caused by various pathological conditions including intrauterine infection, uterine ischemia, uterine overdistention, abnormal allergic reactions, cervical disease, and endocrine disorders [6]. Among them, intrauterine infection and/or inflammation are known to be the main risk factors related to spontaneous preterm labor, accounting for approximately 40% of cases [7,8]. In these vulnerable states, the human amniotic epithelial cells are easily activated by external stimuli, such as hormones, mechanical traction, and lipopolysaccharide (LPS), and subsequently secrete pro-inflammatory cytokines, including interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α [9,10]. These pro-inflammatory cytokines increase the production of prostaglandins (PG) through various pathways and stimulate cyclooxygenase-2 (COX-2), which is indispensable for PG synthesis [6,11]. To maintain a normal pregnancy, suppression of the inflammatory response continues during the pregnancy period, and spontaneous delivery occurs as the increase of PG, the main inflammatory substance [12]. Bacterial endotoxins and various inflammatory stimuli induce COX-2 activation, resulting in increased synthesis of PGs leading to uterine contraction and cervical ripening [6,13]. Among PGs, prostaglandin E₂ (PGE₂) has been found to be the main prostanoid produced by the amnion [6,14].

Even in pregnant women, dental surgery may require sedation or general anesthesia when abscesses (due to increased inflammation), fractures (due to trauma), and oral cancer occur. Dexmedetomidine (DEX), a selective α -2 agonist, is a preferred sedative in the dental field because of its low respiratory depression [15,16]. In pregnant women, DEX can be used as an analgesic during labor when epidural or spinal anesthesia is rejected or to

complement inadequate epidural analgesia [17]. Some studies have investigated the effects of DEX on uterine contractions. Karamon et al. [18] demonstrated that DEX increased spontaneous contractions in the gravid rat myometrium. Sia et al. [19] reported that DEX increased uterine contractility at plasma concentrations of 1×10^{-9} g/mL in the pregnant human myometrium. However, research on the effect of DEX on uterine contractions, especially on the expression of inflammatory substances that can cause uterine contractions is still lacking.

In this study, we explored the effect of DEX and LPS co-treatment on the production of inflammatory substances using human amnion-derived WISH cells, which are a good model for the analysis of PGE₂ release induced by various agents [14]. In addition, we investigated the signaling pathways associated with the effect of DEX and LPS co-treatment on the inflammatory response in WISH cells.

METHODS

1. Cell culture

Amnion-derived WISH cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (ATCC) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C in an atmosphere of 5% CO₂ in 95% air. The cells were passaged once every three days.

2. LPS stimulation and DEX treatment

A commercially available formulation of dexmedetomidine hydrochloride (KyongBo Pharm, Chungnam, Korea) was used in this study. The formulation was diluted with the culture medium and cells were co-treated with DEX at concentrations of 0.001, 0.01, 0.1, and 1 μ g/mL with 1 μ g/mL LPS (Sigma, St. Louis, MO, USA) for 24 h (Table 1).

Table 1. The experimental groups in this study

Experimental groups					
Control	LPS (1 $\mu\text{g}/\text{mL}$)	LPS + DEX (0.001 $\mu\text{g}/\text{mL}$)	LPS + DEX (0.01 $\mu\text{g}/\text{mL}$)	LPS + DEX (0.1 $\mu\text{g}/\text{mL}$)	LPS + DEX (1 $\mu\text{g}/\text{mL}$)

DEX, dexmedetomidine; LPS, lipopolysaccharide.

3. 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay

WISH cells (1×10^5 cells/well) were seeded into 24-well plates and cultured for 24 h at 37°C in an incubator with an atmosphere of 5% CO₂. The cells were subsequently exposed to 1 $\mu\text{g}/\text{mL}$ LPS and/or DEX at concentrations ranging from 0.001–1 $\mu\text{g}/\text{mL}$ for 24 h. Following drug treatment, MTT (Affymetrix Inc. USB, Cleveland, OH, USA) assay was performed by addition of 100 μL MTT solution (5 mg/mL in phosphate buffered saline at pH 7.4) to each well and incubation of the plate at 37°C. The medium was removed after 1 h and 100 μL of dimethyl sulfoxide (DMSO; Biosesang, Seongnam, Korea) was added to each well. The plate was gently rotated on an orbital shaker for 15 min to dissolve the precipitate. Absorbance was measured at 540 nm using a microplate reader (Bio-Rad Model 680; Bio-Rad, Hercules, CA, USA). All experiments were performed at least three times.

