Original Article

Evaluation of Microflora (Viral and Bacterial) in Subgingival and Placental Samples of Pregnant Women with Preeclampsia with and without Periodontal Disease: A Cross-Sectional Study

Swetha Tanneeru[1], Jaideep Mahendra[2], Mahaboob Vali Shaik[3]

[1]Department of Periodontology, Narayana Dental College and Hospital, Nellore, Andhra Pradesh, [2]Department of Periodontology, Meenakshi Ammal Dental College & Hospital, Meenakshi Academy of Higher Education & Research, Chennai, Tamil Nadu, [3]Department of Advanced Research Centre, Narayana Medical College, Nellore, Andhra Pradesh, India

Received : 20-08-19.
Revised : 13-11-19.
Accepted : 15-12-19.
Published : 10-03-20.

ABSTRACT

Aim: Previous studies showed associated periodontal disease with various systemic ailments. This research work was aimed at studying the presence and role of periodontal microflora on preeclampsia during pregnancy. **Materials and Methods:** A cross-sectional study was designed on pregnant women with preeclampsia with and without chronic periodontitis, attending Narayana Medical College and Hospital, Nellore, Andhra Pradesh, India, for prenatal checkups. After obtaining consents, 445 women were recruited in the study. On the basis of systemic and periodontal health, subjects were grouped into Group 1 (women with preeclampsia with chronic periodontitis) and Group 2 (women with preeclampsia without chronic periodontitis). Clinical parameters such as plaque index, bleeding on probing, probing depth, and clinical attachment level were recorded. Quantification of periodontopathic bacteria (*Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola*), Epstein–Barr virus (EBV), cytomegalovirus (CMV), and herpes simplex virus (HSV), were detected using real-time polymerase chain reaction in subgingival samples at one point of time and later compared in placental tissue after parturition. **Results:** *T. forsythia*, *T. denticola*, *F. nucleatum*, *P. intermedia*, EBV, CMV, and HSV were expressed more in Group 3 compared to those in Groups 2, 4, and 1, in their subgingival and placental samples. **Conclusion:** Elevated levels of bacteria and viruses were expressed in subgingival and placental samples in women with preeclampsia with chronic periodontitis compared to those in women with preeclampsia without chronic periodontitis. This shows that chronic periodontitis is a risk factor for preeclampsia. The results concluded that periodontal flora is not only localized to periodontal tissues but can also enter uterine cavity and may elicit their pathological response on mother and developing fetus.

Keywords: *Chronic periodontitis, microflora, periodontal infection, preeclampsia*

Introduction

Periodontal disease is a chronic polymicrobial infection affecting periodontal tissues. Chronic periodontitis is one of the very common chronic diseases worldwide, and is caused mainly by gram-negative anaerobic bacteria.[1] This disease is associated with the accumulation of microflora at the dentogingival margin, although the causal relationship of specific organisms is not fully clear. It is believed that host bacterial interactions are

Access this article online

Quick Response Code:

Website: www.jispcd.org

DOI: 10.4103/jispcd.JISPCD_341_19

Address for correspondence: Dr. Jaideep Mahendra, Department of Periodontology, , Meenakshi Ammal Dental College & Hospital, Meenakshi Academy of Higher Education & Research, Madhuravoyal, Chennai 600095, Tamil Nadu, India.
E-mail: swethatanneeru@yahoo.com

How to cite this article: Tanneeru S, Mahendra J, Shaik MV. Evaluation of Microflora (Viral and Bacterial) in Subgingival and Placental Samples of Pregnant Women with Preeclampsia with and without Periodontal Disease: A Cross-Sectional Study. J Int Soc Prevent Communit Dent 2020;10:171-176.

responsible for the destruction of host periodontal tissues, and they lead to clinical manifestations of the disease.[1]

The host tissue responds to the microbial challenge by generating inflammatory cell infiltrate in the tissue subjacents. Periodontal inflammation may not be limited to periodontal tissues, but it is well documented that periodontal diseases can affect systemic ailments, including adverse pregnancy outcomes.[1]

Periodontal diseases experience frequent bacteremia resulting in the colonization of specific organisms or their products in uterine cavity.[2] Bacteria associated with periodontal disease are not dissimilar to those known to be associated with genitourinary bacterial infections and adverse pregnancy outcomes. Many studies have shown the translocation of *Fusobacterium nucleatum*, *Prevotella nigrescens*, *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Treponema denticola* to the feto-placental unit, whereby a maternal or fetal response has been shown, resulting in premature birth or low birth weight.[2,3] Since then more attention has been focused on oral infections and their effects on pregnancy.

