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CLINICAL RESEARCH ARTICLE

Association between Pathogens and Periodontal Status of Chinese Women during Pregnancy and within One Year after Delivery

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Abstract

Background: The aim of this study is to compare the detection of periodontal pathogens in the oral cavity of pregnant and non-pregnant women in the childbearing age and to understand the influence of pregnancy status on the ecology of periodontal pathogens.

Methods: Non-stimulated whole saliva samples were collected from a total of 84 pregnant women (including 29 during the first trimester, 29 in the second trimester, 26 at the end of pregnancy), 33 postpartum women, who were chosen randomly. The plaque index (PII), sulcus bleeding index (SBI), periodontal probing depth (PPD) and attachment lost (AL) were recorded. Periodontal microorganisms including porphyromonas gingivalis (Pg), prevotella intermedia (Pi), prevotella nigrescens (Pn) were detected by using 16S rRNA based polymerase chain reaction (PCR) method.

Results: Pn was more frequently detected in the pregnant group than in the postpartum group (45.6% *vs.* 12.1%, $P < 0.01$). The prevalence of Pn in the end of pregnancy group was also significantly higher than from subjects in the 1st (57.7% *vs.* 48.3%, $P < 0.05$) and 2nd trimester group (57.7% *vs.* 31.0%, $P < 0.01$). As the pregnancy progressed, the detection rate of Pg gradually decreased (69.0%, 44.8%, 38.5%), while the rate of Pg in women after delivery was significantly higher (81.8%) than that in pregnant women ($P < 0.01$). Pi detection rate after delivery was lower than that in pregnancy, but there was no significant difference between the two groups (24.2% *vs.* 29.2%, $P > 0.05$). Periodontal status in the Pg positive group were poorer than the Pg negative group, the difference of SBI was statistically significant (1.80 ± 0.92 *vs.* 1.35 ± 0.81, $P < 0.01$).

Conclusions: The high detection rate of Pn in pregnant women implied the effect of pregnant status on microbiota of oral cavity. Pn might be the dominant bacteria in the oral cavity of the pregnant women when compared with Pi and Pg.

Abbreviations

PII: Plaque Index; SBI: Sulcus Bleeding Index; PD: Periodontal Probing Depth; AL: Attachment Lost; Pg: Porphyromonas Gingivalis; Pi: Prevotella Intermedia; Pn: Prevotella Nigrescens; PCR: Polymerase Chain Reaction

Background

Periodontal diseases are chronic infection caused by interactions between microorganisms and the immune system of chronic bacterial infection, environmental, behavioral, and/or genetic factors [1]. Pregnant condition, as an environmental factor, increases the response of gingival tissue to microbial plaque, which is prone to causing gingivitis during pregnancy. It has been reported that the prevalence of gingivitis in pregnancy is 36-100% [2,3]. Gingivitis in pregnancy may be healed or alleviated after delivery [4]. The increase in hormone activity during pregnancy aggregates the existed gingival chronic inflammation, so the gums easily bleed, swell, and even form tumor-like changes that affect the chewing, digestion and absorption of pregnant women [5,6]. Study by Offenbacher, et al. has shown that pregnant patients with periodontitis have a high degree of inflam-

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mation and destruction, who are 7.5-7.9 times more likely to have preterm and low birth weight infants than pregnant women with healthy periodontal status [7]. But the role of pathogen in the severity of gingivitis of women during pregnancy is still unclear. In addition, whether these microbial involved periodontal diseases would be able to self-heal after delivery is also debated.

The identification of predominant microorganisms in periodontal diseases of pregnant women requires suitable detection method on various clinical samples. In addition to the direct physiological effects on periodontal tissue, the increase in estrogen levels during pregnancy appears to be associated with increased oral intraoral growth of certain Gram-negative anaerobes [2,8,9]. It is generally accepted that *Prevotella intermedia sensu lato* (formerly Bacteroides intermedius) is a hormone-specific pathogen of gingivitis in pregnancy [2,10,11]. *In vitro* experiments have confirmed that some kinds of bacteria can use estrogen instead of vitamin K to promote its growth [12]. The development of molecular biology over the past two decades has led to a tremendous shift in the classification of Bacteroides species, forming two new genus *Porphyromonas* and *Prevotella* [12,13]. Previous *Bacteroides intermedius* included two strains of the same phenotype, *Prevotella intermedia* (Pi) and *Prevotella nigrescens* (Pn) [14], both of which can be easily and reliably separated [15,16] by molecular biology methods such as 16S rDNA-based PCR at present. However, since the new classification, few studies reported which of these two bacteria is predominant in pregnant women. Only one study from Finnish scholars reported Pn is the dominant in Finnish pregnant patients. It's uncertain that if bacterial distribution would be consistent among different ethnic groups and continents. Therefore, it's worth studying the predominant bacteria in the oral cavity of Chinese pregnant women to elucidate the role of microorganisms in pregnancy.

