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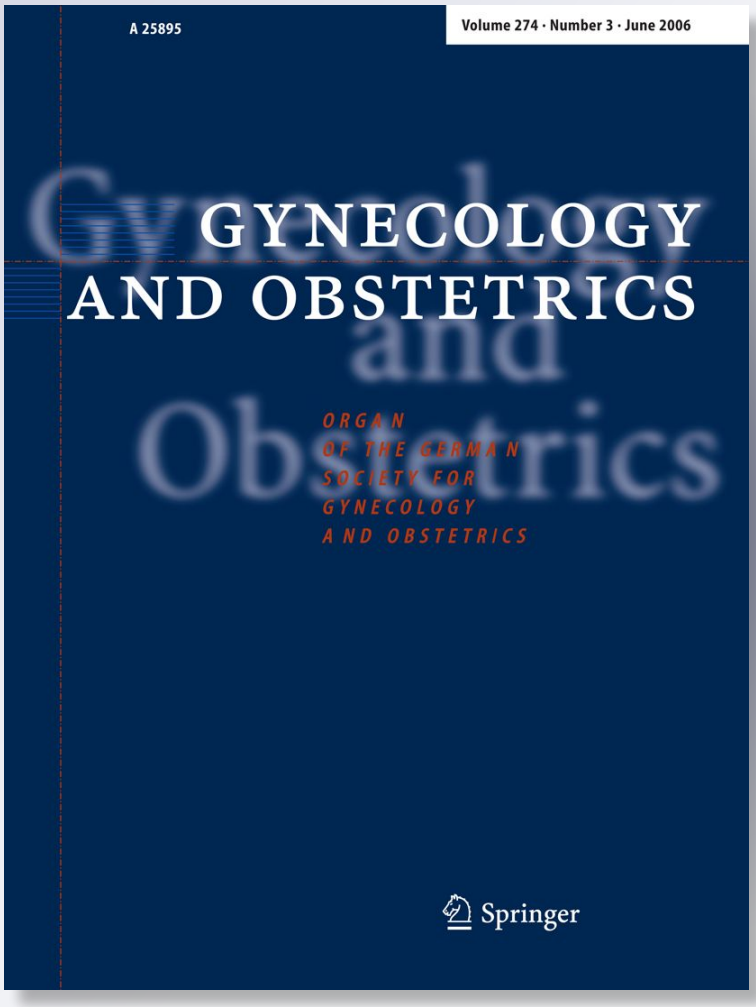

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MATERNAL-FETAL MEDICINE

Simultaneous detection of periodontal pathogens in subgingival plaque and placenta of women with hypertension in pregnancy

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Abstract
Background There are many studies documenting increased prevalence of periodontal infection in women with preeclampsia. But, very few studies have attempted to establish causal relationship between the two.
Objective To find out causal circumstantial evidence by isolating specific periodontal pathogens in oral and placental samples.
Materials and methods Antenatal periodontal screening and subgingival plaque collection was carried out in ten women with hypertension in pregnancy and ten normotensive controls on their hospital admission at term for cesarean delivery. Placental biopsy was obtained after aseptic placental collection at the time of elective cesarean delivery. Subgingival plaque and placental biopsy were studied for *Porphyromonas gingivalis, Fusobacterium nucleatum, Treponema denticola, Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* using quantitative polymerase chain reaction technique.
Periodontist and laboratory personnel were unaware of case or control status. Periodontal status was not informed to the obstetrician recruiting the cases and laboratory. Microbiology report was not revealed till end of the study.
Results Periodontal pathogens were found to be high in the group with hypertension than the controls. *P gingivalis* was found in all the samples from subgingival plaque and placenta, irrespective of the periodontal disease status.
Conclusion In cases with hypertension, periodontal pathogens are present in higher proportion in subgingival plaque and placenta.

Keywords Periodontal disease · Preeclampsia · Subgingival plaque · Placenta · Periodontal pathogens

Introduction

Periodontal disease is a common chronic disorder of infectious origin leading to progressive destruction of supportive tissues of the teeth. It is initiated by infection predominantly with gram negative, anaerobic organisms [1].