Preeclampsia is a maternal syndrome characterized by proteinuria and hypertension and involves multi-organ tissue destruction and has become one of the main causes of maternal and fetal mortality.[4] Many authors have postulated that periodontal disease is a possible risk factor for complications in pregnancy, including preeclampsia.[5,6] However, this association has yet to be proved.[7]

This study was aimed at the identification of microflora (viral and bacterial) in subgingival and placental samples of pregnant women with preeclampsia with and without periodontal disease, and to coincide their percentages in subgingival plaque samples and placental samples to check the presence or absence of periodontal microbial involvement in placental tissues.

Materials and Methods

The proposed study design was approved by the Institutional Ethics Committee, Narayana Dental College and Hospital with reference number Rc. no. ndc/pg/2015–16/ec/2015/01. Women attending prenatal checkups in Narayana Medical College and Hospital were screened for the study. After obtaining written consents, 200 preeclamptic subjects who met the eligibility criteria were recruited and categorized into two groups based on the presence and absence of chronic periodontitis: Group 1 (women with preeclampsia with periodontal disease, $n = 100$) and Group 2 (women with preeclampsia without periodontal disease, $n = 100$). Clinical parameters such as plaque index, bleeding on probing, probing depth, and clinical attachment level were recorded. Quantification of periodontopathic bacteria *P. gingivalis*, *P. intermedia*, *F. nucleatum*, and *Tannerella forsythia* and viruses such as herpes simplex virus (HSV), Epstein–Barr virus (EBV) and human cytomegalovirus (HCMV) were detected in subgingival samples at one point of time and later compared in placental tissue after parturition using 16S ribosomal ribonucleic acid (rRNA)-based real-time polymerase chain reaction (RT-PCR).

Sample collection and storage

The sampling site was isolated using cotton rolls, and supragingival plaques were removed with the help of sterile cotton, collected using sterile Gracey curette, and suspended in 100 µL of Tris–HCl buffer. The samples were immediately incubated at 50°C for 10 min and then stored at -20°C freezer till further processing.

Placental samples were also collected and stored for the same patients and stored as aforementioned.

All the plaque samples and placental samples were subjected to PCR, which was carried out at advanced research center (Narayana Medical Institutions, Nellore, Andhra Pradesh, India).

Materials

1. Tris EDTA (TE) buffer (pH 8.0)
2. 10% SDS
3. Proteinase K
4. Phenol–chloroform mixture
5. 5 M sodium acetate (pH 5.2)
6. Isopropanol
7. 70% ethanol
8. 2 mL Eppendorf tubes
9. Micropipette: 1–10 µL, 20–100 µL, and 200–1000 µL
10. Microtips: 1–10 µL, 20–100 µL, and 200–1000 µL
11. Micro-centrifuge
12. ×10 Luna Universal quantitative polymerase chain reaction Master Mix (contains deoxynucleoside triphosphate (dATP, dTTP, dCTP, and dGTP; $MgCl_2$; Taq buffer; SYBR Green; polymerase enzyme)

Isolation of deoxyribonucleic acid from plaque samples

- Samples from deep freezer (-80°C) were allowed to thaw at room temperature.
- Plaque samples in microcentrifuge tubes were centrifuged at 10,000 rpm for 5 min.
- A total of 875 µL of TE buffer was added to the pellet and resuspended in the buffer by gentle mixing.
- A total of 100 µL of 10% SDS and 5 µL of Proteinase K were added to the cells.

- The aforementioned mixture was mixed well and incubated at 37°C for 1 h in incubator. A total of 1 mL of phenol–chloroform mixture (3:1) was added to the contents, mixed well by inverting, and incubated at room temperature for 5 min and centrifuged at 10,000 rpm for 10 min at 4°C.
- The highly viscous jellylike supernatant was collected using Q-tips and was transferred to a fresh tube.
- The process was repeated once again with phenol–chloroform mixture, and the supernatant was collected in a fresh tube.
- A total of 100 µL of 5 M sodium acetate was added to the contents and was mixed gently.
- A total of 2 mL of isopropanol was added and mixed gently by inversion till white strands of DNA precipitates out, and it was centrifuged at 5000 rpm for 10 min.
- The supernatant was removed and 1 ml 70% ethanol was added and again centrifuged at 10,000 rpm for 10 min. After air-drying for 5 min, 200 µL of TE buffer or distilled water was added to the pellet.

