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Viral load on Preeclamptic pregnant women with and without periodontal disease and its effect on pregnancy outcomes- A Cross-sectional study.

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Abstract

Background: Viral load has its own significance in the pathophysiology of various systemic ailments. Aim of the present study was to quantify the viral load in normal pregnant women and preeclamptic pregnant women with and without periodontal disease and relating the same with pregnancy outcomes.

Materials and methods: A Cross-Sectional study is designed on pregnant women with and without periodontal disease and preeclampsia, who are attending Narayana hospital, Nellore for prenatal checkups. 445 women were recruited in the study after obtaining the consents. Based on systemic and periodontal health, subjects were grouped into Group-1 (systemically healthy and periodontally healthy), Group-2 (systemically healthy with periodontal disease), Group-3 (preeclamptic women with periodontal disease) and Group-4 (preeclamptic women without periodontal disease). Clinical parameters like plaque index, bleeding on probing, Probing depth and clinical attachment level were recorded. Quantification of viruses like Ebstein Barr Virus, Herpes Simplex Virus Cytomegalovirus was done in subgingival plaque and placental tissue. Information related to type of newborn delivered (preterm lowbirth weight, normal birth) was obtained and recorded for further comparision.

Results: Women with preeclampsia and periodontal disease had more adverse outcomes and expressed more viral load compared to other groups, that is group 4. 2 and 1 respectively in both subgingival plaque and placental tissue samples.

Conclusions: There was a significant association between preeclampsia and periodontal diseases with pregnancy outcomes. Viral load has a definite role in the development of preeclampsia, periodontal disease and pregnancy outcomes as well.

Keywords: adverse pregnancy outcomes, Ebstein-Barr virus, herpes infections, lowbirth weight infants, maternal infections, periodontal disease, preeclampsia, preterm low-birth weight.

Introduction

Adverse pregnancy outcomes like preterm low-birth weights (PTLBW), still births (SB) Preeclampsia (PE) has gained importance globally. Preterm (PT) delivery is delivery before the end of 37 weeks of gestation, low birth weight (LBW) birth weight of new born being less than 2500 grams.1 PE is a serious disorder characterized by hypertension and proteinuria with ≥300 mg/day which may lead to maternal and fetal morbidity and mortality.2 Preterm low birth weight (PLBW) are PT infants who were born with LBW, these adverse pregnancy outcomes pose increased risk of mortality among infants born with these conditions1, PE being common risk factor for infant and mother morbidity and mortality.1Despite World Health Organization (WHO) aiming at reduction of the number of PT LBW deliveries a known predictor of childhood morbidity and mortality, adverse pregnancy outcomes have become growing risks of pandemics throughout the globe with more incidence facing developing countries.2 While this complication of pregnancy has major medical, social, and economic consequences, the cause of spontaneous preterm birth is unknown. It is said that maternal infections play a major role in the development of these adverse pregnancy outcomes.2 Concept of oral infections has gained acceptance in the development of these adverse pregnancy outcomes. Many studies have shown association periodontal infection with PTLBW3,4 and PE5.Periodontitis is a microbial inflammatory disease affecting the tooth supporting tissues. Microbes like bacteria, virus and fungi play a major role in the pathophysiology of periodontal diseases, in which periodontal tissues serves as a chronic reservoir of endotoxins and inflammatory cytokines6,7 that initiate and exaggerate atherogenesis and thrombogenesis.8 Periodontal disease has been considered recently to trigger unwanted effects during pregnancy and may induce inflammatory changes locally and systemically and alter host immune response.9Evidence suggests that these immunological alterations may contribute to PE development. Though etiology of PE is not well known, but is believed that it involves poor placental invasion, inappropriate development of placenta and elevated oxidative stress resulting in abnormal trophoblastic invasion.8 Evidence suggests that periodontal diseases and PE were associated with elevated levels of inflammatory cytokines like TNF-α, interleukin-1, 6 which resulted in vascular damage in turn leading to endothelial alterations8.Viruses like Herpes, Ebstein-Bar, Human cytomegalo viruses can cause severe acute oral, orofacial disease and systemic infections and have a definitive role on pregnant women and developing fetus10,11. Therefore, it is necessary to understand the role of viral infection and its relevance to preeclampsia and periodontal infections on developing fetus. The present cross-sectional study was aimed to quantify the viral load in normal pregnant women and preeclamptic pregnant women with and without periodontal disease and relating the same with pregnancy outcomes.

