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Effect of female sex hormones on *Campylobacter rectus* and human gingival fibroblasts

M. Yokoyama[1], D. Hinode[1], K. Masuda[1], M. Yoshioka[1], D. Grenier[2]
[1]Department of Preventive Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770–8504, Japan, [2]Groupe de Recherche en Écologie Buccale, Faculté de Médecine Dentaire, Université Laval, Quebec City, Quebec, Canada

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Recent studies have suggested a relationship between maternal *Campylobacter rectus* infections and preterm low birth weight. The purpose of this study was to investigate the effect of female sex hormones, estradiol and progesterone, on *C. rectus* and human gingival fibroblasts (HGF). The growth of *C. rectus* was significantly enhanced by incorporating either estradiol or progesterone in the culture medium. The production of vascular endothelial growth factor (VEGF), interleukin (IL)-6 and IL-8 by HGF increased following stimulation with estradiol or progesterone, at concentrations comparable to those present in the plasma of pregnant women. In addition, a significantly higher secretion of VEGF by HGF treated with the combination of *C. rectus* and estradiol was observed in comparison with a treatment with *C. rectus* alone. Stimulation of HGF with VEGF resulted in production of IL-6 and IL-8 in a dose-dependent manner. The capacity of female sex hormones to enhance both *C. rectus* growth and VEGF, IL-6, and IL-8 production by HGF has the potential to contribute to periodontal disease progression during pregnancy.

Key words: *Campylobacter rectus*; female sex hormones; human gingival fibroblasts; vascular endothelial growth factor

Daisuke Hinode, Department of Preventive Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770–8504, Japan
Tel.: +81 88 6337337;
Fax: +81 88 6337338;
e-mail: hinode@dent.tokushima-u.ac.jp
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Immunological changes have long been considered to be, at least in part, responsible for periodontal conditions observed during pregnancy (14, 27). Female sex hormones have also been suggested to play an important role in periodontal disease progression in pregnant women (17, 28). ElAttar (4) reported an increased synthesis of oral prostaglandin E_2 (PGE_2) when female sex hormones are present at high concentrations during pregnancy. Female sex hormones were also reported to stimulate growth of *Prevotella intermedia* (11) and may thus contribute to the fact that level of this periodontopathogen increases in periodontal sites of pregnant women (10).

Campylobacter rectus is a gram-negative motile rod that has been associated with periodontal diseases (15, 23). *C. rectus* is known to produce a variety of putative virulence factors, including lipopolysaccharide, crystalline surface layer (S-layer) and GroEL-like protein (GroEL), which can enhance the expression of various inflammatory mediators by host cells (8, 21, 30, 31, 33). More specifically, *C. rectus* lipopolysaccharide stimulated the production of PGE_2, interleukin (IL)-1 beta and IL-6 by gingival fibroblasts (21, 30), whereas *C. rectus* S-layer enhanced the expression and secretion of IL-6, IL-8, and tumor necrosis factor alpha by HEp-2 cells derived from human pharyngeal cancer (33). The expression of IL-6 and IL-8 by human gingival cells stimulated with *C. rectus* GroEL, known as 64-kDa heat shock protein, was also reported (8, 31).

Recent studies suggested that maternal periodontal diseases may represent a significant risk factor for preterm low birth weight (PLBW) (20) and that *C. rectus* might be associated with PLBW (16, 18). More specifically, it was reported that the prevalence of fetal immunoglobulin M positive to *C. rectus* was significantly higher for preterm than for full-term neonates (16). In addition, PLBW mothers had significantly higher levels of *C. rectus* in their subgingival plaque than women with normal birth outcome (18). In this study, we hypothesized that female sex hormones (estradiol and progesterone), through their effects on *C. rectus* and human gingival fibroblasts (HGF), may contribute to periodontal disease progression during pregnancy.