Our previous studies using samples from Chinese women show the inflammation and destruction of periodontal tissues of mothers with low birth weight of their infants is worse than the mothers with normal birth weight [17-20]. This finding is consistent with the result from Offenbacher, et al. [7]. We also found that the oral Pg detection rate in the former is also significantly higher than the normal mother. Therefore, it is worth studying the dominancy of Pg, Pn, and Pi in the development of periodontal inflammation among Chinese pregnant women. The impact of the Chinese traditional view on pregnant women prevents them from regular daily dental care [21]. The aim of this study is to detect Pi, Pn, and Pg in the oral cavity of pregnant women and women who have given birth within one year, to understand the interactions between pregnancy stages and these three periodontal pathogenic bacteria, and to determine whether Pi or Pn increase during pregnant gingivitis.

Methods

Subjects

This study was approved by the Biomedical Ethics Committee in Peking University. Patients from four hospitals were selected for this study, including two urban hospitals, the Third Hospital affiliated to Peking Univer-

Table 1: Clinical characteristic of study subjects.

		1st trimester	2nd trimester	3rd trimester	Post partum
Age (yr)		29.31	29.17	28.81	29.76
Height (cm)		160.10	161.00	160.12	161.12
Weight (kg)		56.62	57.62	57.62	57.03
Monthly salary (in RMB)	< 1000	13.8%	20.7%	11.5%	9.1%
	1000-3000	34.5%	55.2%	46.2%	36.4%
	3000-5000	13.8%	6.9%	38.5%	21.2%
	5000-8000	13.8%	10.3%	0	15.2%
	8000-10000	13.8%	3.4%	3.8%	9.1%
	> 10000	10.3%	3.4%	0	9.1%
Teeth brush per day (time)	>= 2 times	65.5%	72.4%	88.5%	81.8%
	1 time	31.0%	27.6%	7.7%	18.2%
	0	3.5%	0	3.8%	0
Teeth brush time (min)	<= 1	17.2%	3.4%	11.5%	3.0%
	1-2	58.6%	72.4%	53.8%	60.6%
	2-3	6.9%	17.2%	34.6%	30.3%
	>= 3	17.2%	6.9%	0	6.1%
Teeth cleaning interval	< 1 year	6.9%	3.4%	0	18.2%
	> 1 year	6.9%	10.3%	7.7%	9.1%
	Never	86.2%	86.2%	92.3%	72.7%

sity and Haidian District Maternal and Child Health Hospital of Beijing, and two suburban and township hospitals, Miyun County Hospital and Miyun County Maternal and Child Health Hospital. The subjects were selected from initial pregnancy women and postpartum women (3 months to 1 year after delivery) in these four hospitals on during 2007-2008. All selected patients were healthy and between 18 to 40-years-old, a full mouth periodontal examination was conducted after filling out the questionnaire. The questionnaire included age, height, weight, economic status, cultural level, alcohol and tobacco preferences, general health Status and other background information. A total of 117 women were finally selected in this study, including 33 postnatal women (after 0.5-1 years) and 84 pregnant women which have 29 in the first trimester (12-14 weeks), 29 in the second trimester (25-27 weeks), and 26 in the third trimester. The average age of these patients is 29.11 ± 3.85, with the maximum age of 38 years and the minimum age of 21-years-old (Table 1).

Periodontal clinic examination: According to the diagnostic standard for periodontal examination, each patient's probing depth (PD), plaque index (PLI), bleeding index (BI) and attachment loss (AL) were recorded.

Sample collection and processing: Each patient's non-irritating whole saliva was collected (sample processing was completed within 4 hours). 0.5 ml of saliva was pipetted into a 1.5 ml EP tube and centrifuged for 5 minutes at 13000 rpm (rotor radius = 5.5 cm). The supernatant was discarded with caution and 0.5 ml TE buffer was added to the pellet and rinse thoroughly. The sample was then centrifuged at 13000 rpm (with the same rotor) for 5 minutes. Precipitate was subsequently washed with 0.5 ml TE buffer for 4 times. The rinsed precipitate was frozen at -20 °C for DNA extraction.