Isolation of deoxyribonucleic acid from placental samples

- Samples from deep freezer (-80°C) were allowed to thaw at room temperature.
- After thawing, placental tissues were homogenized by standard protocol with homogenizer. Homogenized samples were added with 100 µL of lysis buffer and 100 µL of 10% SDS and 5 µL of Proteinase K solution.
- The aforementioned mixture was mixed well and incubated at 37°C for 1 h in incubator. A total of 1 mL of phenol–chloroform mixture (3:1) was added to the contents, mixed well by inverting, and incubated at room temperature for 5 min.
- The contents were centrifuged at 10,000 rpm for 10 min at 4°C.
- The highly viscous jellylike supernatant was collected using Q-tips and was transferred to a fresh tube.
- The process was repeated once again with phenol–chloroform mixture, and the supernatant was collected in a fresh tube.
- A total of 100 µL of 5 M sodium acetate was added to the contents and was mixed gently.
- A total of 2 mL of isopropanol was added and mixed gently by inversion till white strands of DNA precipitates out, and it was centrifuged at 5000 rpm for 10 min.
- The supernatant was removed and 1 mL 70% ethanol was added and centrifuged at 10,000 rpm for 10 min.
- After air-drying for 5 min, 200 µL of TE buffer was added to the pellet.

Measurement of deoxyribonucleic acid concentration

The concentration of DNA was determined using a NanoDrop (Thermo Scientifics, Waltham, Massachusetts, USA) spectrophotometer at 260/280 nm. The remaining samples were stored for PCR experiment.

This procedure was used to determine the amount, concentration, and purity of the DNA sample. Turn on the NanoDrop, click on ultraviolet (UV) measure option in NanoDrop software. Take 1 µL TE buffer to measure blank. Measure all the DNA samples (1 µl) separately.

The quality and yield obtained was measured by using a NanoDrop spectrophotometer. Quantitation of DNA was carried out of 1 µl of all plaque DNA samples using NanoDrop 2000/2000c spectrophotometers at absorbance at 260 and 280 nm. The quality and yield obtained was measured by the ratio of 260 and 280 nm using a NanoDrop spectrophotometer.

Polymerase chain reaction procedure

All the mix was prepared in hard-shell PCR plate 96 well WHT-CLR (cat. no. HSP 9601) (Bio-Rad Laboratories, Hyderabad, Telangana, India) with seal plates of optically transparent film (Bio-Rad Laboratories). Care was taken to seal the plate edges and corners to prevent artifacts caused by evaporation. PCR amplification was performed in a real-time thermocycler (Bio-Rad-CFX100; Bio-Rad Laboratories). SYBR Green channel of the real-time instrument Bio-Rad-CFX100 (Bio-Rad Laboratories) was used for the quantification of *P. gingivalis* using Luna Universal Master Mix.

Putative periodontopathic bacteria (*P. gingivalis*, *F. nucleatum*, *P. intermedia*, *T. forsythia*, and *T. denticola*) were detected using 16S rRNA-based PCR. The primers of these microorganisms were designed based on the relevant literature and National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) and then synthesized by Bioserve Biotechnologies and IRA Biotech, Hyderabad, Telangana, India. Primer sequences are shown in Table 1.

Luna Universal qPCR Master Mix and other reaction components were kept at room temperature to set them at room temperature, and then placed on ice. After thawing completely, all reagents were mixed by inversion, pipetting, or gentle vortexing.

Reaction setup

Component	25 µL reaction	Final concentration
Luna Universal qPCR Master Mix	10 µL	×1
Forward primer (10 µM)	1 µL	0.4 µM
Reverse primer (10 µM)	1 µL	0.4 µM
Template DNA	Variable	<100 ng
Nuclease-free water	Up to 25 µL	

For each batch of samples, negative control was set for PCR amplification using sterile deionized water. Duplicates were performed for all samples.