Material and methods

Determination of growth stimulating effect

C. rectus ATCC 33238 (American Type Culture Collection, Rockville, MD) and

796 (clinical isolate from periodontitis patient) were grown in Trypticase soy broth (Becton Dickinson and Company, Cockeysville, MD) supplemented with 0.2% yeast extract, 0.3% Phytone peptone, 0.2% NaCl, 0.3% ammonium formate, 0.4% sodium fumarate and 0.4% L-asparagine, adjusted to pH 7.8. *Porphyromonas gingivalis* ATCC 33277, *Actinobacillus actinomycetemcomitans* ATCC 29522, *Fusobacterium nucleatum* ATCC 23726 and *P. intermedia* ATCC 25611 were grown in brain heart infusion broth (Difco Laboratory, Detroit, MI) supplemented with 0.5% yeast extract, 0.001% vitamin K_1 and 0.0005% hemin, adjusted to pH 7.8. Growth was carried out at 37°C in an anaerobic chamber (80% N_2, 10% H_2 and 10% CO_2) for 24–36 h. Cultures were maintained by 1 : 500 dilution in their respective culture medium. Estradiol (Upstate Biotechnology, Inc., Lake Placid, NY) and progesterone (Calbiochem, La Jolla, CA), known as typical female sex hormones, were dissolved in dimethylsulfoxide (DMSO), and then diluted to 100 ng/ml in phosphate-buffered saline (PBS). One hundred µl of each bacterial culture and 100 µl of PBS containing estradiol, progesterone or 0.005% (v/v) DMSO (as control) were added into each well of 96-well microplates (Corning Glass Works, Corning, NY). Bacterial growth was monitored by measuring the absorbance at 655 nm (A_{655}) after 12, 24, 36, and 48 h of incubation using a microplate reader (Model 550, Bio-Rad, Hercules, CA). Bacterial cell counts of the cultures were determined (triplicate) by microscopy using a Petroff-Hausser counting chamber (Hausser Scientific Co., Horsham, PA) in order to confirm that increases in A_{655} values were correlated with increased cell yield.

Determination of VEGF, IL-6, and IL-8 production by HGF

Gin-1 cells (HGF cell line ATCC CRL-1292) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B and passaged by trypsinization. Cells, washed with DMEM to remove FBS and antibiotics, were used at passages 6–10.

Estradiol and progesterone were adjusted to 0.01, 0.1, 1, 10 or 100 ng/ml and 0.1, 1, 10, 100 or 1000 ng/ml in DMEM, respectively, and then 1 ml of each solution was added onto confluent monolayers of HGF in the wells of 24-well flat-bottom tissue culture plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan). Following incubation at 37°C for 24 h in a CO_2 atmosphere (5% CO_2, 95% air and 100% humidity), the concentrations of VEGF, IL-6 and IL-8 in the HGF culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using commercial assay kits (CytoSets, BioSource International, Camarillo, CA), according to the manufacturer's protocol.

VEGF production by HGF treated with the combination of female sex hormones and periodontopathogenic bacteria was also measured. Bacteria were harvested by centrifugation (10,000 *g* for 10 min) and suspended in DMEM to an absorbance at 650 nm equal to 0.1. One hundred µl of each bacterial sample, 500 µl of 100 ng/ml female sex hormones (estradiol or progesterone) or 0.005% (v/v) DMSO (as control), and 400 µl of DMEM were added onto confluent monolayers of HGF in wells of 24-well flat-bottom tissue culture plates. After 24 h of incubation, concentrations of VEGF in the conditioned media were measured as described above.

Production of IL-6 and IL-8 by HGF treated with VEGF was also investigated as above. Human recombinant VEGF, purchased from Endogen (Woburn, MA), was used at 62.5, 125, 250, 500 or 1000 pg/ml in DMEM.

Determination of HGF viability

Cell viability of HGF treated as above was measured using the colorimetric Cell Counting Kit-8 (Dojin Laboratories, Kumamoto, Japan). The culture supernatants were removed for quantitative analysis of VEGF, IL-6 and IL-8, and then 490 µl of DMEM and 10 µl of Cell Counting Kit-8 reagent were added onto HGF. After incubation in a CO_2 incubator for 1 h, 200 µl of each sample was transferred into wells of a 96-well microplate. The absorbance of these samples was measured using a microplate reader at two wavelengths, 450 and 655 nm (for the reference), against a background control as blank.