It is proposed that the bacterial pathogens, antigens, endotoxins, and inflammatory cytokines of periodontal disease may induce systemic responses that lead to clinical manifestation of variety of diseases in susceptible individuals. In recent years there is an increase in research evidence suggesting associations between periodontal disease and increased risk of systemic diseases such as atherosclerosis, myocardial infarction, stroke, diabetes mellitus, and adverse pregnancy outcomes. Adverse pregnancy outcomes that have been linked to periodontal diseases include preterm birth, low birth weight, miscarriage or early pregnancy loss, and preeclampsia [2–7].

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It is reported that there is an increased prevalence of periodontitis in patients with preeclampsia, a multisystem disorder of hypertension specific to pregnancy. Preeclampsia is variously named as pregnancy induced hypertension, gestational hypertension, or hypertensive disorder of pregnancy. Acute atherosis, the pathognomonic finding seen in placenta and other vital structures of these patients is similar to the clinical and pathological alterations of atherosclerotic vascular changes. There are evidences to correlate these conditions with periodontal disease, since periodontal pathogens have been detected in atheromas of patients with atherosclerotic vascular disease and placenta of patients with preeclampsia [8].

There are very few studies establishing causal relationship between periodontal disease and preeclampsia. And the association between the two conditions is not universal with some studies failing to find any such association [9].

Present case control study is an effort to find out causal circumstantial evidence for periodontal disease as one of the etiologies for pregnancy hypertension by simultaneously isolating DNA of specific periodontal pathogens in subgingival plaque and placental samples.

Materials and methods

For the purpose of the study ten women with hypertension in pregnancy and ten women with no hypertension, scheduled for elective cesarean delivery at term were recruited. Recruitment of the cases and their age, socioeconomic status and pregnancy duration matched controls was made by a single obstetrician.

Hypertension in pregnancy was defined as onset of sustained hypertension in pregnancy after 20 weeks of gestation with or without associated proteinuria. Woman was considered to be hypertensive if the resting blood pressure was 140/90 mm Hg or greater [10]. It was considered sustained if the hypertension is recorded at least on two occasions, 6 h apart without any treatment.

Women with any associated medical disorders (like preexisting hypertension, renal disease, anemia, diabetes mellitus including gestational diabetes), history of intake of systemic antimicrobial therapy in last 3 months, and receipt of periodontal treatment in last 6 months were not considered for recruitment to study.

Periodontal examination was carried out at the bedside in ante-partum ward by a single periodontist (PS), who was not aware of the case–control status of the recruited. Periodontal infection was considered to be present if any of the following were present: Oral hygiene index-simplified >3 (poor)/Gingival index >1 (moderate to severe gingivitis)/Periodontal pocket depth >4 mm/Loss of attachment >3 mm [11]. Severity of periodontal disease was classified based on the AAP classification system proposed by Armitage et al. 1999 [12].

In all of them, sub-gingival plaque was collected from the site with deepest probing depth on standardized perio-paper strips at the time of periodontal examination. The paper used for collection was inserted to the base of the pocket and kept in place for 30 s before sealing in eppendorf transportation tube.

Placental biopsy specimens were collected in the operation room at the time of cesarean delivery, under aseptic conditions. Placental samples (1 cm^3 each) were taken from four sites—one near center and the other near periphery on the maternal (two samples) and fetal (two samples) sides, collected in sterile glass tubes and capped.

Placental biopsy and sub-gingival plaque samples were transported to polymerase chain reaction (PCR) section within 4 h of collection. The samples were stored at −20°C until PCR analysis.

For processing of the samples from subgingival plaque and placenta, 50 µl of milliQ water was transferred to the eppendorf tube containing the paper strip. It was then placed in boiling water for 10 min and 10 µl of supernatant used for PCR.

PCR analysis included initial denaturation step at 95°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 67°C for 50 s, an extension at 72°C for 50 s, and a final extension at 72°C for 10 min. The amplified PCR product (200 bp) was then run on 0.8% agarose gel and analyzed. The PCR product was then cloned in order to obtain isolated clones and to validate the presence of *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Treponema denticola*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*.

The ligated product was transformed into prepared competent cells (DH10B) and plated on to agar plates impregnated with ampicillin. These plates were incubated overnight at 37°C for growth of recombinant clones. These clones were picked randomly from each of the plates and cultured overnight in 1.5 ml of 2XYT media. Plasmid was then isolated using miniprep and was checked on 0.8% gel for purity. 2 µl of plasmid was used for PCR using same condition and the amplicon was run on 2% gel along with appropriate marker. Subsequent restriction digestion analysis was carried out.

