FEMALE SEX HORMONES DECREASE MMP-1 AND MMP-8 EXPRESSIONS IN *Porphyromonas gingivalis* LPS-STIMULATED HUMAN GINGIVAL FIBROBLASTS

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Abstract

Elevated female sex hormone concentrations are associated with gingival inflammation during pregnancy. This study aimed to investigate the effects of female sex hormones and *Porphyromonas gingivalis* lipopolysaccharide (LPS) on the expression of matrix metalloproteinase (MMP)-1 and MMP-8 in human gingival fibroblasts, in vitro. Primary cultures of human gingival fibroblasts (HGFs) were incubated with β-estradiol (0.1-10 µg/ml), or progesterone (1-100 µg/ml), or LPS from *P. gingivalis* (0.1-10 µg/ml) for up to 48 h. The cell viability of HGFs was examined by cell proliferation assay. The expression of MMP-1 and MMP-8 was investigated using conventional and real-time reverse transcription polymerase chain reaction (RT-PCR). B-estradiol (0.1-10 µg/ml) and progesterone (1-100 µg/ml) did not alter the number of cells of HGFs after 24 h incubation. Stimulation of HGFs with LPS from *P. gingivalis* up-regulated the expression of MMP-1 and MMP-8, whereas β-estradiol and progesterone inhibited both MMP-1 and MMP-8 expression. Pre-treatment of gingival fibroblasts with either estrogen or progesterone decreased the effect of LPS from *P. gingivalis* on MMP-1 and MMP-8 expression by gingival fibroblasts. The effects of estrogen and progesterone on the expression of MMP-1 and MMP-8 by human gingival fibroblasts may play a role in pregnancy gingivitis.

Keywords: Sex hormones, gingival fibroblasts, MMPs, inflammation

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Introduction

Hormonal imbalance is thought to be a major determinant that is responsible for periodontal inflammation observed during pregnancy (Armitage, 2013). Indeed, it has been postulated that the pathogenesis of pregnancy gingivitis is initiated by bacterial biofilms and exacerbated by endogenous sex hormones (Mealey and Moritz, 2003). Increased levels of estradiol and progesterone in serum and saliva are associated with the severity of gingival inflammation (Figuero *et al*., 2010). Alterations in the immune-inflammatory process by elevated female sex hormone levels have been proposed to explain the pathobiology of the interaction between pregnancy and periodontal disease, including depression of the immune system (Raber-Durlacher *et al*., 1994), increased vascularity and vascular flow (Jitprasertwong *et al*., 2013), and cellular changes and changes in oral biofilms (Markou *et al*., 2009). However, the precise mechanistic links between pregnancy and gingival changes are not fully understood.

Female sex hormones play a vital role in the function of immune and non-immune cells (Whitacre, 2001). The sex hormone receptor is expressed in neutrophils, monocytes, lymphocytes, and fibroblasts (Gilliver, 2010). Both estrogen and progesterone can regulate the secretion of a myriad of cytokines and inflammatory mediators (Jain *et al*., 2004; Gilliver, 2010). However, studies show that estradiol and progesterone can also have immunosuppressive effects (Su *et al*., 2009). For example, progesterone and estrogen inhibit the production of interleukin-1β (IL-1β) from human peripheral monocytes (Morishita *et al*., 1999), and estrogen reduced the expression of chemokine (C-C motif) ligand 3 (CCL3) mRNA by periodontal ligament cells (Nebel *et al*., 2010). A recent study suggested that modulation of immune responses by β-estradiol and progesterone may promote a disbiotic inflammatory environment of periodontal disease by down-regulating the toll-like receptor 2 (TLR2) and cluster of differentiation 14 (CD14) expression and inhibiting *Porphyromonas gingivalis* lipopolysaccharide (LPS)-stimulated cyclooxygenase-2 (COX-2) mRNA expression and prostaglandin E2 (PGE2) production in primary human monocytes (Jitprasertwong *et al*., 2016). In addition, progesterone was found to significantly reduce IL-1β-stimulated matrix metalloproteinase (MMP)-1, MMP-3, and MMP-10 production by human gingival fibroblasts (HGFs) (Lapp *et al*., 2003).