Statistical analysis

All experiments were performed in triplicate except for the study on the bacterial growth stimulating effect of estradiol and progesterone (n=10). The values represented the mean ± standard deviation (SD). Statistical significance was evaluated by unpaired Student's *t*-test, analysis of variance (ANOVA)/Scheffe's *F*-test or Pearson's correlation coefficient, at 5% significance level using the SPSS 11.0 for windows statistical software package (SPSS Inc., Chicago, IL).

Results

Growth stimulatory effect of estradiol and progesterone

As shown in Fig. 1, A_{655} values reflecting growth of both strains of *C. rectus* (ATCC 33238 and 796) at time 12, 24, 36 and 48 h in the culture medium supplemented with 50 ng/ml estradiol were found to be 1.4–3.7-fold higher ($P < 0.01$) than those obtained in the control medium. Similarly, A_{655} values for growth of *P. intermedia* in the presence of estradiol were 1.3–2.3-fold higher ($P < 0.01$) than the control. On the other hand, A_{655} values for *P. gingivalis*, *A. actinomycetemcomitans,* and *F. nucleatum* were not statistically different for growth in the presence compared with the absence of estradiol. When progesterone was used to supplement culture media, results comparable to those recorded with estradiol were obtained (data not shown).

The correlation between A_{655} values and bacterial cell counts determined with a Petroff-Hausser counting chamber was established for growth of *C. rectus* ATCC 33238 in the culture medium with estradiol. After 12, 24, 36, and 48 h of incubation, cell counts were $3.33 \pm 0.61 \times 10^7$/ml, $3.87 \pm 1.01 \times 10^7$/ml, $5.33 \pm 1.01 \times 10^7$/ml and $7.87 \pm 1.01 \times 10^7$/ml, respectively, which were positively correlated with the A_{655} ($r = 0.90$, $P < 0.01$). Similarly, positive correlations were also observed for growth of the other bacteria in spite of the presence of female sex hormones (data not shown).

Induction of VEGF, IL-6, and IL-8 production by HGF treated with estradiol and progesterone

The cell viability of HGF treated with estradiol or progesterone was decreased dose-dependently (Fig. 2A, B). A significant ($P < 0.01$) loss of viability was observed with estradiol at 100 ng/ml and progesterone at 1000 ng/ml. Production of VEGF by HGF stimulated with estradiol was dose-dependent and reached a value of 245 ± 36.3 pg/ml at 100 ng/ml estradiol, which was significantly higher ($P < 0.05$) than the unstimulated control (102 ± 10.2 pg/ml) (Fig. 2C). Stimulation of HGF with progesterone resulted in an optimal production of VEGF