The clones were sequenced to confirm the presence of pathogen specific DNA using ABI genetic analyzer 3130 automated DNA sequence.

The primers evaluated for the organisms in the study were done by enzymes selected using the web based tool NEB cutter 2.0 after providing the PCR product sequences of the five pathogens in question.

All products were subjected to digestion with selected restriction enzymes. The PCR product sequence of pathogenic

- **PCR Products:**
- > Fusobacterium nucleatum subsp. nucleatum ATCC 25586 - nucleotides 452484-452658 (175 bp)

TTACCGCGGCTGCTGGCACGTATTTAGCCGTCACTTCTTCTGTTGGTACCGTCATTTTTTTCTTCCCAAC
TGAAAGCACTTTACATTCCGAAAAACGTCATCGTGCACACAGAATTGCTGGATCAGACTCTCGGTCCATT
GTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGT

- > Porphyromonas gingivalis W83 - nucleotides 119897-120087 (191)

ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGGCGAGAGCCTGAACCAGCCAAGTCGCGTGA
AGGAAGACAGTCCTAAGGATTGTAAACTTCTTTTATACGGGAATAACGGGCGATACGAGTATTGCATTGA
ATGTACCGTAAGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAA

- >Treponema denticola ATCC 35405 - nucleotides 610521-610717 (197)

ACTCCTACGGGAGGCAGCAGCTAAGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCGACGCCGTGTGA
ATGAAGAAGGCCGAAAGGTTGTAAAATTCTTTTGCAGATGAAGAATAAGAAGAAGAGGGAATGCTTCTTT
GATGACGGTAGTCATGCGAATAAGCCCCGGCTAATTACGTGCCAGCAGCCGCGGTAA

- >Aggregatibacter actinomycetemcomitans pcr product size 196 bp

ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGCAACCCTGACGCAGCCATGCCGCGTGAATGAAGAAGGCCTTCGGGTTGT
AAAGTTCTTTCGGTATTGAGGAAGGTTGGTGTGTTAATAGCATGCCAAATGACGTTAAATACAGAAGAAGCACCGGCTAACTCCGTGCCA
GCAGCCGCGGTAA

- >Prevotella intermedia (T); ATCC 25611; X73965 pcr product 192 bp

ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGCAACCCTGACGCAGCCATGCCGCGTGAATGAAGAAGGCCTTCGGGTTGT
AAAGTTCTTTCGGTATTGAGGAAGGTTGGTGTGTTAATAGCATGCCAAATTGACGTTAAATACAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAA

Fig. 1 List of the primers and PCR product sequence of pathogenic specific DNA clones

specific DNA clones evaluated in the study are given in the figure (Fig. 1).

To minimize the bias, all the participants recruited were coded. Recruitment was made by the resident obstetrician. Until the end of the study—'case' or 'control' status of the participants was not revealed to the periodontist and laboratory personnel conducting microbiological evaluation; the obstetrician was not informed of the periodontal status; and microbiological report (PCR status) was not available to periodontist or obstetrician till the end of study.

The study had the approval by the Institution Ethics Committee.

Fisher exact test was used for determining the probability and significance on VasserStats calculator accessed from http://faculty.vassar.edu/lowry/VassarStats.html. A probability value of <0.05 was considered as statistically significant.

Results

The participant attributes is shown in Table 1. The PCR analysis of periodontal pathogenic organisms demonstrated that simultaneous presence of all the organisms studied was higher in the group with hypertension than the normotensive controls, irrespective of the periodontal disease status. Samples were positive for *P gingivalis* in all the ten cases, while so in only six of the normotensive controls ($p = 0.17$). *P intermedia* and *A actinomycetemcomitans* were detected in nine of the ten hypertensive cases, while the former was found in five and the latter organism in four specimen from ten controls, respectively. *F nucleatum* was demonstrated in eight of ten hypertensives and five of ten normotensives. Similarly, *T denticola* was also noted in higher number of samples from hypertensive women (7 of 9) than in normotensive controls (4 of 10) (Table 2). When presence of these organisms was looked for only in placental specimens, it was found that in hypertensive women *P gingivalis* of red complex was detected in eight, the orange complex organisms *F nucleatum* and *P intermedia* in seven each, and *A actinomycetemcomitans* of green complex in six specimens. Whereas, in normotensives only three each of placental specimens demonstrated *P gingivalis* and *P intermedia*, and there were two each of the specimens with *F nucleatum* and *A actinomycetemcomitans*.