The reaction conditions of *P. gingivalis*, *F. nucleatum*, and *T. forsythia* were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a last extension at 72°C for 5 min.

The reaction conditions of *P. intermedia* and *T. denticola* were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s, with a last extension at 72°C for 5 min.

The relative quantification of bacterial load was achieved by comparison with a standard amplification curve obtained from the (standard) genomic DNA corresponding to 6×10^6 colony forming units (CFUs) of bacterial pure cultures. The standard bacterial DNA concentrations are 1 × 109, 1 × 107, 1 × 105, 1 × 103, and 1 × 101, respectively. Finally, the expected amplicon was analyzed along with standard 1 kb DNA ladder on 1.5% agarose gel under UV transillumination.

Results

Table 1 shows that high levels of microbes were observed both in subgingival and placental samples of Group 1 subjects compared to those in Group 2.

Statistical analysis

The descriptive statistics (mean, standard deviation, or percentage) of all variables were recorded. Chi-square tests or two sample *t* tests were performed to compare differences in periodontal clinical parameters and the prevalence of periodontopathic microorganisms between the case group and the control group. The quality and yield obtained was measured by using a NanoDrop spectrophotometer. The NanoDrop data given mean DNA yield obtained was 120 ng/µL (range, 51–225 ng/µL) and purity (A260/A280 ratios) ranged between 1.55 and 1.90.

Polymerase chain reaction data analysis

In our approach, each bacterial species was targeted by individual qPCR reactions containing specific primer pairs. PCR experiment with primers of putative periodontopathic bacteria (*P. gingivalis*, *F. nucleatum*, *P. intermedia*, *T. forsythia*, and *T. denticola*) and viruses were performed by amplifying the 16S rRNA gene set up with the extracted genomic DNA from each sample. RT-PCR experiment using serial diluted templates over four orders of magnitude indicate the absence of PCR inhibitors in the extracted DNA.

The RT-PCR using the SYBR Green chemistry was applied to the clinical plaque samples, which has been described in "Methods" section. The original 10 µL of extracted DNA samples was run in the assay. The 16S rRNA gene in the plaque samples was evaluated by the SYBR Green assay using the standard of the DNA of each bacteria.

Table 1: Comparison of virus in both subgingival plaque and placental samples within Groups

Microbes	Subgingival samples			Placental samples		
	Group 1 Preeclamptic pregnant with periodontitis (*n* = 100)	Group 2 Preeclamptic pregnant without periodontitis (*n* = 100)	*P* value	Group 1 Preeclamptic pregnant with periodontitis (*n* = 25)	Group 2 Preeclamptic pregnant without periodontitis (*n* = 25)	*P* value
Epstein–Barr virus	79 (79%)	20 (20%)	<0.0001*	10 (40%)	5 (20%)	0.123
Human cytomegalovirus	8 (8%)	2 (3%)	0.052	2 (8%)	2 (3%)	1.00
Herpes simplex virus	6 (6%)	2 (2%)	0.149	8 (32%)	2 (8%)	0.034*
Porphyromonas gingivalis	88 (88%)	48 (48%)	<0.0001*	16 (64%)	4 (16%)	0.001*
Fusobacterium nucleatum	83 (83%)	45 (45%)	<0.0001*	13 (52%)	10 (40%)	0.395
Prevotella intermedia	68 (68%)	58 (58%)	0.143	16 (64%)	12 (48%)	0.255
Tannerella forsythia	91 (91%)	79 (79%)	0.0175*	14 (56%)	5 (20%)	0.009*
Treponema denticola	56 (56%)	25 (25%)	<0.0001*	12 (48%)	10 (40%)	0.569

*Significant

In our approach, each microbial species was targeted by individual qPCR reactions containing specific primer pairs. The Bio-Rad CFX96 software computed the *Ct* values that were compared with the *Ct* inferred from the amplification curve of a standard sample containing genomic DNA equivalent to 6×10^6 CFUs of each microorganism. The target amplicon corresponding to *P. gingivalis* has 404 basepair lengths, *F. nucleatum* has 817 basepair length, *P. intermedia* has 259 basepair length, *T. forsythia* has 641 basepair length, and *T. denticola* has 314 basepair lengths. This feature could impact the amplification efficiency due to their increased size that enhances the assay sensitivity. However *Ct* values estimated in the early cycles were adequately comparable for the study's purpose.