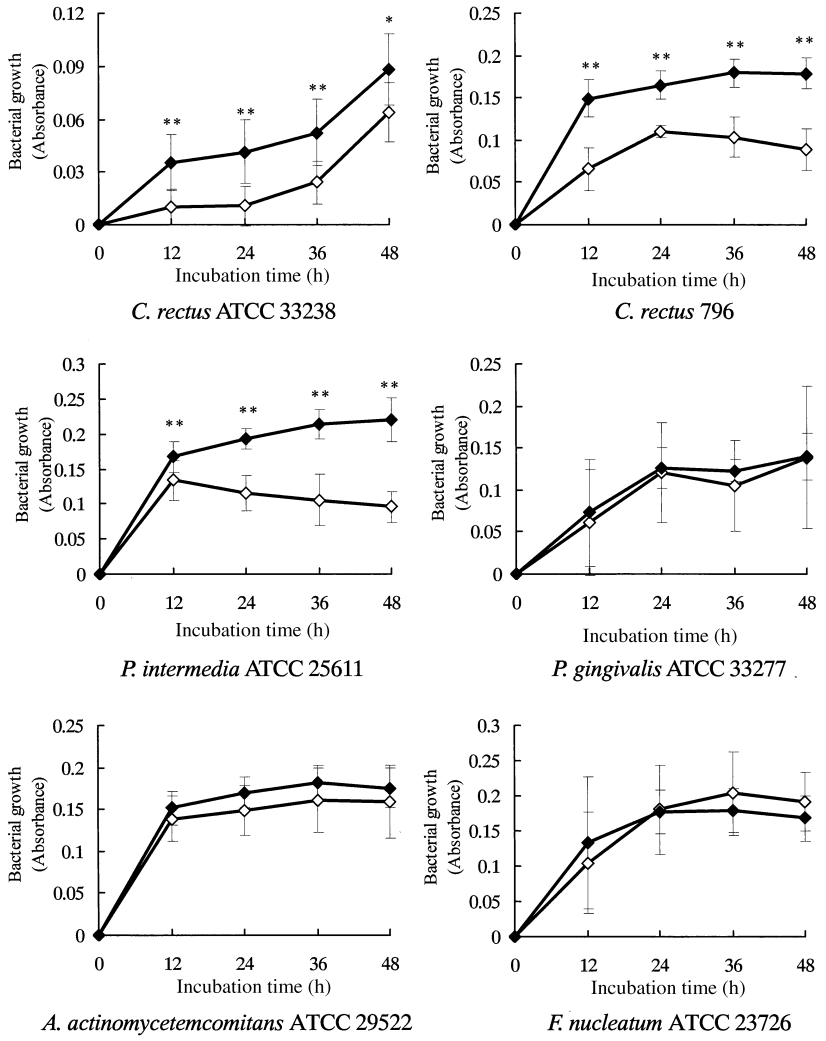

Fig. 1. Growth of periodontopathogenic bacteria with (◆) or without (◇) estradiol at 50 ng/ml. Horizontal axis shows the incubation time in hours. Vertical axis shows the absorbance values measured at 655 nm, which reflect bacterial growth. The values represent the means ± SD (n=10). Statistical significance was evaluated by unpaired Student's t-test at the 5% significance level: **$P < 0.01$, *$P < 0.05$.

(263 ± 11.0 pg/ml) at a concentration of 10 ng/ml ($P < 0.05$) (Fig. 2D). The secretion of IL-6 and IL-8 by HGF reached a plateau with both estradiol and progesterone at concentrations of 10–100 ng/ml (Fig. 2E–H).

The amounts of VEGF secreted by HGF treated with *C. rectus* alone or *C. rectus* in association with estradiol were 1336 ± 38.7 pg/ml and 1497 ± 75.1 pg/ml, respectively, which were significantly different at $P < 0.01$. When the other periodontopathogenic bacteria were tested in association with estradiol, no statistically significant increase were observed. Similarly, no statistically significant differences were observed when all bacteria were tested in association with progesterone (data not shown).

HGF were then treated with VEGF to investigate production of IL-6 and IL-8. The concentration of VEGF used for the treatment of HGF was positively correlated with the secreted levels of IL-6 (Fig. 3A) and IL-8 (Fig. 3B). The cell viability of HGF treated with VEGF (up to 1000 pg/ml) was not affected (data not shown).

Discussion

C. rectus is a member of the consortium of bacteria for which evidence suggests a role in periodontitis (15, 23). This bacterial species has also been associated with PLBW (16, 18). In this study, we showed that estradiol and progesterone can significantly stimulate the growth of *C. rectus*. In addition, we confirmed the results of Kornman & Loesche (11) indicating the growth stimulatory effect of female sex hormones on *P. intermedia*. The correlation obtained between A_{655} values and bacterial cell counts indicates that increases in absorbance reflect bacterial growth and not clumping or aggregation of bacteria. Aspartate and asparagine may serve as natural electron acceptors for *C. rectus* in periodontal lesions in which proteolytic bacteria are often predominant (22). Since large amounts of formate are produced by *P. intermedia* and this compound stimulates the growth of *C. rectus* (7), female sex hormones may also indirectly stimulate growth of *C. rectus*. Hormonal steroids may thus influence the microbial ecology of the gingival sulcus and be responsible for changes observed in subgingival bacterial flora of pregnant women (10). Further research is needed to clarify the mechanisms by which estradiol and progesterone support the growth of *C. rectus* and whether hormonal steroids can enhance tissue adherence of periodontopathogens as observed for *Chlamydia trachomatis* (1).