DNA extraction and PCR amplification: DNA was extracted with commercial bacterial DNA extraction kit provided by Shanghai Huashun Bioengineering Co., Ltd. The primers of 16S rDNA of Pg, Pi and Pn were designed according to Ashimoto [22] and Baumgartner's [23] report (Table 2). The PCR system was set in a total reaction system of 25 µl that contains 2 µl of sample, 10 mmol/L Tris-HCL, 50 mmol/L KCL, final concentration of dNTP 0.2 mmol/L, final primer concentration 0.4 µmol/L, Taq DNA polymerase 1U, $MgCl_2$ concentration of 1.5 mmol/L for Pg samples, and $MgCl_2$ concentration of 1.0 mmol/L for Pi and Pn samples. PCR reactions were carried on GeneAmp PCR system 2700 instrument. The reaction cycles for Pg samples were set as 2 cycles of pre-denaturation at 95 °C, 36 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 2 minutes. With regard to Pi and Pn samples, the PCR reaction cycles were set as after pre-denaturation at 95 °C for 2 minutes, denaturation at 94 °C for 30 seconds, annealing at 55 °C for 1 minute, extension at 72 °C for 2 minutes, and finally extension at 72 °C for 10 minutes after 36 cycles were performed. PCR products were later detected by 1.5% agarose gel electrophoresis. Regarding the quality control of PCR reactions, both the positive control and negative control were set up for each batch of PCR amplification. The positive control was the DNA extract of the corresponding microorganism standard strain (the standard strain was provided by Tokyo Medical Dental University and Capital Medical University Institute of Stomatology) Pg (ATCC33277), Pi (ATCC 25611), and Pn (ATCC33563). Sterile deionized water was used as the negative control. Repeated tests were conducted on randomly selected 50% samples, and the repeatability was 97.3%. When the results from repeated test were inconsistent with the original one. A third round of test was conducted on these samples.

Statistical analysis: All statistical analyses were conducted on SPSS19.0 software package. The measurement data were expressed as mean ± standard deviation (M ± SD), and the counted data was expressed as a percentage. The normally distributed data was analyzed by one-way ANOVA and t-test. Multiple comparison and pair wise comparisons by Kruskal-Wallis Test were conducted for data that violate normality assumption. Counted data were compared by chi-square test and multiple-sample chi-square test.

Results

Periodontal status of women during pregnancy and after childbirth

All groups were similar respect to age, height, weight, socioeconomic status and education level when being compared with each other. As shown in Table 3, with the increase of pregnancy stage, the status of PLI, SBI and PD gradually became worse. Compared with the entire childbirth stages, the periodontal parameters PLI,

Table 2: Species-specific PCR primers for periodontal pathogens detection.

Strain	Oligonucleotides (5'→3')	Amplicon size (bp)
Pg	AGG CAG CTT GCC ATA CTG CG	404
	ACT GTT AGC AAC TAC CGA TGT	
Pi	TTT GTT GGG GAG TAA AGC GGG	575
	TCA ACA TCT CTG TAT CCT GCG T	
Pn	ATG AAA CAA AGG TTT TCC GGT AAG	804
	CCC ACG TCT CTG TGG GCT GCG A	

Table 3: Comparing periodontal clinic measurement in different groups.

Trimester #	PLI	SBI	PD	AL
1st (n = 29)	1.39 ± 0.25	1.07 ± 0.67	1.74 ± 0.36	0.39 ± 0.26
2nd (n = 29)	1.66 ± 0.48	1.32 ± 0.77	2.07 ± 0.58	0.39 ± 0.29
3rd (n = 26)	1.77 ± 0.49	1.64 ± 0.62	2.19 ± 0.18	0.45 ± 0.41
Post partum (n = 33)	1.72 ± 0.47	2.34 ± 0.92	2.35 ± 0.42	0.38 ± 0.22
P value	0.224	0.013*	0.000**	0.741

*, $p < 0.05$; **$p < 0.01$, compared with Pg + group in same column.

Table 4: Comparing periodontal clinical measurement in Pg Pi Pn positive and negative groups.

	PLI	SBI	PD	AL
Pg + (n = 70)	1.68 ± 0.48	1.80 ± 0.92	2.15 ± 0.53	0.39 ± 0.39
Pg - (n = 47)	1.56 ± 0.39	1.35 ± 0.81**	2.02 ± 0.35	0.31 ± 0.27
Pi + (n = 32)	1.73 ± 0.46	1.59 ± 0.93	2.13 ± 0.44	0.40 ± 0.36
Pi - (n = 85)	1.60 ± 0.45	1.63 ± 0.90	2.08 ± 0.48	0.34 ± 0.35
Pn + (n = 42)	1.61 ± 0.38	1.46 ± 0.72	1.98 ± 0.46	0.36 ± 0.31
Pn - (n = 75)	1.65 ± 0.49	1.70 ± 0.99	2.16 ± 0.46	0.35 ± 0.37

**$p < 0.01$, compared with Pg + group in same column.

Table 5: Detection of three periodontal pathogens in different stages of pregnant women.

