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Relationship between oral malodor and the menstrual cycle

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Background and Objective: Sex hormones have been suggested to be important modifying factors that may influence the pathogenesis of periodontal disease. This study examined changes in volatile sulfur compounds (VSC) levels, clinical parameters and bacterial levels during the menstrual cycle.

Material and Methods: The study group consisted of 10 female subjects with periodontitis and 12 periodontally healthy female subjects. Clinical and bacterial measurements were performed for all subjects during the ovulation and follicular phases of the menstrual cycle.

Results: Bleeding on probing (BOP) was significantly increased in the ovulation phase in periodontitis subjects but not in healthy subjects. The VSC levels in subjects with periodontitis increased 2.2-fold in the ovulation phase compared with the follicular phase. In the ovulation phase, VSC levels and BOP were significantly higher in subjects with periodontitis than in healthy subjects. The number, and salivary levels, of *Prevotella intermedia* in subjects with periodontitis were significantly higher in the ovulation phase than in the follicular phase.

Conclusion: The present study indicated changes in VSC, BOP and *P. intermedia* during the menstrual cycles of women with periodontitis.

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Periodontitis is an inflammatory disorder that results in the destruction of the structures that support the teeth. It is initiated by an accumulation of predominantly anaerobic gram-negative bacteria. However, links have also been shown between systemic factors and disease prevalence, progression and severity (1). Among these, sex hormones have been suggested to be important modifying factors that may influence the pathogenesis of periodontal disease (2–4). Sex hormones have significant biological actions that can affect many organ systems, including the oral cavity (5–10). Many women report an increase in gingival inflammation and discomfort in association with their menstrual cycle. In particular, around ovulation, the gingival inflammation index was found to increase, although no significant change in plaque index was detected (11,12). Levels of gingival exudate were found to increase during ovulation and to decrease at menstruation (13). A sudden and marked increase in the production of estrogen occurs at ovulation. Lu *et al.* (14) found a sharp rise in salivary estradiol immediately at the ovulation phase. Tonzetich *et al.* (15) also showed that volatile sulfur compounds (VSC) levels increased by at least twofold at the ovulation phase compared with the follicular phase. Volatile sulfur compounds such as hydrogen sulfide, methyl mercaptan and dimethyl sulfide play important roles in oral malodor (16). Volatile sulfur compounds are produced from food debris, cells, saliva and blood in the oral cavity, primarily through microbial putrefaction (17,18). Oral microbes, especially gram-negative bacteria, are the pathogens primarily responsible for oral malodor (19,20). Several studies have reported that periodontal disease-associated bacteria, such as *Porphyromonas gingivalis* and *Prevotella intermedia*, produce VSC (20–23). However, little evidence supports a relationship between oral malodor and the menstrual cycle. Therefore, the objective of this study was to examine changes in VSC levels, clinical parameters and bacterial levels over the menstrual cycle.

Material and methods

Subject selection

The study group consisted of 10 female systemically healthy subjects with periodontitis, mean age 29.7 years (range 22–37 years), who first visited Nihon University School of Dentistry Dental Hospital for oral examination, periodontal treatment or conservative dental treatment. The clinical criteria for periodontitis were standard measurements of clinical probing depths. Periodontitis patients had at least two sites showing probing depths of ≥ 4 mm in the follicular phase of the menstrual cycle. Twelve female dental staff members from the Nihon University Dental Hospital with clinically healthy periodontia served as controls (mean age 25.8 years, range 20–36 years). All subjects had a history of normal, regular menstrual cycles and exhibited no evidence of systemic pelvic pathology such as pelvic inflammatory disease. No subject had received professional cleaning, antibiotic medication, or oral contraceptives in the 3 mo before the start of the study. All subjects were nonsmokers. Subjects were instructed to record their basal body temperature on a chart.

This protocol was approved by the Nihon University School of Dentistry Institutional Review Board. Informed consent was obtained from each subject.

Generally, the menstrual cycle is divided into four phases: (i) ovulation phase (the day with the lowest basal body temperature after menses); (ii) luteal phase (the period of time from ovulation to menses, which is the high phase of basal body temperature); (iii) menses, which starts on the day with the lowest basal body temperature after the luteal phase; and (iv) follicular phase (the period of time from menses to ovulation, which is the low phase of basal body temperature) (24).

Tonzetich *et al.* (15) showed that VSC levels increased by at least twofold at ovulation compared with the follicular phase. The following measurements were performed in all subjects in the ovulation and follicular phases. On the day of the examination, subjects were requested to refrain from oral activities, including drinking, eating, chewing, mouth rinsing and brushing for 2 h before data collection. Measurements in all subjects were made on a same time zone in both the ovulation phase and the follicular phase.

VSC

Volatile sulfur compounds were measured using a portable industrial sulfur monitor (Halimeter®; Interscan Corp., Chatsworth, CA, USA) zeroed on ambient air before each measurement. A disposable plastic straw was attached to the air inlet, and subjects were instructed to bring their mouths slightly opened over the straw so that it extended approximately 4 cm into the oral cavity. Subjects were then asked to breathe through their nose during the measurements. The peak VSC level was determined in parts per billion (p.p.b.) sulfide equivalents (25).