VEGF, also known as vascular permeability factor (26), is member of the angiogenic factor family (13). VEGF is expressed during the process of placental angiogenesis in pregnant women (24) and the concentration of maternal serum VEGF increases remarkably during pregnancy (5). VEGF is also thought to play an important role in pathological angiogenesis such as tumors, rheumatoid arthritis and diabetic retinopathy (19). There are several findings about VEGF production by human gingival fibroblasts. Doan et al. (3) and Yoshino et al. (35) reported, respectively, that VEGF production was stimulated in human gingival fibroblasts by ultrasound and cyclic tensile stretching. Yuan et al. (36) reported that the pyogenic granuloma group expressed significantly more VEGF than healthy gingiva and periodontitis, and that this VEGF was mostly localized in the cytoplasm of macrophages and fibroblasts. Lastly, Suthin et al. (29) reported that human gingival fibroblasts produced VEGF and that levels were significantly enhanced by stimulation with vesicles and outer membrane proteins from *A. actinomycetemcomitans* and *P. gingivalis*.

During pregnancy, the concentrations of estradiol and progesterone in plasma increase to approximately 30 and 300 ng/ml, respectively, both of which are 1000 times higher than the levels observed during follicular phase of menstrual cycle

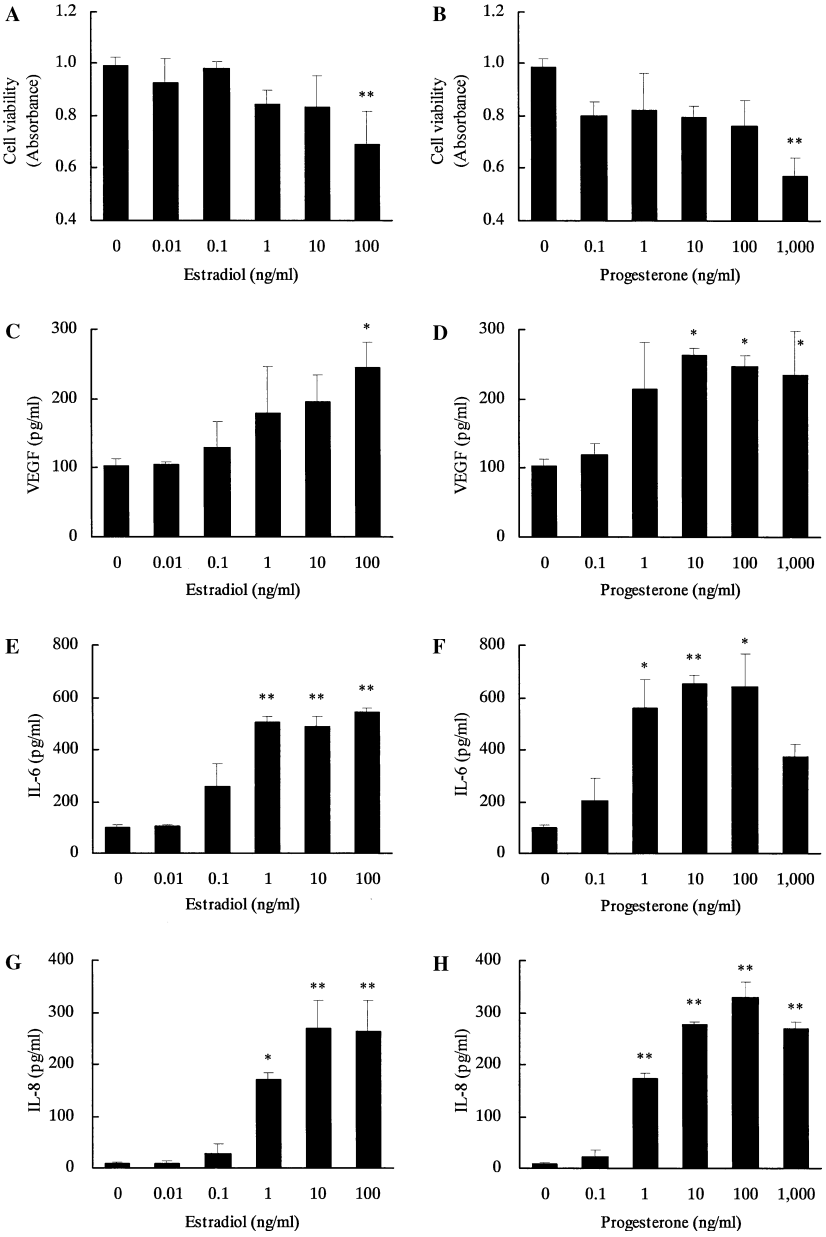

Fig. 2. Production of VEGF, IL-6, and IL-8 by HGF treated with either estradiol or progesterone. Horizontal axis shows the concentration of estradiol (A, C, E and G) or progesterone (B, D, F and H) used to stimulate cells. Vertical axis in panels A and B shows the absorbance value, obtained at 450 and 655 nm (for reference), which reflects the cell viability. Vertical axis in panels C and D, E and F, and G and H shows the secretion levels of VEGF, IL-6, and IL-8, respectively, by HGF. All experiments were performed in triplicate. The values represent the means ± SD. Statistical significance was evaluated by ANOVA/Scheffe's *F*-test at the 5% significance level: $^{**}P < 0.01$, $^{*}P < 0.05$.