Materials and methods

After ethical approval from Narayana Dental College & Hospital, a cross-sectional study was performed between June 2015 to July 2018 in the department of Periodontology, Narayana Dental College & Hospital on pregnant women attending Department Of Obstetrics and Gynaecology, Narayana Medical College & Hospital, Nellore, Andhra Pradesh for prenatal checkups.

Inclusion and Exclusion criteria: Pregnant women above 18 and below 30 yrs without systemic diseases or

syndromes other than periodontal disease and who were not using systemic antibiotics were included in the study.

Periodontal screening: Intraoral examination was done with the help of artificial light source, mouth mirror, William's periodontal probe, and cotton pliers. Clinical parameters like Plaque Index, Papillary Bleeding Index and Probing Pocket Depth have been examined to evaluate the clinical signs of periodontal disease. Subgingival plaque was collected with sterile Gracey curette. Samples were screened for the identification of Ebstein Barr Virus, Herpes Simplex Virus and Cytomegalovirus in subgingival plaque and placental tissue (after parturition).

Plaque Index: 16, 12, 24, 36, 32, 4 disto-facial, mesio facial, facial surfaces were examined for plaque. Papillary Bleeding Index*:* The gingival bleeding was determined dichotomously by gentle probing of the gingival crevice with the William's periodontal probe. Papillary bleeding index by Saxer and Muehleman .

Probing Pocket Depth: The distance between the base of the pocket and the gingival margin was measured at six deepest sites in a subject.

Medical data: Demographic data, medical history, and detailed information on events during pregnancy and delivery were obtained from patients' medical records.

Women were diagnosed with preeclampsia if they had the following:

Blood pressure ≥140/90 mm Hg and urine protein concentration ≥0.30g/dl. (obtained from medical records)

Methodology: 900 pregnant women were screened for the study 210 subjects were excluded due to factors like age and systemic conditions, 245 women had refused to participate in the research study. After obtaining consents remaining 445 subjects who met the eligibility criteria were recruited for the study and were categorized into 4 groups based on the presence and absence of preeclampsia and periodontal disease. Based on systemic and periodontal health, the subjects were grouped in to 4 groups after obtaining informed consents.

Group 1: (Controls) systemically and periodontally healthy women. N=145

Group 2: Systemically healthy women with periodontal disease. N=100

Group 3: Preeclamptic women with periodontal disease. N=100

Group-4: Preeclamptic women without periodontal disease. N=100

Virus Detection: The EBV, HSV, CMV viruses DNA have been isolated from both subgingival plaque samples and placental tissue and its concentration and purity of the DNA sample is determined using a NANODROP (Thermo Scientifics, US) spectrophotometer at 260/280 nm. The remaining samples are stored for PCR experiment.

Turn on the Nanodrop, click on UV measure option in Nanodrop software. Take 1 ul TE buffer to measure blank. Measure all the DNA samples (1 ul) separately. The quality and yield obtained was measured by using a NanoDrop Spectrophotometer. Quantitation of DNA carried out 1µl of all plaque DNA samples using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific, US) at absorbance at 260 nm and 280 nm. The quality and yield obtained was measured by ratio of 260 nm and 280 nm using a NanoDrop Spectrophotometer.

PCR Procedure: All mix was prepared in hard-shell PCR plate-96 well WHT-CLR(cat.no. HSP 9601)(Bio-Rad Laboratories Inc, US) with seal plates of optically transparent film(Bio-Rad Laboratories Inc, US). 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 (BIORAD-CFX100 (BIORAD, USA)). EBV, HCMV, and HSV in the plaque and placental samples were detected using nested PCR. The primers and BLAST search and then synthesized by IRA biotechnology, Hyderabad, India. Primer sequences were shown in Table 1. Luna Universal qPCR Master Mix and other reaction components were kept at room temperature to set room temperature, and then placed on ice. After thawing completely, all reagents were mixed by inversion, pipetting or gentle vortexing. For each batch of samples, negative control was set for PCR amplification using sterile deionized water. Duplicates were was performed for all samples.

The reaction conditions of the HCMV were as follows: initial denaturation at 94^0 C for 5 min, followed by 25 cycles of denaturation at 94^0 C for 30 sec, annealing at 59^0 C for 30 sec, and extension at 72^0 C for 30 sec, with a last extension at 72^0 C for 3 min. The reaction conditions of the EBV were as follows: initial denaturation at 94^0 C for 3 min, followed by 35 cycles of denaturation at 94^0 C for 30 sec, annealing at 63^0 C for 15 sec, and extension at 72^0 C for 30 sec, with a last extension at 72^0 C for 1 min.