	Pg detection	Pi detection	Pn detection
1st trimester (n = 29)	20 (69.0%)	8 (27.6%)	14 (48.3%)
2nd trimester (n = 29)	13 (44.8%)	4 (13.8%)	9 (31.0%)
3rd trimester (n = 26)	10 (38.5%)	12 (46.2%)	15 (57.7%)
Post partum (n = 33)	27 (81.8%)*	8 (24.2%)	4 (12.1%)*

*compared with different trimester stages, $p < 0.01$.

SBI and PD of women after childbirth were even worse than those of pregnant women. The differences of SBI and PD between pregnant women and women after childbirth were significant.

Relationship between periodontal clinical indexes and detection of different pathogenic bacteria

As illustrated in Table 4, all the periodontal clinical indexes of PLI, SBI, PD, AL in Pg positive subjects were lower than Pg negative subjects, with SBI index significantly low. But there was no statistically significant difference of periodontal clinical indexes between Pi and Pn-positive and -negative subjects.

Three periodontal pathogens detected in women at different childbirth stages

As can be seen from Table 5, with the increase of pregnancy stage, Pg detection rate decreased gradually, but after the childbirth, the detection rate of Pg became significantly higher than all groups in pregnancy ($p < 0.01$). The detection rate of Pi was not significantly different within all pregnant groups and between subjects in pregnancy and those after childbirth, though the detection rate in the group after childbirth was lower than that at the end of pregnancy ($p > 0.05$). Pn detection rate in subjects after childbirth was significantly lower than all pregnant groups including pre-pregnancy, mid-pregnancy and late pregnancy. Among all observations in pregnant groups, the Pn detection rate was the highest in the late pregnancy group ($p < 0.01$).

Discussion

Microorganisms are initial cause of gingivitis during pregnancy. But it's acknowledged that the traditional methods limit the gingivitis bacterial identification [24] in pregnant and postpartum women, as well as the relationship to the periodontal healthy condition. In this study, salivary rather than gingival crevicular fluid were selected because microorganisms in saliva also come from the sulcus, tongue back, and other places in the oral and is easily colonized in various parts of the periodontal tissue.16S rDNA fragments were amplified in the present study to identify the microbiota from pregnant and postpartum women collected from four hospitals in Beijing Municipality of north China. All the periodontal clinical indexes of PLI, SBI, PD, AL in Pg positive subjects were lower than Pg negative subjects, of which SBI index is significantly low, indicating Pg richness represents the severity of pregnancy gingivitis throughout the entire pregnancy and postpartum stage in these women.

In 1999, Socranskyet, et al. [25] who studied 13261 subgingival plaque samples from 185 subjects by using

whole-genome DNA probes and checkerboard DNA-DNA hybridization, determined the 40 kinds of common subgingival bacteria and their impact levels on subgingival plaque. According to their aggregation characteristics and the relationship with the periodontal status, the bacteria were divided into five major microbial complexes, and Pg was identified as the red complex, i.e. defined as a complex strikingly related to periodontitis and periodontal clinical parameters, especially with the depth of the periodontal pocket and exploration of bleeding. Pi and Pn belong to the orange complex, which is closely related to the development of periodontitis. The orange complex is also related to the depth of periodontal pocket. There is a close relationship between the red and orange complexes, which would help immensely in the diagnosis of periodontal disease. Therefore, changes in hormones during pregnancy lead to increase the Pn level, which subsequently causes the decrease of Pg by suppression Pg for Pn's colonization. Once hormones decreased to the normal level after the baby is delivered, Pg levels could immediately increase.

Pi and Pn have similar virulence factors to destroy periodontal tissue. But due to the previous mixed studies on Pi and Pn, as well as the shortage of the conventional phenotypic biochemical methods distinguishing Pi and Pn, previous studies show contradictory results. Our study provides more information on the detection of Pi and Pn during pregnancy and finds that Pn is the dominant bacteria while in the past it was well acknowledged that Pi was the dominant bacteria in the gingivitis during pregnancy. Pn's dominancy during pregnancy could attribute to the increased gestational progesterone during pregnancy as progesterone can be used by Pn to meet its growth need instead of Vitamin K, resulting in its significant increase in pregnancy, and decrease after the baby delivery when progesterone level has declined.