Human gingival fibroblasts constitute the major cellular part of the periodontal tissue. HGFs produce large amounts of MMPs, which are a family of zinc-containing and calcium-dependent endopeptidases (Graves, 2008). Matrix metalloproteinase-mediated periodontal tissue remodeling plays an important role in the pathogenesis of periodontal diseases including pregnancy gingivitis (Franco *et al*., 2017). Gingival inflammation during pregnancy is associated with a production and activation of several MMPs, such as MMP-1, MMP-2, MMP-8, and MMP-9 (Gursoy *et al*., 2010b). Lipopolysaccharide from a key periodontal pathogen, *P. gingivalis*, is known to induce the production of several MMPs which contribute to periodontal tissue destruction (Sorsa *et al*., 2006). In addition, elevated MMP levels and activity are detected in the gingival tissue and gingival crevicular fluid of gingivitis and periodontitis subjects (Stadler *et al*., 2016).

A key periodontal pathogen, *P. gingivalis*, activates the periodontal cells via toll-like receptors (TLRs), as shown in Figure 1. It is known that HGFs respond to *P. gingivalis* stimuli by increased MMPs' production (Bozkurt *et al*., 2017). However, the role of sex hormones combined with *P. gingivalis* LPS in the regulation of MMPs' expression in HGFs is still lacking. With this background, we hypothesized that increased levels of estradiol and progesterone during pregnancy may decrease gingival fibroblast immune responses to periodontal infection by *P. gingivalis*. The purpose of the present study was therefore to investigate the effects of β-estradiol and progesterone on MMP-1 and MMP-8 expressions in human gingival fibroblasts stimulated with *P. gingivalis* LPS.

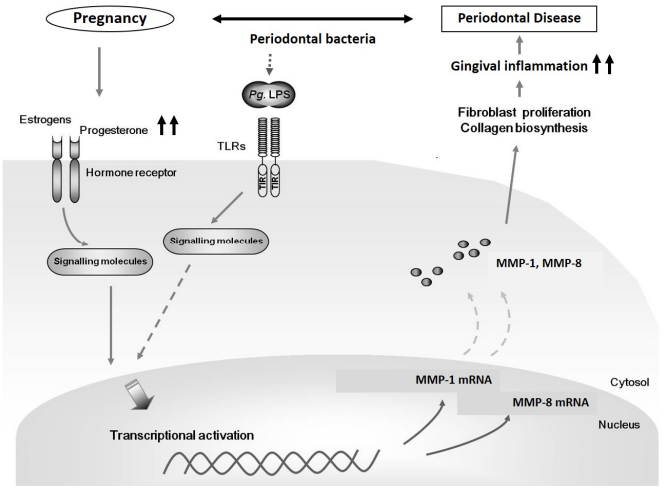

Figure 1. The schematic diagram explaining the proposed mechanisms of how female sex hormones and *P. gingivalis* LPS mediate gingival inflammation during pregnancy

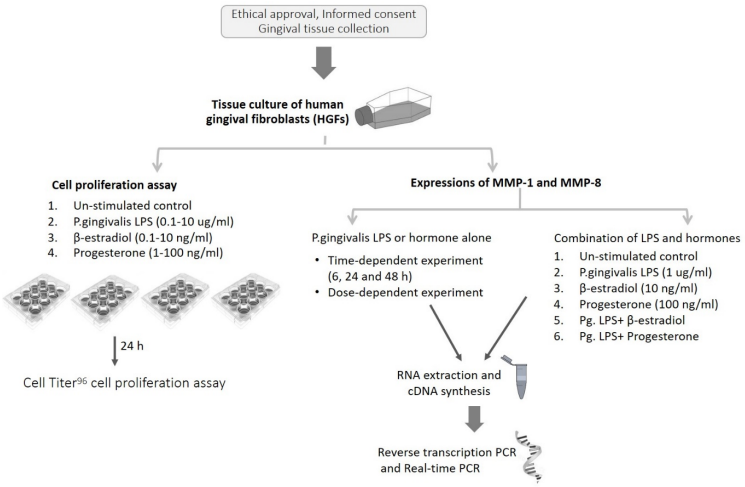

Figure 2. Summary of the methodology of the conceptual framework of the present study

Materials and Methods

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin G (100 U/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine (Gibco Laboratory, Grand Island, NY, USA) was used throughout the study. Progesterone (P7556) and β-estradiol (E4389) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Ultrapure LPS from *P. gingivalis* were purchased from Invivogen (San Diego, CA, USA).