Table 1 Participant characteristics

Characteristic	Hypertensive cases ($N = 10$)	Normotensive controls ($N = 10$)	Statistical significance
Age (Mean ± SD, years)	27 ± 4.47	27 ± 3.55	$t = 1.0.$; $p = 0.36$
Gestation (Mean ± SD, weeks)	34.1 ± 4.33	38.8 ± 1.13	$t = 0.003$; $p = 0.006$
Primigravida (n, %)	6 (60)	6 (60)	$\chi^2 = 2.46$; $p = 0.11$
≤Middle socio-economic status (n, %)	9 (90)	7 (70)	$\chi^2 = 2.07$; $p = <0.0001$
<Secondary education (n, %)	4 (40)	2 (20)	$\chi^2 = 0.95$; $p = 0.8875$
Housewife (n, %)	7 (70)	7 (70)	$\chi^2 = 0$; $p = 1$
Positive family history (n, %)	6 (60)	2 (20)	$\chi^2 = 4.53$; $p = 0.6$
Body mass index (Mean ± SD)	23.02 ± 3.55	21.53 ± 3.16	$t = 3.17$; $p = 0.001$
Neonatal weight (g, Mean ± SD)	3.05 ± 0.55	2.2 ± 0.51	$t = 19.46$; $p = 0.0$

Table 2 Simultaneous presence of periodontal organisms in SGP and PB

Organism	Case status	Present in SGP and PB		Fisher exact probability test (2-tailed)
		Number	Percent	
Porphyromonas gingivalis	Case	7	70	0.17
	Control	3	30	
Fusobacterium nucleatum	Case	6	60	0.16
	Control	2	20	
Treponema denticola	Case	3	30	1.0
	Control	2	20	
Prevotella intermedia	Case	5	50	0.65
	Control	3	30	
Aggregatibacter actinomycetemcomitans	Case	6	60	1.0
	Control	2	20	

SGP subgingival plaque, *PB* placental biopsy

In one hypertensive and two normotensive women despite periodontal disease being absent, organisms were found in placenta though they were not present in subgingival plaque (Table 3).

Discussion

It has been hypothesized that women with active periodontal disease during pregnancy may have transient translocation of oral bacteria to the maternal blood circulation, inciting placental inflammation or oxidative stress early in pregnancy, ultimately producing placental damage and the clinical manifestations of hypertension in pregnancy [3, 13].

In the present study the presence of periodontal pathogens was significantly high in the group with hypertension than normotensive controls. Similar findings were presented by Contreras et al. [14]. They demonstrated the presence of members of red, orange and green complex of periodontal pathogens in the gingival crevicular fluid in higher proportions in preeclamptic women, suggesting that hematogenous spread of these organisms may be responsible for causation of preeclampsia.

Since periodontal disease appear to be caused by relatively finite group of periodontal pathogens acting alone or in combination out of more than 500 species detected, these bacteria are grouped into microbial complexes and each one is assigned a color designation for convenience of discussion [15]. The 'blue', 'green', 'yellow' and 'purple' clusters include mainly bacteria that colonize the periodontal sulcus in the early stages of dental plaque formation. As the biofilm matures and becomes more pathogenic, organisms of the 'orange' cluster (*Campylobacter rectus, F. nucleatum, Peptostreptococcus micros, P. intermedia and Prevotella. nigrescence*) appear and provide the necessary habitat for the subsequent colonization and establishment of the more aggressive bacteria of the 'red' cluster (*P. gingivalis, Tannerella forsythus and T. denticola*). Because of this reason, the present study focused on these five organisms.

Although the exact role of each of these bacterial species in the progression of periodontal disease is not fully under-