The reaction conditions of the HSV were as follows: initial denaturation at 94^0 C for 1 min, followed by 30 cycles of denaturation at 94^0 C for 1 min, annealing at 55^0 C for 1 min, and extension at 72^0 C for 30 sec, with a last extension at 72^0 C for 1 min. The relative quantification of viral load was achieved by comparison with a standard amplification curve obtained from the (standard) genomic DNA. The viral DNA concentrations used: 1 x 10^9, 1 x 10^7, 1 x 10^5, 1x 10^3, and 1 x 10^1 respectively. Finally, the expected amplicon was analyzed along with standard 1 kb DNA ladder on 1.5% agarose gel under ultraviolet (UV) transillumination. The quality and yield obtained was measured by using a NanoDrop Spectrophotometer.The NonoDrop data given mean DNA yield obtained was 120 ng/µl (range 51-225 ng/µl) and purity (A260/A280 ratios) ranged between 1.55 to 1.90.

Results

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 prevalence of periodontopathic microorganisms between the case group and the control group. The difference of the average viral counts among the study groups analysed by ANOVA test. $P < 0.05$ was considered statistically significant and all P-values were two-sided. Statistical analysis was performed using SPSS version 23.0 (IBM, Armonk, NY, USA).

Comparison of means of maternal age, gestational age was done between the groups using one-way ANOVA and no significance was found.

Table 1 shows comparison of viral load in subgingival plaque samples among four groups. In the study elevated levels of virus are detected n group3 compared to group 4, 2 and 1 respectively. Which implies virus is detected more in women with preeclamptic women with periodontal disease and preeclampsia. (with more relevance to EBV and then HSV)

Table 2 shows comparison of viral load in placental samples of women among four groups. In the study, group3 women had more viral levels compared to group 4, 2 and 1 respectively (with more relevance to EBV being 40% and HSV 32%,)

Table 3 shows comparison of pregnancy outcomes between the groups. High levels of adverse pregnancy outcomes were seen in group3 and 4 compared 2 and 1 respectively.

Discussion

The possible relationship between periodontal infection and preterm birth was first reported in 1996, [3] since then, the idea that periodontal disease may have adverse effects on pregnancy induced several researchers to investigate the association between periodontal diseases and preterm birth, low birth weight, and preeclampsia. And strong epidemiologic evidence have been shown that pregnant women are at higher risk of severe illness and mortality from viral infections.[11,12, 13] Furthermore, viral infection may predispose the pregnancy to preterm labor and preterm delivery by infection with other superimposed microorganisms[14,15,16]. Infact viral infection contributes in the development of many periodontal diseases like chronic periodontitis, localized and generalized aggressive periodontitis, HIV-associated periodontitis and acute NUG.[17]

Viruses play a key role in periodontal pathogenesis by various mechanisms.

They trigger inflammatory cells such as polymorphonuclear, leukocytes, lymphocytes, macrophages, and other cells such as fibroblasts, endothelial cells, even bone cells and results in the release of inflammatory cytokines. Which in turn stimulates cells to produce chemicals like MMP's MMP's are involved in cellular components degradation (collagen, elastin, proteoglycans, and laminins) and are considered to be of great clinical importance in periodontal pathogenesis. Viruses are able to activate latent forms of effector

proteins such as antimicrobial peptides, chemokines and cytokines.[17, 18, 19]

Virus also interferes with the immune system of the host. Studies have shown EBV infecting periodontal B-lymphocytes, and CMV infecting periodontal monocytes / macrophages and T-lymphocytes in periodontitis lesions[19]. HCMV can interfering with cytotoxic T-lymphocyte recognition through the down-regulating cell surface expression of major histocompatibility complex class I and class I molecules and inhibits immunity by suppressing antigen-specific cytotoxic responses. Thus, may lead to global impairment of cell-mediated responses, inturn inducing more tissue breakdown and impaired healing. [20]

Evidence suggest that virus enhance the bacterial adhesion to host as seen in herpes infection, virus infected cells act as nidus for bacterial adherence, which results in aggravated periodontal infection and might lead to systemic dissemination of inflammatory products.[20]

The present cross-sectional study focused on

1: The associating periodontal diseases, preeclampsia and viral load in preeclamptic pregnant women with and without periodontal disease.

2: Relation of PE, PD and viral load in sub gingival plaque and placental samples with pregnancy outcomes.