Human Gingival Fibroblast Cell Culture

Healthy human gingival tissues were obtained with informed donor consent from 3 individuals during their crown lengthening procedure for prosthetic purposes. Both the research protocol and the written informed consent were reviewed and approved by the Institution Review Board, prior to study initiation. The certificate of ethical approval number is IRB. No. 350/58. The method of explanting fibroblasts from the gingival tissues was that described by Mahanonda *et al.* (2008). Briefly, the tissues were washed with DMEM supplemented with gentamycin (20 µg/mi), penicillin G (50 U/ml), streptomycin (50 µg/ml), and fungizone (2.5 µg/ml) and then were cut into small fragments approximately 1-3 mm^3 and placed in a 60-mm tissue culture dish. The tissues were cultured in DMEM supplemented with 10% FCS and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every 3 days. After a confluent monolyer of cells was reached, HGFs were trypsinized with 0.25% trypsin-EDTA for 2 min, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passage were used in this study.

Stimulation of HGF with Hormones and LPS

An overview of the experimental design is shown in Figure 2. Unless otherwise stated, all experiments were conducted in duplicate cultures from at least 3 independent healthy donors. Human gingival fibroblasts (2×10^5 cells) were seeded in 6-well plates (Corning Inc., Corning, NY, USA). Upon confluency, cells were serum starved for 18 h before stimulation with varying concentrations of hormones or LPS for up to 48 h. The concentrations of β-estradiol (0.1-10 µg/ml) and progesterone (1-100 µg/ml) used in experiments are based on their serum levels during pregnancy (Mealey and Moritz, 2003; Figuero *et al*., 2010) and the LPS concentrations are based on a previous study applying a comparable experimental setup (Herath *et al*., 2013). Then, cells were harvested for analysis of MMP-1 and MMP-8 mRNA expression using conventional and real-time RT-PCR.

Cell Proliferation Assay

The effects of hormones and LPS on cell proliferation of primary human gingival fibroblasts were evaluated by cell proliferation assay (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Briefly, gingival fibroblasts (1×10^4 cells/well) were cultured in duplicate and were stimulated with hormones or LPS for 24 h. An 8-point standard curve of cells with 2×10^5 cells/ml as the highest standard was produced using a 2-fold dilution series in fresh cell culture medium. After 1h incubation with Owen's reagent, absorption was measured at 460 nm on a spectrophotometer (Microplate Fluorescence Reader, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a reading at 645 nm was subtracted to adjust for plate background. Linear standard curve fitting was created to calculate the cell numbers of the samples.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA isolation was performed using RNeasy® Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. Concentration of RNA was measured using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Wilmington, DE, USA). One microgram of total RNA was used for the reverse transcription reaction by iScriptTM cDNA Synthesis kit (Bio-Rad Laboratories, Inc.). MMP-1 and MMP-8 mRNA expressions were detected by RT-PCR using i-TaqTM PCR Master (iNtRON Biotechnology, Inc., Seoul, South Korea). The primer sets were purchased from iNtRON Biotechnology, Inc., and the PCR conditions are shown in Table 1.

Quantitative Real-Time PCR

MMP-1 and MMP-8 mRNA expressions were also quantified using LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany). The β2 microglobulin (β2M) was used as the reference

gene. Data were analyzed by the comparative Ct method ($2^{-\delta\delta}$Ct) to calculate the relative fold changes between stimulations.