4. Quantitative real-time polymerase chain reaction (real-time qPCR)

WISH cells were seeded in 12-well cell culture plates at a density of 5×10^5 cells/well. The cells were treated with LPS alone or with DEX for 24 h. Following drug treatment, total ribonucleic acid (RNA) was isolated using 500 μL TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total messenger RNA (mRNA) per sample was reverse transcribed into cDNA using an oligo (dT) PrimeScript[™] 1st strand cDNA Synthesis Kit (TaKaRa Clontech, BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. RT-PCR was performed using a SimpliAmp[™] thermal cycler (Applied Biosystems, Life Science Technologies, CA, USA). The primers used for PCR were as follows: IL-1 β , 5'

-CTCGCCAGTGAAATGATGGCT-3' (forward) and 5' -GTCGGAGATTCGTAGCTGGAT-3' (reverse); TNF- α , 5'-CCAGGCAGTCAGATCATCTTC-3' (forward) and 5' -GTTATCTCTCAGCTCCACGC-3' (reverse); β -actin, 5' -GACCTGACTGACTACCTCATG-3' (forward) and 5' -CGCTCATTGCCAATGGTGATG-3' (reverse). The mRNA expression levels were normalized to that of β -actin. IL-1 β / β -actin was amplified using 35 cycles of PCR at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension was performed at 72°C for 7 min. TNF- α / β -actin was amplified using 35 cycles of PCR at 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, with a final extension step at 72°C for 10 min. Real-time qPCR was performed in triplicate. The PCR products were separated on a 1.5% agarose gel. They were assayed using the Gel Doc ImageQuant LAS 500 System (GE Healthcare Biosciences AB, Uppsala, Sweden). The data were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

5. Western blot analysis

Proteins were isolated from cells using chilled RIPA buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 5 mM DTT, 0.2 mM sodium orthovanadate, 100 mM NaF, and 1 mM PMSF) containing 1 \times protease inhibitor/phosphatase inhibitor cocktail (Cell Signaling Technology). Proteins (25 $\mu\text{g}/\text{well}$) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). Membranes were blocked with TBS-0.1% Tween-20 (TBST) containing 3% skim milk for 1 h. The membranes were subsequently incubated overnight with α -tubulin (1: 1000; Santa Cruz, CA, USA), PGE synthase 2 (A-2) (1: 1000; Cell Signaling Technology), COX-2 (D5H5) rabbit mAb (1: 1000; Santa Cruz), p38 MAPK

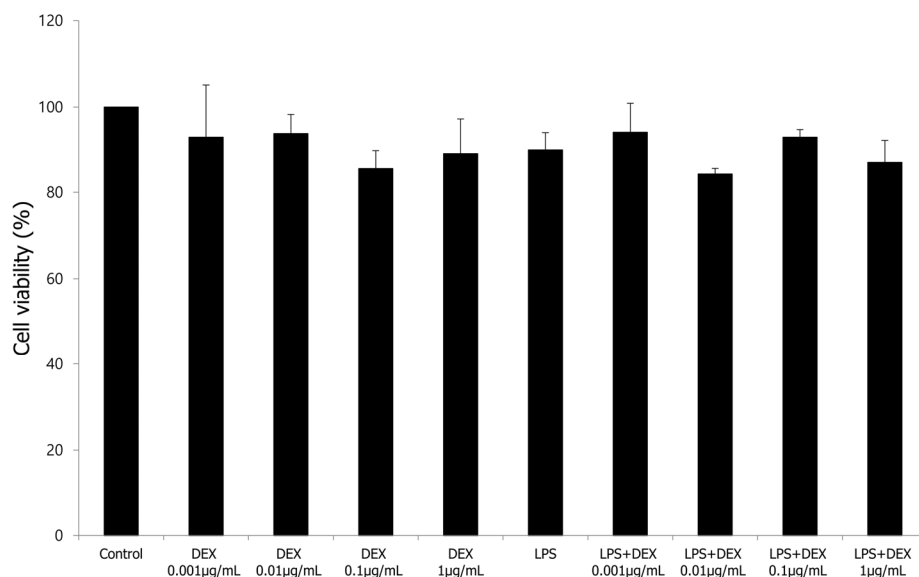


Fig. 1. Effect of treatment with LPS or co-treatment with DEX and LPS on cytotoxicity in WISH cells was measured by MTT assay. Values are presented as mean \pm standard deviation (SD). All experiments were repeated three times. DEX, dexmedetomidine; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide.