Table 3 Microbiogram according to source and case status

Sample	Case/ Control Status	Periodontal disease	Subgingival plaque	Placenta
1	Case	Present	*Pg; Fn; Td; Pi; Aa*	*Pg; Fn; Td; Pi; Aa*
7	Case	Present	*Pg; Fn; Td; Pi; Aa*	*Pg; Fn; Td; Pi; Aa*
9	Case	Present	*Pg; Fn; Td; Pi; Aa*	*Pg; Fn; Td; Pi; Aa*
12	Case	Present	*Pg; Fn; Td; Aa*	*Pg; Fn; Aa*
13	Case	Present	*Pg; Fn; Td; Aa*	*Fn; Pi; Aa*
14	Case	Present	*Pg; Fn; Pi; Aa*	*Pg; Fn; Td; Pi; Aa*
15	Case	Present	*Pg; Fn; Pi; Aa*	*Pi*
16	Case	Present	*Pg; Pi; Aa*	*Pg; Fn*
17	Case	Absent	*Pg; Fn; Pi*	*Pg*
20	Case	Absent	-	*Pg; Td; Pi*
2	Control	Present	*Pg; Fn; Pi; Aa*	*Pg; Fn; Pi; Aa*
3	Control	Present	*Pg; Fn; Td; Pi; Aa*	-
4	Control	Absent	-	-
5	Control	Present	*Pg; Td; Pi*	*Pg; Td; Pi*
6	Control	Present	*Pg; Pi*	-
8	Control	Absent	*Fn; Td*	-
10	Control	Absent	*Pg*	*Aa*
11	Control	Absent	-	*Fn; Td*
18	Control	Absent	-	-
19	Control	Absent	-	*Pg; Fn; Pi*

Pg Porphyromonas gingivalis, *Fn Fusobacterium nucleatum*, *Td Treponema denticola*,*Pi Prevotella intermedia*, *Aa Aggregatibacter actinomycetmcomitans*

stood, it is clear that the presence of a large group of bacteria somehow is necessary for the overall pathogenic effect. As periodontal disease progresses, the host's immune system responds by producing antibodies against the various bacterial species.

The cysteine proteinases produced by *P gingivalis* are incriminated in activating coagulation factors and platelet aggregation [16, 17]. Other virulence factor of *P gingivalis*, lipopolysaccharide can activate spleen cells and peripheral blood monocytes, resulting in the release of proinflammatory cytokines [18, 19]. Other microorganisms also possess similar pathogenic property albeit in different virulence. Contreras et al. [14] hypothesized that the virulence factors of various periodontal pathogens may in part explain the possible mechanisms involved in preeclampsia.

Barak et al. [20] have provided evidence that periodontal pathogens are present in placenta and have reported significantly increased cell counts in women with preeclampsia. Having found *T forsythensis*, *P gingivalis*, *A actinomycetemcomitans*, *P intermedia* and *F nucleatum* in placenta they suggested that these organisms may have transmitted hematogenously and took part in the formation of atherosis of preeclampsia. Since acute atherosis of preeclampsia shares similar pathology as atherosclerosis and the very same organisms were also demonstrated in atheromatous plaques [21–23], it is reasonable to correlate the role of periodontal disease in pregnancy hypertension.

Contreras et al. [14] provided the microbiological evidence in subgingival tissue in patients with pregnancy hypertension, and Barak et al. [20] demonstrated these organisms in placental tissue of patients with hypertension.

However, in the present study we have looked at both of these sources and demonstrated that periodontal pathogens are present simultaneously in subgingival plaque and placenta. The periodontal organisms in higher proportion of women with hypertension may suggest that the virulence attributability of these organisms could be responsible for hypertension in pregnancy. Although periodontal organisms have been detected in their placental tissues, it does not imply that etiology of pregnancy hypertension is understood. It is possible that localization at feto-placental interface may further lead to inflammatory response with the production of more inflammatory cytokines inflicting the injury. The unresolved question is whether preventing or treating periodontal disease will reduce severity or occurrence of pregnancy hypertension. Highest level of evidence to support the concept that periodontal disease is a possible reversible cause of pregnancy hypertension can be obtained from intervention trials.

In the present study periodontal pathogens were detected in placenta of two of normotensive controls who did not have periodontal disease. It is possible that these organisms may have gained access through genital tract as ascending infection or retrogradely translocated from the peritoneal cavity through fallopian tubes. We do not have information about lower genital tract infection and peritoneal factors in these patients.

Although the results of this study are devoid of bias at all levels (of recruitment, examination and laboratory) and that application of q-PCR technique to identify the organisms has rendered the results more specific, we feel that studies with more number of cases from different regions is required to infer firmly.

It is concluded that in women with hypertension in pregnancy there is significantly higher presence of periodontal pathogens simultaneously in their subgingival plaque and placenta.

Conflict of interest Authors certify that no actual or potential conflict of interest in relation to this exists.

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