Statistical Analysis

Results were expressed as means ± SD from 3 independent experiments. Statistical analysis of real-time RT-PCR data was performed on ΔCt values (Jitprasertwong *et al*., 2014) using the SPSS program (version 17.0, SPSS Inc., Chicago, IL, USA). Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed prior to ANOVA. P-values were corrected for multiple comparisons with the Bonferroni-Holm method. A *p*-value of < 0.05 was considered significant.

Results and Discussion

B-estradiol and Progesterone have No Cytotoxic Effects on Human Gingival Fibroblasts

The viability of primary human gingival fibroblasts after stimulation with β-estradiol (0.1-10 μg/ml), or progesterone (1-100 μg/ml), or *P. gingivalis* LPS (0.1-10 μg/ml) for 24 h is shown in Figure 3. Cell proliferation was performed using Cell Titer96 (Promega Corp.) cell proliferation assay. The graph represents the mean ± SD from duplicate cultures of 3 experiments (n=6). Both β-estradiol and progesterone had no cytotoxic effect on primary human gingival fibroblasts. There are no significant differences in the total number of cells between those stimulated with hormones and the unstimulated controls ($p > 0.05$). We also found that *P. gingivalis* LPS (0.1-10 μg/ml) had no cytotoxic effect on human gingival fibroblasts after 24 h stimulation.

Effect of *P. Gingivalis* LPS on MMP-1 and MMP-8 mRNA Expression in Human Gingival Fibroblasts

The regulation of MMP-1 and MMP-8 mRNA expression by *P. gingivalis* LPS in human gingival fibroblasts was examined by conventional and real-time RT-PCR. HGFs (2×10^5 cells) were stimulated with 1 μg/ml of *P. gingivalis* LPS for 6, 24, and 48 h. The data are expressed as the means of duplicate cultures of 3 independent experiments (n=6). * indicates a statistically significant increase compared with the controls, $p \leq 0.05$. As shown in Figure 4(a), human gingival fibroblasts constitutively expressed MMP-1 and MMP-8 mRNA. Compared to the controls, *P. gingivalis* LPS significantly up-regulated MMP-1 and MMP-8 mRNA expression in a time-dependent manner. The results from real-time RT-PCR (Figure 4(b-c)) also demonstrated that *P. gingivalis* LPS (1 μg/ml) significantly induced the up-regulation of MMP-1 and MMP-8 in human gingival fibroblasts after 24-48 h stimulation ($p < 0.05$).

B-estradiol and Progesterone Down-Regulate Human Gingival Fibroblast MMP-1 and MMP-8 Expression

HGFs (2×10^5 cells) were stimulated with β-estradiol (0.1-10 μg/ml), or progesterone (1-100 μg/ml), or *P. gingivalis* LPS (0.1-10 μg/ml) for 24 h. Total RNA was isolated and the mRNA expressions of MMP-1 (A, B) and

Table 1. Primers used in the polymerase chain reaction expression analyses

Gene	Primer sequence (5'-3')	Annealing temp. (°C)	Product size (bp)
MMP-1	F: 5'-CATCCAAGCCATATATGGACGTTCC-3' R: 5'-TCTGGAGAGTCAAAATTCTCTTCGT-3'	*60*	*611*
MMP-8	F: 5'-AGCTGTCAGAGGCTGAGGTAGAAA-3' R: 5'-CCTGAAAGCATAGTTGGGATACAT-3'	*65*	*359*
β2M	F: 5'-ACCCCCACTGAAAAAGATGA-3' R: 5'-CTTATGCACGCTTAACTATC-3'	*60*	*435*

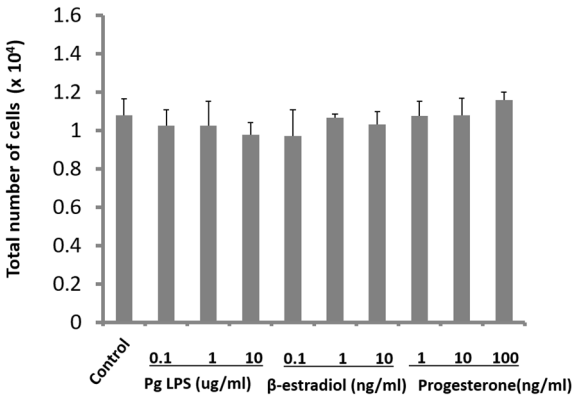

Figure 3. Effects of β-estradiol and progesterone on cell proliferation of human gingival fibroblasts