(1: 1000; Cell Signaling Technology), phospho-p38 MAP kinase (1: 500; Cell Signaling Technology), NF- κ B p65 (1: 1000; Santa Cruz), and phospho-NF- κ B p65 27. Ser 536 (1: 500; Santa Cruz) antibodies in TBST with 3% skim milk at 4°C. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (1: 1000; Enzo Life Sciences, Plymouth Meeting, PA, USA) or anti-mouse (1: 1000; Santa Cruz Biotechnology) antibodies for 1 h at room temperature. The membranes were subsequently washed three times with TBST, and the protein bands were visualized using enhanced chemiluminescence (ECL) detection reagents (Promega, Madison, WI, USA). Protein expression levels were normalized to those of α -tubulin. The target protein bands were normalized relative to the control band using the ImageJ software.

6. Statistical analyses

Data are presented as mean \pm standard deviation (SD). All experiments were repeated at least three times. Statistical analyses were performed using SigmaPlot v10 software. Statistically significance was set at $P < 0.05$.

RESULTS

1. Cytotoxicity of DEX and LPS co-treatment in WISH cells

We performed an MTT assay to examine the effects of LPS, DEX, and DEX + LPS co-treatment on the cytotoxicity of WISH cells. WISH cells were treated with 0.001, 0.01, 0.1, and 1 μ g/mL DEX, and 1 μ g/mL LPS or with DEX and LPS co-treatment. In our study, LPS and DEX showed no cytotoxicity in the WISH cells. In addition, there was no difference in the cell viability following co-treatment with various concentrations of DEX and LPS (Fig. 1).

2. Effect of DEX and LPS co-treatment on the expression of pro-inflammatory cytokines (IL-1 β and TNF- α) in WISH cells

Real-time qPCR was performed to evaluate the effect of DEX and LPS co-treatment on the expression of pro-inflammatory cytokines such as IL-1 β and TNF- α . As shown in Fig. 2, treatment with 1 μ g/mL LPS significantly increased the mRNA expression of IL-1 β and TNF- α compared to those in the control. The mRNA