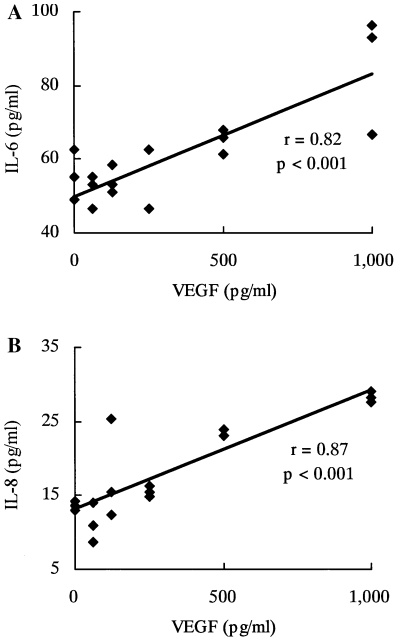

Fig. 3. Production of IL-6 and IL-8 by HGF treated with VEGF. Horizontal axis shows the concentration of VEGF added. Vertical axis shows the secretion levels of IL-6 (A) and IL-8 (B) by HGF. All experiments were performed in triplicate. Statistical significance was evaluated by Pearson's correlation coefficient at the 5% significance level.

(32). Furthermore, the levels of female sex hormones in saliva increase during pregnancy (37). In this study, we hypothesized that the increased amounts of female sex hormones produced by pregnant women might contribute to periodontal disease progression through enhancement of VEGF production in the periodontium. We showed that production of VEGF by HGF was enhanced significantly, in spite of the decrease of cell viabilities, by stimulation with estradiol and progesterone at concentrations comparable to those found in plasma of pregnant women. Interestingly, the amounts of VEGF secreted by HGF treated with *C. rectus* in association with estradiol were higher than with *C. rectus* alone. This reflects an additive effect of *C. rectus* and estradiol. Ishihara et al. (9) demonstrated that the 16S rRNA detection rates of *C. rectus* and *P. gingivalis* in coronary artery plaque samples were positively correlated with their presence in subgingival plaque. This finding suggests that *C. rectus* might penetrate gingival tissues and enter blood stream as well as *P. gingivalis* (12, 25). Therefore, the additive effect of *C. rectus* and estradiol against HGF seems to be meaningful.