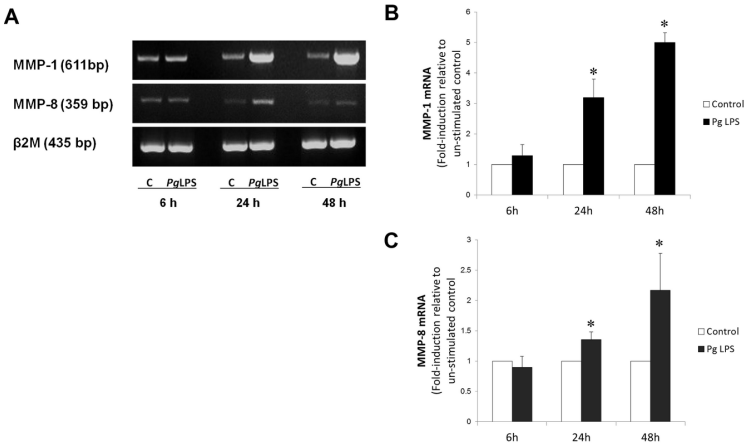

Figure 4. Time-dependent effect of lipopolysaccharide (LPS) on MMP-1 and MMP-8 mRNA expression in human gingival fibroblasts

MMP-8 (A, C) were analysed by conventional and real-time RT-PCR, as shown in Figure 5. The data are expressed as the means of duplicate cultures of 3 independent experiments (n=6). * indicates a statistically significant difference compared with the controls, $p \leq 0.05$. Conventional RT-PCR analysis demonstrated that *P. gingivalis* LPS induced a significant increase of MMP-1 and MMP-8 mRNA expression by stimulated cells, whereas down-regulations of MMP-1 and MMP-8 mRNA in fibroblasts treated with β-estradiol and progesterone were observed (Figure 5(a)). Significantly, real-time RT-PCR results (Figure 5(b-c)) showed that β-estradiol and progesterone reduced the expression of MMP-1 and MMP-8 mRNA by human gingival fibroblasts in a dose-dependent manner (p <0.05).

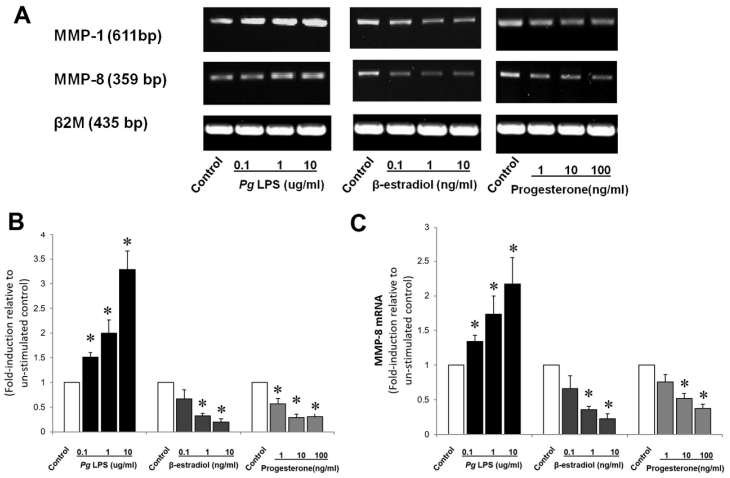

Figure 5. Dose-dependent effect of hormones and LPS on MMP-1 and MMP-8 mRNA expression in human gingival fibroblasts