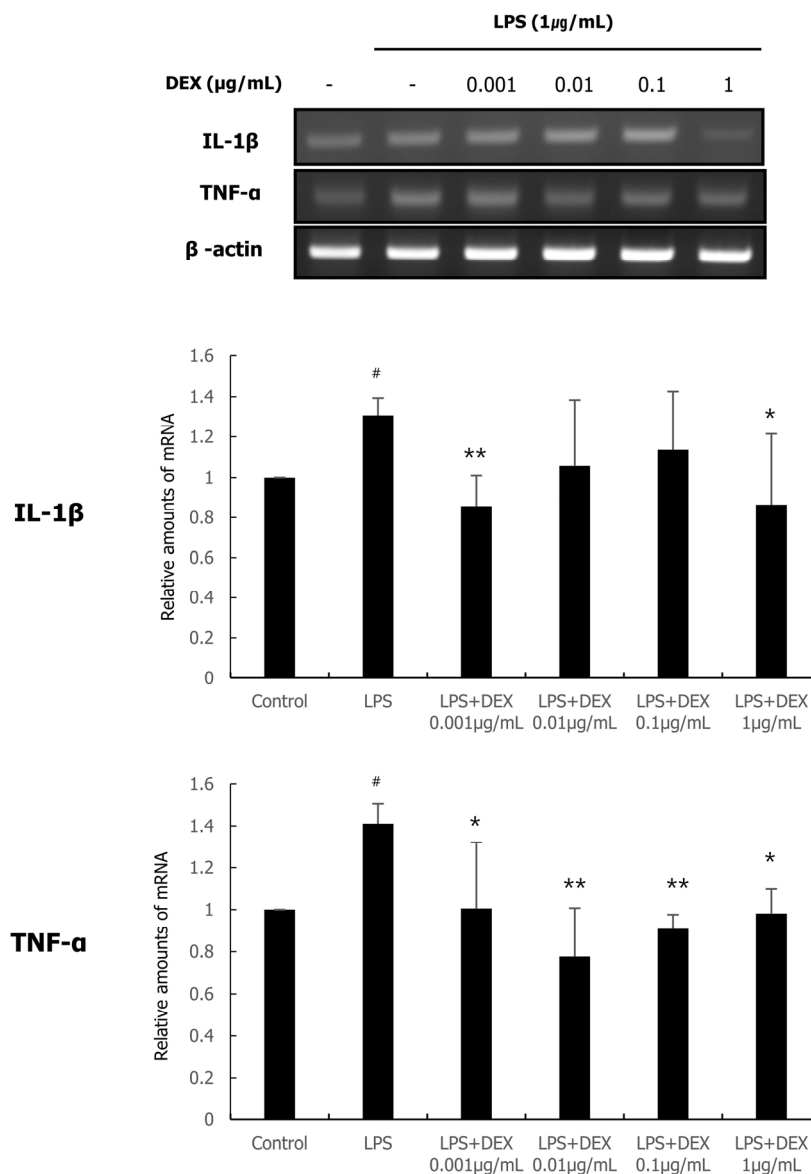


Fig. 2. The mRNA expression of IL-1 β and TNF- α following DEX and LPS co-treatment of WISH cells was measured using quantitative real-time PCR. Relative mRNA level was normalized to that of β -actin and presented as mean \pm standard deviation of three independent experiments. # P < 0.05 versus control group; *P < 0.05, **P < 0.01 versus LPS group. DEX, dexmedetomidine; IL, interleukin; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; TNF, tumor necrosis factor.

expression of IL-1 β was significantly decreased by DEX (0.001 and 1 μ g/mL) and LPS co-treatment. The mRNA expression of TNF- α was significantly decreased by DEX and LPS co-treatment at all concentrations compared to that in the LPS treatment group.

3. Effect of DEX and LPS co-treatment on COX-2 and PGE₂ expression in WISH cells

The protein expression levels of COX-2 and PGE₂ were

analyzed by western blotting. As shown in Fig. 3, LPS treatment increased the protein expression of COX-2 and PGE₂ in WISH cells compared to that in the control. DEX (0.1 and 1 μ g/mL) and LPS co-treatment significantly decreased the protein expression of COX-2. The protein expression of PGE₂ was significantly decreased at all concentrations of DEX compared to that in the LPS treatment group.