Pro-inflammatory cytokines such as IL-6 and IL-8 are believed to be major pathological mediators in periodontal disease (34). These cytokines have also been suggested to be involved in the regulation of placental growth during later stages of pregnancy (2). Greig et al. (6) reported that, at term, women in labor had significantly elevated median maternal serum IL-6 concentrations compared with those at term but not in labor. We showed that production of IL-6 and IL-8 by HGF was enhanced significantly by stimulation with female sex hormones at high concentrations, which were comparable to those found in plasma of pregnant women. This result suggests that large amounts of female sex hormones in pregnant women may directly stimulate the production of these cytokines by gingiva as well as placenta. Furthermore, we also showed

that the concentration of VEGF used to stimulate HGF was positively correlated with the levels of IL-6 and IL-8 secreted by those cells. This result suggests that the increased concentration of VEGF in the oral cavity of pregnant women may favor periodontal inflammation by both accelerating the vascular permeability and stimulating the production of IL-6 and IL-8.

In summary, our findings suggest that the enhancement of both *C. rectus* growth and VEGF, IL-6, and IL-8 production by HGF mediated by female sex hormones has the potential to contribute to periodontal disease progression during pregnancy. Further research is needed to clarify the definitive molecular mechanisms leading to the effects observed in this study.

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References

1. Bose SK, Goswami PC. Enhancement of adherence and growth of *Chlamydia trachomatis* by estrogen treatment of HeLa cells. Infect Immun 1986: **53**: 646–650.
2. Bowen JM, Chamley L, Keelan JA, Mitchell MD. Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition. Placenta 2002: **23**: 257–273.
3. Doan N, Reher P, Meghji S, Harris M. *In vitro* effects of therapeutic ultrasound on cell proliferation, protein synthesis, and cytokine production by human fibroblasts, osteoblasts, and monocytes. J Oral Maxillofac Surg 1999: **57**: 409–420.
4. ElAttar TM. Prostaglandin E_2 in human gingiva in health and disease and its stimulation by female sex steroids. Prostaglandins 1976: **11**: 331–341.
5. Evans PW, Wheeler T, Anthony FW, Osmond C. A longitudinal study of maternal serum vascular endothelial growth factor in early pregnancy. Hum Reprod 1998: **13**: 1057–1062.
6. Greig PC, Murtha AP, Jimmerson CJ, Herbert WN, Roitman-Johnson B, Allen J. Maternal serum interleukin-6 during pregnancy and during term and preterm labor. Obstet Gynecol 1997: **90**: 465–469.
7. Grenier D, Mayrand D. Nutritional relationships between oral bacteria. Infect Immun 1986: **53**: 616–620.
8. Hinode D, Yoshioka M, Tanabe S, Miki O, Masuda K, Nakamura R. The GroEL-like protein from *Campylobacter rectus*. immunological characterization and interleukin-6 and -8 induction in human gingival fibroblast. FEMS Microbiol Lett 1998: **167**: 1–6.
9. Ishihara K, Nabuchi A, Ito R, Miyachi K, Kuramitsu HK, Okuda K. Correlation between detection rates of periodontopathic bacterial DNA in coronary stenotic artery plaque [corrected] and in dental plaque samples. J Clin Microbiol 2004: **42**: 1313–1315.
10. Kornman KS, Loesche WJ. The subgingival microbial flora during pregnancy. J Periodontal Res 1980: **15**: 111–122.
11. Kornman KS, Loesche WJ. Effects of estradiol and progesterone on *Bacteroides melaninogenicus* and *Bacteroides gingivalis*. Infect Immun 1982: **35**: 256–263.
12. Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A. *Porphyromonas gingivalis* invasion of gingival epithelial cells. Infect Immun 1995: **63**: 3878–3885.
13. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989: **246**: 1306–1309.