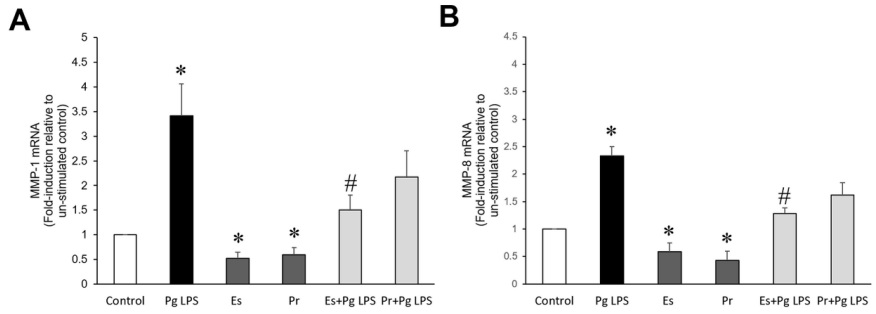

Figure 6. Effects of β-estradiol and progesterone on *P. gingivalis* LPS-induced MMP-1 and MMP-8 mRNA expression by human gingival fibroblasts

B-estradiol and Progesterone Reduce *P. gingivalis* LPS-induced MMP-1 and MMP-8 mRNA Expression by Human Gingival Fibroblasts

HGFs (2×10^5 cells) were pre-treated with either β-estradiol (10 μg/ml) or progesterone (100 μg/ml) for 24 h, followed by stimulation with *P. gingivalis* LPS for 24 h. Total RNA was isolated and the mRNA expression of MMP-1 (A) and MMP-8 (B) were quantified by real-time RT-PCR, as shown in Figure 6. The data are expressed as the means of duplicate cultures of 3 independent experiments (n=6). * indicates a statistically significant difference compared with the controls, $p \leq 0.05$. # indicates a statistically significant decrease compared with *P. gingivalis* LPS alone, $p \leq 0.05$. Both progesterone and β-estradiol were found to significantly inhibit MMP-1 and MMP-8 mRNA expression by human gingival fibroblasts (Figure 6(a-b)). Pre-treating human

gingival fibroblasts with β-estradiol (1 μg/ml) for 24 h significantly inhibited the effect of *P. gingivalis* LPS on MMP-1 and MMP-8 mRNA expression. In addition, the effect of *P. gingivalis* LPS on MMP-1 and MMP-8 expression was also reduced when cells were pre-incubated with progesterone (10 μg/ml), but these findings did not reach a statistical significance.

Exploring the underlying mechanism of how pregnancy contributes to periodontal disease highlights the link between these 2 conditions. Alteration of maternal immune responses during pregnancy may modulate the initiation and progression of periodontal disease (Armitage, 2013; Mariotti and Mawhinney, 2013). The levels of progesterone and estrogen are significantly increased throughout the 3 trimesters of pregnancy (Edelstam *et al*., 2007; Figuero *et al*., 2010) and play a vital role in the increase in gingival inflammation. Previous studies have identified the role of estradiol and progesterone as important immune modulators (Gilliver, 2010; Garcia-Gomez *et al*., 2013;).The findings of this study suggested that estradiol and progesterone have an inhibitory effect on the cellular response by human gingival fibroblasts to *P. gingivalis* LPS. We found that estradiol and progesterone significantly inhibited MMP-1 and MMP-8 mRNA expression and modulated gingival fibroblast responses to *P. gingivalis* LPS, a key pathogen in periodontal disease. Interestingly, pre-treatment HGFs with estradiol or progesterone reduced the effect of *P. gingivalis* LPS on the expression of MMP-1 and MMP-8 mRNA by human gingival fibroblasts.

Plasma estrogen and progesterone levels during pregnancy may reach 1-30 μg/ml and 10-300 μg/ml, respectively (Yokoyama *et al*., 2005). The selected concentrations of β-estradiol (0.1-10 μg/ml) and progesterone (1-100 μg/ml) in the present study could mimic the elevated levels of estradiol and progesterone in pregnant women. The results from the cell proliferation assay showed that both estradiol (0.1-10 μg/ml) and progesterone (1-100 μg/ml) had no proliferative or cytotoxic effects on human gingival fibroblasts after 24 h stimulation. Therefore, any hormones-induced MMP expressions were not due to changes in fibroblast cell numbers. Previous studies reported that incubation of HGFs with progesterone at concentrations of 50 and 100 μg/ml for 72 h significantly reduced cellular growth in fibroblast cultures, whereas estradiol (10, 50, and 100 μg/ml) did not have a significant effect on fibroblast cellular growth (Christoffers *et al*., 2003). Another study demonstrated that estradiol (1 nM) can induce cellular proliferation of HGFs after 5 days of incubation (Mariotti, 2005). These findings likely suggested that higher concentrations of estradiol and progesterone exert their proliferative and cytotoxic effect on human gingival fibroblasts when cells were incubated for up to 3-5 days. In addition, these discrepancies may be also due to the diverse immunological assay variations, different concentrations of hormones, or different incubation times.