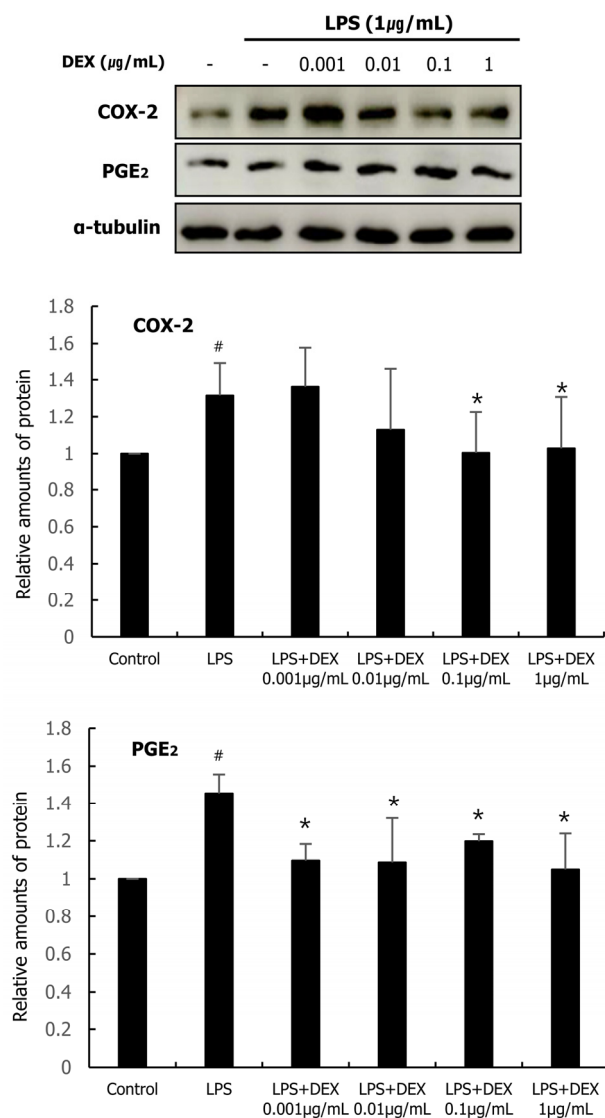


Fig. 3. Western blot analysis was conducted to analyze the protein expression of COX-2 and PGE₂ in WISH cells were treated with DEX (0.001 – 1 µg/mL) and/or 1 µg/mL LPS. The effect of DEX and LPS co-treatment on the protein expression of COX-2 and PGE₂ were evaluated. Relative density analysis was performed using NIH Image program and normalized to that of α-tubulin. Data are presented as mean ± standard deviation of three independent experiments. # P < 0.05 versus control group; *P < 0.05 versus LPS group. COX, cyclooxygenase; DEX, dexmedetomidine; LPS, lipopolysaccharide; PGE, prostaglandin. DEX, dexmedetomidine; LPS, lipopolysaccharide.

4. Effect of DEX and LPS co-treatment on the phosphorylation of p38 and NF-κ B in WISH cells

To evaluate the role of MAPK p38 and NF-κB in the inhibition of the expression of inflammatory substances following DEX and LPS co-treatment in WISH cells, we investigated the protein expression of p38 and NF-κB, and their activated forms phospho-p38 and phospho-NF-κB, respectively using western blot analysis. LPS treatment significantly increased the protein expression of

phospho-p38 and phospho-NF-κB in WISH cells compared to that in the control. The protein expression of phospho-p38 in the DEX (0.01, 0.1, and 1 µg/mL) and LPS co-treatment group was significantly reduced compared to that in the LPS treatment group (Fig. 4). In addition, the protein expression of phospho-NF-κB was significantly decreased following DEX (0.1, and 1 µg/mL) and LPS co-treatment compared to that in the LPS treatment group (Fig. 5). Our results suggest that the inhibitory effect of DEX and LPS on the expression of