Table 1 shows that Group 1 subjects have more bacterial and viral counts compared to Group 2 subjects.

Discussion

Periodontal diseases are initiated with microflora including bacteria and viruses. It is believed that host tissue damage is mediated by inflammatory response of the host.[1] This inflammatory response may not be limited to the periodontal tissues but may induce systemic response by either microbes or their endotoxins in the systemic circulation, which may induce pro-inflammatory cytokine production. These cytokines then further activate the inflammatory response, which results in a chronic low-grade systemic upregulation of the inflammatory cytokines (such as interleukin [IL]-6, tumor necrosis factor (TNF)-α, and C-reactive protein) that further induce systemic activation of the inflammatory response and upregulate chronic low-grade systemic inflammation.

Cota LO *et al.*[8] have postulated that the host response to a long-term exposure of periodontal pathogens may provoke systemic maternal and placental pro-inflammatory endothelial activation and dysfunction, representing a significant risk for vascular diseases (such as preeclampsia) and premature rupture of membranes, posing a threat to fetal–placental unit. Many studies evidenced fetal antibody seropositivity to oral organisms and exposure of fetus to these organisms or their end products. And it has been identified that fetal seropositivity to oral organisms is more frequently associated with preterm babies. These findings point to a blood-borne infectious pathway leading to direct fetal exposure as a major pathogenic mechanism of periodontitis associated with prematurity.[3,9,10]

Bacteria associated with periodontal disease are not dissimilar to those known to be associated with genitourinary bacterial infections and adverse pregnancy outcomes. Many studies have shown the translocation of *F. nucleatum*, *P. nigrescens*, *P. intermedia*, *P. gingivalis*, and *T. denticola* to the feto-placental unit, whereby a maternal or fetal response has been detected, resulted in premature birth or low birth weight.[9]

The primary outcome of this study was more number of both bacteria (*T. forsythia*, *T. denticola*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *F. nucleatum*) and viruses (EBV, CMV, and HSV) in both subgingival plaque and placental samples in preeclamptic chronic periodontitis (Group 1) compared to women with preeclampsia without periodontal disease (Group 2).

The role of subgingival microbiota in the development of periodontal diseases has been extensively documented. More frequently higher levels of *Actinobacillus actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, and *T. denticola* are observed in periodontitis sites.[1] A periodontal microbe, *F. nucleatum*, has been linked with adverse pregnancy outcomes.[2] Many inflammatory markers such as C-reactive protein, IL-1, 6, and TNF-α markers of systemic inflammation are associated with periodontal disease. Therefore, these chemical mediators might be responsible intermediaries in developing adverse pregnancy outcomes.[10] In addition, increased gingival bleeding due to hormonal influence will aid in the entry of bacteria into the blood stream, which may enter uterus.[10,11]

A 5-year prospective study showed that periodontal disease is significantly associated with a higher prevalence rate of preterm births. Authors found 2.9-fold higher rate of immunoglobulin M seropositivity for one or more organisms of the Orange or Red complex in preterm babies compared to term babies.[12]

The secondary outcome of this research was that chronic periodontitis acts as a risk factor in patients with preeclampsia during pregnancy. This was well discussed in a recent review that bacterial pathogens, antigens, endotoxins, and pro-inflammatory cytokines produced during periodontal disease can cross the placental barrier, resulting in maternal-fetal unit disturbance, which could contribute to immune and inflammatory changes during pregnancy and might contribute to adverse pregnancy outcomes such as preterm low-birth weight.[13]

The strength of this study was that it was designed in a large group of subjects. In scientific studies, sample size is a crucial consideration for quality research. In terms of statistics, larger sample size provides accurate mean

values and allows the researcher to obtain better data determination and avoid errors. The limitation of the study would be that the samples were recruited from single center (Narayana Medical College and Hospital). From the current research, future research directions to be taken into consideration would be as follows: (1) studies in this relevance were suggested to be performed in multiple centers and (2) studies to be performed with large sample size, to reduce the center-based bias and errors.