Women aged 18 – 35 years were recruited as maternal ages of ≤18 and ≥35 years are at more risk for PTLBW[3,4, 6] and PE.[5]

PTLBW were seen more in group 3 than 4, 2 and 1 respectively implying that the both PE & PD are individual risk factors for adverse pregnancy. Our results were consistent with other studies who have positively related PD and PTLBW[3,4,6] and PD with PE[5]. Pitiphat et al. [23] concluded that maternal Periodontal Status is associated with adverse pregnancy outcomes. Whereas, other study showed that PTB was 4.2 times higher for women with periodontitis as compared to those without periodontitis.[24] Though many studies have evidenced the role of PD in adverse pregnancy outcomes[25, 26, 27, 28] there are studies which contradict this statement. [29, 30]

In the present study subjects subgingival plaque and placental samples were examined for identification of virus like EBV, HCMV & HSV through PCR & Nanodrop techniques. Viruses are one of the smallest forms of microorganism consisting of the "naked," or "enveloped" nucleocapsid, within a lipoprotein sheath derived from the host cell membrane measuring 10–100 nm, which gets multiplied only inside living cells. In Our study viruses were significantly identified more in number in subgingival and placental samples of women with both PE and periodontal disease (PD) than women with PE alone and PD alone. These results showed that virus have a definitive role either in the development of PD or PE, or may aggravate the present systemic condition and lead to adverse pregnancy outcomes. Many studies have speculated virus have positive role in the development of PD.[11, 15, 19] Chronic periodontal infections can produce local and systemic host responses leading to transient bacteremia or viremia. Lipopolysaccharide (LPS) endotoxins and other bacterial substances can gain access to gingival tissue, initiate and perpetuate local inflammatory reactions, and consequently produce high levels of proinflammatory cytokines. Such activations of maternal inflammatory cell responses and cytokine cascades play important roles in the pathophysiological processes of preterm labour, low birthweight, and preeclampsia.[3,6, 27, 28]

In addition, LPS, bacteria from subgingival plaque, and proinflammatory cytokines from inflamed periodontal tissue can enter the bloodstream, reach the maternal– fetal interface, trigger or worsen maternal inflammatory response, and increase plasma levels of prostaglandin and cytokines (e.g. tumour necrosis factor).[8–10, 32] Thus, it appears that periodontal disease may play a nonspecific role in various adverse pregnancy outcomes.

Studies suggested an association between periodontal disease and pre-eclampsia.[5] However, no such association was identified when periodontal disease was determined prior to the 26 weeks of gestation.[35]

Conclusion

The results of this case–control study provided convincing evidences that virus have a definite role in the outcomes of pregnancies. Subgingival plaque as well as placental samples have evidenced increased viral load in subjects with preeclampsia and with periodontal diseases. This clearly states that EBV, HCMV and HSV which are associated with periodontal disease development might have a definite role in the development of preeclampsia and adverse pregnancy outcomes (PTB and LBW). Future studies that investigate specific characteristics of Virus as periodontal pathogens and host's immune and inflammatory response, and their relation in the development of preeclampsia with large sample size may help to move this literature forward.While the exact mechanisms by which PE occurs still remain unknown, knowing how different components of the immune system individually affect the progression and development of PE will be useful to improve the outcomes of mothers and babies affected by the disease. Further, prevention and management of periodontal diseases should be among the priorities in public health planning and programs. We believe that for the pregnant women in particular, educational and screening programs and awareness of PE should be implemented by health care providers for a better knowledge about periodontal health maintenance during pregnancies.

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Table I: Comparison of virus load in subgingival plaque between groups

Virus	Group 1	Group 2	Group 3	Group 4
Epstein-Barr Virus	10(6.9%)	32(32%)	79(79%)	20(20%)
Human Cytomegalovirus	4(2.75%)	6(6%)	8(8%)	2(3%)
Herpes Simplex Virus	2(1.38%)	4(4%)	6(6%)	2(2%)

Table II: Comparison of virus load in placental tissue samples in between groups.

Virus	Group 1	Group 2	Group 3	Group 4
Epstein-Barr Virus	2(5%)	10(40%)	10(40%)	5(20%)
Human Cytomegalovirus	1(2.5%)	3(12%)	2(8%)	2(3%)
Herpes Simplex Virus	2(5%)	4(16%)	8(32%)	2(8%)

Table III: Comparison of pregnancy outcomes in between groups

Pregnancy outcomes	Group 1	Group 2	Group 3	Group 4
NB	87%	76%	40%	48%
PTB	7%	14%	32%	29%
LBW	6%	9%	20%	21%
IUGR	0%	1%	8%	2%