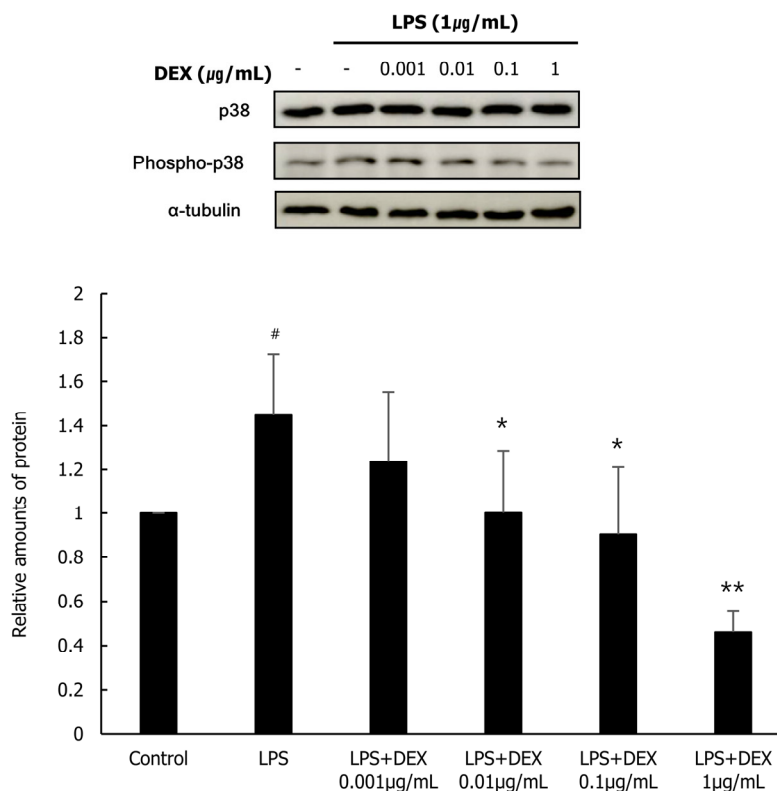


Fig. 4. Phosphorylation of p38 following co-treatment of WISH cells with DEX and LPS was evaluated using western blot analysis. Relative amounts of phospho-p38 protein were analyzed in triplicate experiments and normalized to that of p38. # P < 0.05 versus control group; *P < 0.05 versus LPS group. DEX, dexmedetomidine; LPS, lipopolysaccharide.

inflammatory substances in WISH cells is mediated via the suppression of p38/NF- κ B activation.

DISCUSSION

Preterm labor and birth increase neonatal mortality and morbidity in children under the age of five, which not only impacts the family affected but also presents considerable socioeconomic burden [7]. Therefore, studies on the inhibition of inflammatory substances that have the greatest influence on preterm labor are valuable in the medical and social sciences. In this study, we found that co-treatment with DEX and LPS inhibited the expression of inflammatory substances in amnion-derived WISH cells. This result is consistent with that of a previous study that showed that pretreatment with DEX inhibited the LPS-induced increase in inflammatory substances in WISH cells [20]. Based on these findings,

DEX inhibits the production of inflammatory substances that increase the risk of preterm labor when used for sedation and general anesthesia during pregnancy.

Our results showed that co-treatment of WISH cells with DEX and LPS resulted in a decrease in the expression of IL-1 β and TNF- α compared to treatment with LPS alone. IL-1 β is one of the most important members of the IL-1 family and is the first pro-inflammatory cytokine known to be involved in preterm labor [6]. Previous studies have reported that IL-1 β induces premature contractility of the myometrium in mice and pregnant rhesus monkeys, and systemic administration of IL-1 β significantly increases the production of other pro-inflammatory cytokines including IL-6, TNF- α , and PGs [21,22]. It is also known that IL-1 β activates COX-2 [23]. TNF- α is a pro-inflammatory cytokine belonging to the TNF superfamily and is involved in the regulation and stimulation of PG synthesis during preterm labor, like IL-1 β [24]. In addition, an