Conclusion

Evidence of periodontopathic microflora in placental tissue proves that there is definite dissemination of periodontal microflora (bacteria and virus) through blood. This research strengthens the analysis of previous studies, mentioning chronic periodontitis as an individual risk factor during pregnancy and an additional risk factor in preeclampsia. This is thought to be due to the systemic dissemination of periodontal infection, and the chemical mediators thus released would play a major role in the pathogenesis of adverse pregnancy outcomes.

Acknowledgements

I would like to acknowledge Dr Vijay Chava for his valuable suggestions during the research work.

Financial support and sponsorship

The study is self funded.

Conflicts of interest

The authors have no conflicts of interest to declare.

Author contributions

Dr Swetha is principal investigator who collected subject's data, samples and analysed.

Dr Jaideep Mahendra is Guide and contributed in design of the tudy and manuscript writing. Dr Md Vali Basha is co-guide for the research work and helped in microbiological as well as statistical part of the study. Finally, all the authors approved the final version of the manuscript for publication.

Ethical policy and institutional review board statement

"Statement that all the procedures have been performed as per the ethical guidelines laid down by The Institutional Ethical Board, Narayana Dental College and hospital, Nellore, Andhra Pradesh, India, approved the study with RC.no.NDC/staff/2015-16/EC/2015/01.

Patient declaration of consent

All the participants were well informed and consent letters were obtained from participants for the research work as well as publishing the same.

Data availability statement

Dataset can be made available after embargo period due to commercial restrictions.

References

1. Michael G. Newman DDS, Henry Takei DDS, MS, Perry R. Klokkevold DDS, MS, Fermin A. Carranza Dr. ODONT. *Carranza Clinical Periodontology*, 10th edn. Published by Elsevier Health Sciences, Clinical diagnosis 543-48.
2. Offenbacher S, Jared Hl, O ' Reilly Pg, Wells Sr, Salvi Ge, Lawrence Hp *et al*. Potential pathogenic mechanisms of periodontitis associated pregnancy complications. Annals of Periodontology 1998;3:233-50.
3. Shlomi B, Orit Oettinger-B, Eli EM, Hannah S, Gonen O. Evidence of periopathogenic microorganisms in placentas of women with preeclampsia. Periodontol. J Periodontol 2007;78:670-76.
4. Girija J, Prathibha AN, Sanu S, Kiran N. Maternal periodontal disease and preeclampsia in Jaipur population. J Indian Soc Periodontol. 2018;22:50-4.
5. Canakci V, Canakci CF, Canakci H, Canakci E, Cicek Y, Ingec M, *et al*. Periodontal disease as a risk factor for pre-eclampsia: a case control study. Aust NZJ Obstet Gynaecol. 2004;44:568-73.
6. Boggess KA, Lieff S, Murtha AP, Moss K, Beck J, Offenbacher S. Maternal periodontal disease is associated with an increased risk for preeclampsia. Obstet Gynecol. 2003;101:227-31.
7. Yaghini J, Mostajeran F, Afshari E, Naghsh N. Is periodontal disease related to preeclampsia?. Dent Res J (Isfahan). 2012;9:770-73.
8. Cota LO, Guimaraes AN, Costa JE, Lorentz TC, Costa FO. Association between maternal periodontitis and an increased risk of preeclampsia. J Periodontol. 2006;77:2063-69.
9. Han YW, Wang X. Mobile microbiome: oral bacteria in extra-oral infections and inflammation. J Dent Res 2013;92:485-91.
10. Bhumanapalli Venkata Ramesh Reddy, Tanneeru S, Chava VK. The effect of phase-I periodontal therapy on pregnancy outcome in chronic periodontitis patients. Journal of Obstetrics and Gynaecology 2014;34:29-32.
11. Ruma M, Boggess K, Moss K, Jared H, Murtha A, Beck J, *et al*. Maternal periodontal disease, systemic inflammation, and risk for preeclampsia. Am J Obstet Gynecol. 2008;198:389 e381-85.
12. Offenbacher S, Lieff S, Boggess KA, Murtha AP, Madianos PN, Champagne CM, *et al*. Maternal periodontitis and prematurity. Part I: Obstetric outcome of prematurity and growth restriction. Ann Periodontol. 2001;6:164-74.
13. Bui FQ, Almeida-da-Silva CLC, Huynh B, Trinh A, Liu J, Woodward J, *et al*. Association between periodontal pathogens and systemic disease. Biomed J. 2019;42:27-35.