The possible reason that hormones would facilitate the bacterial impact could be that the high levels of estrogen during pregnancy would increase the number of blood vessels, the blood flow in the gingival tissues, and the metabolism of connective tissue. These hormones of pregnancy can be utilized by the bacteria, such as the progesterone as a source of nutrition. Some studies have found that estradiol and progesterone are structurally similar to naphthoquinone, which is a group of essential growth factors for Pn. Estradiol and progesterone can substitute for vitamin K, which belongs to naphthoquinone family, to be the bacterial growth factors. As a result, increased secretion of gestagens can promote the growth of these microorganisms.

The former Bacteroides intermedius, currently including *Prevotella intermedia* and *Prevotella nigrescens*, has been associated with hormone-induced pregnancy gingivitis.

Currently, it is generally accepted that *Prevotella intermedia* (formerly *Bacteroides intermedius*) is the predominant periodontal pathogen in patients with gingivitis during pregnancy. However, due to the development of molecular biology, *Bacteroides* was proposed to be reclassified in 1988 [13], and two new genus *Porphyromonas* and *Prevotella* were divided from the old taxonomic system [26]. Thus, in 1992, Shah and Gharbia [14] renamed the former *Bacteroides intermedius* as *Prevotella intermedia sensu lato* and isolated two separate sub-species from it: The so-called narrow sense *Prevotella intermedia sensu stricto*, and *Prevotella nigrescens* (Pn). However, because of the lack of appropriate experimental methods to isolate Pi and Pn at that time, the name of *Prevotella intermedia* was still used for both Pi and Pn in many studies for a long time. Until 2009 [27], in a study of gestational gingivitis in pregnant women in Finland, the researchers used 16S ribosomal DNA PCR technique to isolate Pi and Pn and found that Pn is widespread in women with gingivitis during pregnancy rather than Pi.

In our study on pregnant and postpartum women in China, Pn detection rate in pregnant subjects was significantly higher than in women after childbirth, presenting the Pn predominantly exists in all pregnant women with gingivitis (Table 4). This is similar to the study conducted in Finland. This finding suggests that regardless of race or diet, Pn may widely exist in pregnant gingivitis-infected women, and the richness in gingiva is highly impacted by the aforementioned physiological variations of pregnancy including the high level of estrogen, suppressed immune responses, and a better environment for plaque microorganisms in gingiva caused by gingival bleeding and the increased depth of the periodontal pocket.

In this study, all studied subjects were non-intervened in the natural development of periodontal status. We found that women's periodontal condition did not improve, but worsened, even 0.5-1 years after childbirth and without periodontal intervention. This worsened condition after pregnancy is because the traditional concept of consciousness and poor oral health awareness among Chinese women, especially the mistaken idea revealed from a survey on 264 primipara and 264 of their mothers that over half of primipara and roughly 80% of their mothers believe that women couldn't brush the teeth during pregnancy and postpartum [21]. This finding demonstrates that without any medical intervention, gingivitis occurring in pregnant women will not be self-healed with the reduction of postpartum hormone levels after the childbirth, verifying the importance of periodontal treatment before and during pregnancy, and the necessity of the change of attitudes of both doctors and patients on gingivitis in pregnancy.

Conclusions

From the results of this study, with the increase of pregnancy, the periodontal condition of pregnant wom-

en gradually worsens. The periodontal condition of women within one year after delivery is still not optimistic, and the basic periodontal condition is the same as or slightly worse during pregnancy. As the pregnancy progressed, the detection rate of Pg gradually decreased, but the Pg detection rate of postpartum women was even significantly higher than that of pregnant women, showing that Pg could reflect the severity of gingivitis but is not the predominant bacteria in the oral cavity of pregnant women. The detection rate of Pi in different gestations and before and after childbirth shows no statistical difference, indicating that Pi is not the predominant bacteria in the oral cavity of pregnant women. It is worth noting that the detection rate of Pn is higher in different gestational stages, but the detection rate of Pn in postpartum women is significantly lower than that in pregnant women, showing Pn is the dominant bacteria in the mouth of women during pregnancy. However, without any medical interfere, gingivitis occurring in pregnant women will not be self-healed.

Declaration

Ethics approval and consent to participate

This study had been approved by "Peking University Biomedical Ethics Committee". The reference number is IRB00001052-07104.

Consent for publication

All participants were required to sign an informed consent of participation to show that they consent to publish their clinical details anonymously.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

ZH-Clinical data collection, conducted the experiments and analyzed the results, prepared the manuscript; ZC-Conducted the experiments and analyzed the results; LH-Designed and interpreted the experiments, prepared the manuscript; YS-Designed the experiments; JK-Clinical data collection; All authors reviewed the manuscript.

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