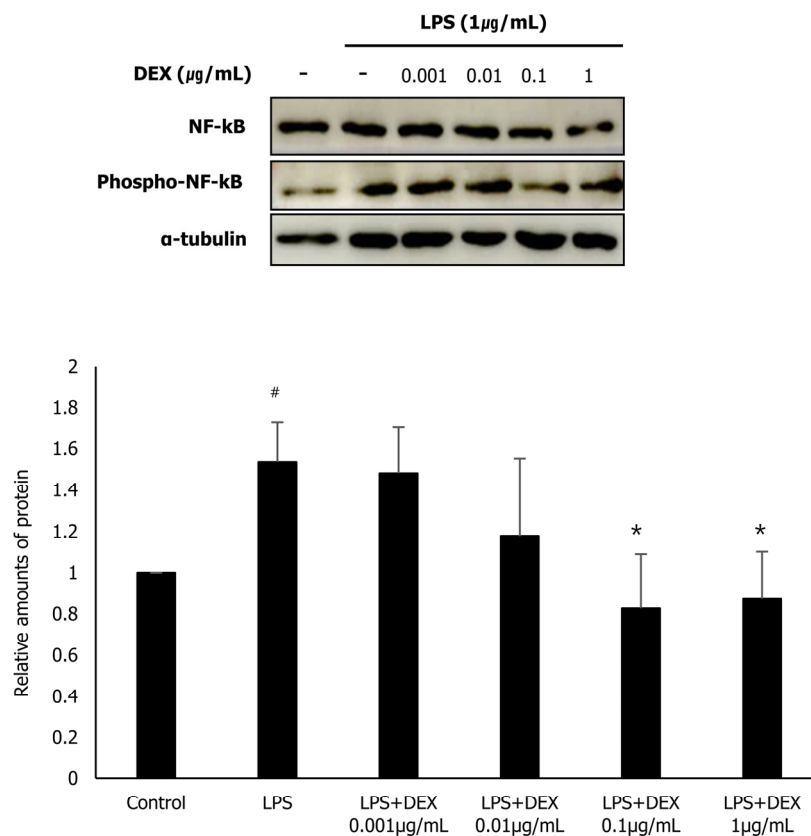


Fig. 5. Phosphorylation of NF- κ B following co-treatment of WISH cells with DEX and LPS was evaluated using western blot analysis. Relative amounts of phospho-NF- κ B protein were analyzed by triplicate experiments and normalized to that of NF- κ B. # $P < 0.05$ versus control group; * $P < 0.05$, ** $P < 0.01$ versus LPS group. DEX, dexmedetomidine; LPS, lipopolysaccharide; NF- κ B, nuclear factor kappa B.

increase in TNF- α in the amniotic fluid activates the NF- κ B pathway, leading to the amplification of the inflammatory processes related to preterm birth [25]. TNF- α stimulates cervical ripening and uterine contraction [6,26].

Many studies on the anti-inflammatory effects of DEX have recently been reported. Xu et al. [27] showed that DEX treatment decreased the serum concentrations of TNF- α and IL-6 in mice with sepsis. Several recent randomized controlled studies have reported that DEX effectively reduced the level of serum inflammatory factors in patients undergoing intestinal surgery and that of C-reactive protein and procalcitonin, the most common inflammatory biomarkers in patients with sepsis [28,29]. However, because these studies did not examine the production of inflammatory substances in the amniotic membrane, it is difficult to correlate their results to the results of our study. Several studies on the effects of DEX

on uterine contractions have reported that DEX increases uterine contractions [30,31]. However, as these studies also did not investigate the effect of DEX on the production of inflammatory substances in the uterus or amniotic membrane, further studies on the effect of DEX on the inflammatory response in the amniotic membrane should be conducted.

The current study demonstrated that the anti-inflammatory effect of DEX and LPS co-treatment on WISH cells was mediated via the suppression of p38/NF- κ B activation. NF- κ B is a transcription factor that plays an important role in the transcription of pro-inflammatory molecules such as COX-2, IL-1 β , and TNF- α [32]. COX-2 is an established NF- κ B-dependent gene, and PGs produced by COX-2 induce cervical ripening, and its increase in activated amnion is known to play a critical role in labor initiation [33]. p38 MAPK is a family of serine/threonine kinases related to pro-inflammatory

signaling cascades in several cell types [34]. p38 MAPK signaling regulates the transcriptional activity of NF- κ B. Previous studies have reported that inhibition of p38 with potent and specific inhibitors leads to the attenuation of the transcriptional activity of NF- κ B in various cell types [35,36]. Our results suggest that co-treatment of WISH cells with DEX and LPS suppresses the transcriptional activity of NF- κ B through inhibition of p38 activation, which reduces the expression of COX-2 and consequently reduces the expression of PGs.

This study has some limitations. In this study we evaluated the effect of DEX on the inflammatory response in WISH cells *in vitro*. Further *in vivo* studies are required to verify this conclusion. In addition, other signaling pathways related to the anti-inflammatory effects of DEX and LPS co-treatment need to be investigated. As we only examined the effects of DEX and LPS co-treatment on the activation of p38 and NF- κ B, additional experiments on the activation of other transcription factors or MAPKs involved in the expression of inflammatory factors are also required.

In conclusion, we demonstrated that DEX and LPS co-treatment suppressed the expression of COX-2 and PGE₂, as well as pro-inflammatory cytokines, including IL-1 β and TNF- α , in WISH cells. In addition, we showed that co-treatment with DEX and LPS mediates anti-inflammatory effects in WISH cells via the inhibition of p38/NF- κ B activation. Although further research on the anti-inflammatory effect and mechanism of DEX is required, this study is significant in that it suggests that DEX reduces the expression of inflammatory substances that increase the risk of preterm labor.

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Ji-Young Yoon: Methodology, Writing - review & editing

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Eun-Ji Choi: Supervision

Yeon Ha Kim: Investigation, Methodology, Software

Eun-Jung Kim: Conceptualization, Supervision, Writing - original draft

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