Matrix metalloproteinases are a family of zinc-dependent proteinases implicated in the pathogenesis of periodontal diseases. HGF is a major source of MMP-1 and MMP-8, whereas MMP-8 is mainly produced by neutrophils (Gursoy *et al*., 2010a). Matrix metalloproteinase-1 (MMP-1) and MMP-8 expressions were significantly up-regulated in HGFs in response to *P. gingivalis* LPS (Sorsa *et al*., 2006; Cavalla *et al*., 2017). In periodontal lesion, MMPs account for the collagenase activity and initiate destruction of the extracellular matrix. In agreement with previous studies (Sorsa *et al*., 2006; Kuo *et al*., 2012), the present study demonstrated that MMP-1 and MMP-8 mRNA is expressed in HGFs and their expression can be induced by inflammatory stimuli such as LPS. Interestingly, both estradiol and progesterone down-regulate MMP-1 and MMP-8 mRNA expression in a dose-dependent manner.

It has been demonstrated that hormonal changes during pregnancy are associated with the reduced expression and activity of several MMPs not only in periodontal tissues but also in saliva (Gursoy *et al*., 2010a; Gursoy *et al*., 2010b). The levels of salivary MMP expression are significantly reduced during

pregnancy, and then levels returned to normal after lactation (Gursoy *et al*., 2013). It could be explained that low levels of MMP-1 and MMP-8 may be due to the inhibitory effect of female sex hormones on the functions of human gingival fibroblasts. Indeed, we demonstrated an inhibitory effect of female sex hormones on fibroblast stimulated with LPS from *P. gingivalis*, a key periodontal pathogen. We showed that treatment with either β-estradiol or progesterone significantly reduced the induction of MMP-1 and MMP-8 mRNA expression by *P. gingivalis* LPS in human gingival fibroblasts.

The anti-inflammatory roles of female sex hormones have previously been demonstrated in many studies (Su *et al*., 2009; Jones *et al*., 2010). In newborn mononuclear cells, estradiol and progesterone strongly inhibited TNF and IL-6 production induced by E. coli LPS (Giannoni *et al*., 2011). In addition, a recent study reported that β-estradiol and progesterone significantly decrease the proinflammatory properties of *P. gingivalis* LPS-induced COX2 expression and PGE2 secretion in human monocytes (Jitprasertwong *et al*., 2016). In HGFs, it has been shown that estradiol and progesterone reduces production of chemokines and MMPs by cultured human GFs in response to interleukin-1 (Lapp *et al*., 2003; Lapp and Lapp, 2005). The reduction of proteinase concentrations in local tissues, including saliva and GCF, may reflect suppressed functions of HGFs during pregnancy, which may partially explain the enhanced susceptibility to gingivitis during pregnancy and may also explain why the periodontal inflammation during pregnancy is limited to gingivitis and does not predispose or proceed to periodontitis. Taken collectively, hormonal changes seem to modulate gingival inflammation during pregnancy in association with immunosuppression and alteration in bacterial biofilm (Carrillo-de-Albornoz *et al*., 2012). These effects may enable the persistence of pathogens and therefore promote a disbiotic inflammatory environment of periodontal disease (Hajishengallis, 2015).

Conclusions

The present study supports that estrogen and progesterone suppress immune-inflammatory responses during pregnancy. The immunosuppressive effect of female sex hormones on MMP-1 and MMP-8 synthesis by human gingival fibroblasts may play a role in the periodontal disease progression in pregnant women.

Acknowledgments

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