14. Loe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand 1963: **21**: 533–551.
15. Macuch PJ, Tanner AC. *Campylobacter* species in health, gingivitis, and periodontitis. J Dent Res 2000: **79**: 785–792.
16. Madianos PN, Lieff S, Murtha AP, Boggess KA, Auten RL Jr, Beck JD, et al. Maternal periodontitis and prematurity. Part II. Maternal infection and fetal exposure. Ann Periodontol 2001: **6**: 175–182.
17. Mascarenhas P, Gapski R, Al-Shammari K, Wang HL. Influence of sex hormones on the periodontium. J Clin Periodontol 2003: **30**: 671–681.
18. Mitchell-Lewis D, Engebretson SP, Chen J, Lamster IB, Papapanou PN. Periodontal infections and pre-term birth: early findings from a cohort of young minority women in New York. Eur J Oral Sci 2001: **109**: 34–39.
19. Mukhopadhyay D, Zeng H, Bhattacharya R. Complexity in the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)-receptors signaling. Mol Cell Biochem 2004: **264**: 51–61.
20. Offenbacher S, Katz V, Fertik G, Collins J, Boyd D, Maynor G, et al. Periodontal infections as a possible risk factor for preterm low birth weight. J Periodontol 1996: **67**: 1103–1113.
21. Ogura N, Matsuda U, Tanaka F, Shibata Y, Takiguchi H, Abiko Y. *In vitro* senescence enhances IL-6 production in human gingival fibroblasts induced by lipopolysaccharide from *Campylobacter rectus*. Mech Ageing Dev 1996: **87**: 47–59.
22. Ohta H, Gottschal JC, Fukui K, Kato K. Aspartate and asparagine as electron acceptors for *Wolinella recta*. Oral Microbiol Immunol 1991: **6**: 76–80.
23. Rams TE, Feik D, Slots J. *Campylobacter rectus* in human periodontitis. Oral Microbiol Immunol 1993: **8**: 230–235.
24. Reynolds LP, Redmer DA. Angiogenesis in the placenta. Biol Reprod 2001: **64**: 1033–1040.
25. Sandros J, Papapanou PN, Nannmark U, Dahlen G. *Porphyromonas gingivalis* invades human pocket epithelium *in vitro*. J Periodontal Res 1994: **29**: 62–69.
26. Senger DF, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983: **219**: 983–985.
27. Silness J, Loe H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. Acta Odontol Scand 1964: **22**: 121–135.
28. Sorry M. Hormonal factors in periodontal disease. Dent Update 2000: **27**: 380–383.
29. Suthin K, Matsushita K, Machigashira M, Tatsuyama S, Imamura T, Torii M, et al. Enhanced expression of vascular endothelial growth factor by periodontal pathogens in gingival fibroblasts. J Periodontal Res 2003: **38**: 90–96.
30. Takiguchi H, Yamaguchi M, Okamura H, Abiko Y. Contribution of IL-1 beta to the enhancement of *Campylobacter rectus* lipopolysaccharide-stimulated PGE_2 production in old gingival fibroblasts *in vitro*. Mech Ageing Dev 1997: **98**: 75–90.
31. Tanabe S, Hinode D, Yokoyama M, Fukui M, Nakamura R, Yoshioka M, et al. *Helicobacter pylori* and *Campylobacter rectus* share a common antigen. Oral Microbiol Immunol 2003: **18**: 79–87.
32. Tulchinsky D, Hobel CJ, Yeager E, Marshall JR. Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. Am J Obstet Gynecol 1972: **112**: 1095–1100.
33. Wang B, Kraig E, Kolodrubetz D. Use of defined mutants to assess the role of the *Campylobacter rectus* S-layer in bacterium–epithelial cell interactions. Infect Immun 2000: **68**: 1465–1473.
34. Wilson M, Reddi K, Henderson B. Cytokine-inducing components of periodontopathogenic bacteria. J Periodontal Res 1996: **31**: 393–407.
35. Yoshino H, Morita I, Murota SI, Ishikawa I. Mechanical stress induces production of angiogenic regulators in cultured human gingival and periodontal ligament fibroblasts. J Periodontal Res 2003: **38**: 405–410.
36. Yuan K, Jin YT, Lin MT. The detection and comparison of angiogenesis-associated factors in pyogenic granuloma by immunohistochemistry. J Periodontol 2000: **71**: 701–709.
37. Zaki K, el Hak R, Amer W, Saleh F, El Faras A, Ragab L, et al. Salivary female sex hormone levels and gingivitis in pregnancy. Biomed Biochim Acta 1984: **43**: 749–754.