Organoleptic measurements

In the organoleptic measurement, subjects remained quiet and kept their mouths closed for 60 s. Subjects were asked to exhale through the mouth briefly, with moderate force, at a distance of 10 cm from the judge. Organoleptic measurements were made by three judges, and the score upon which at least two of them agreed was recorded. In our study, all scores were agreed by two judges. There were no statistically significant differences among three judge scores in Kruskal–Wallis one-way analysis of variance on ranks ($p = 0.870$). Organoleptic malodor scores were recorded independently by each judge on a scale of 0–5, as follows: 0, no appreciable odor; 1, barely noticeable odor; 2, slight, but clearly noticeable odor; 3, moderate odor; 4, strong odor; and 5, extremely foul odor. Judges were blinded regarding the others' scores and their own previous scores (25).

Clinical parameters and saliva sampling

The clinical parameters were probing depth, percentage of sites with bleeding on probing (BOP) and O'Leary's plaque control record. Measurements of these parameters were obtained at six sites around six reference teeth (11, 16, 24, 31, 36, 44) using a PCPUNC15 probe (Hu-Friedy, Chicago, IL, USA). After clinical examination, paraffin wax-stimulated whole saliva was collected, and the samples were stored at −80°C until use.

Quantitative analysis with real-time PCR

To determine the numbers of total bacteria and two anaerobic bacteria – *P. intermedia* and *P. gingivalis* – in saliva, real-time PCR was used with an ABI PRISM 7700 Sequence Detection System (ABI, Foster City, CA, USA).

Table 1. Species-specific primers/TaqMan probes for real-time PCR

		References
Prevotella intermedia		
Forward	CGG TCT GTT AAG CGT GTT GTG	28
Reverse	CAC CAT GAA TTC CGC ATA CG	
Probe	FAM-TGG-CGGACT TGA GTG CAC GC-TAMRA	
Porphyromonas Gingivalis		
Forward	TAC CCA TCG TCG CCT TGGT	29,30
Reverse	CGG ACT AAA ACC GCA TAC ACT TG	
Probe	FAM-GCT AAT GGG ACG CAT GCC TAT CTT ACA GCT-TAMRA	
Universal		
Forward	TCC TAC GGG AGG CAG	31
Reverse	GGA CTA CCA GGG TAT CTA ATC CTG TT	
Probe	FAM-CGT ATT ACC GCG GCT GCT GGC AC-TAMRA	

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Table 2. Change in clinical measurements during the menstrual cycle

	Periodontits subjects (n = 10)			Healthy subjects (n = 12)		
	Follicular phase	Ovulation phase	p-value	Follicular phase	Ovulation phase	p-value
VSC (p.p.b.)	131.9 ± 114.7	293.1 ± 150.0	< 0.001** ††	51.6 ± 30.7	84.0 ± 52.9 †	0.017**
Organoleptic ratings	1.4 ± 0.8	2.5 ± 0.5	0.004** †	1.1 ± 0.7	1.8 ± 0.8	0.016**
BOP (%)	13.3 ± 11.2	23.3 ± 13.1	0.002** ††	8.8 ± 10.5	11.3 ± 9.0	NS
Mean PD (mm)	2.2 ± 0.3	2.3 ± 0.3	NS ††	2.0 ± 0.4	1.9 ± 0.3	NS
Maximum PD (mm)	4.1 ± 0.3	4.3 ± 0.5	NS	2.8 ± 0.4	3.1 ± 0.3	NS
PCR (%)	45.4 ± 22.6	36.6 ± 17.2 ††	NS	34.4 ± 16.9	35.1 ± 17.3	NS
Salivary flow rate (mL)	7.4 ± 2.7	7.0 ± 2.8	NS	8.0 ± 2.9	7.8 ± 3.6	NS

All values are expressed as the mean ± SD.
The Wilcoxon's signed-ranks test was used to determine statistically significant differences between the follicular phase and ovulation, $^{**}p < 0.01$, NS, not significant.
The Mann–Whitney U-test was used to determine statistically significant difference between follicular phase and ovulation phase, $^{††}p < 0.01$, $^{†}p < 0.05$.
BOP, bleeding on probing; PD, probing depth; p.p.b., parts per billion; VSC, volatile sulfur compounds.

The saliva samples were vortexed for 3 min, boiled for 10 min and immediately placed on ice. The samples of saliva were then centrifuged (12,000 *g*, 5 min) to remove large debris, and aliquots of supernatant were harvested for PCR processing (26). PCR amplification was conducted with the following parameters: 5 min at 50°C (one cycle), 10 min at 95°C (one cycle) and 40 cycles of 15 s at 95°C and 1 min at 60°C (27).

The primer and probe sets for the two bacterial species are listed in Table 1 (28–31). To quantify total bacteria, conserved sequences in reported 16S genes were selected. At the same time, total bacterial ribosomal RNA was amplified in a separate reaction under the same conditions as those used for specific amplification of the two bacteria. The probes were labeled at the 5′-end with the reporter dye 6-carboxyfluorescein (6-FAM) and at the 3′-end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). Data were analyzed using the Sequence Detection System software from ABI. The number of bacterial cells was determined using DNA from known amounts of *P. intermedia* ATCC25611 and *P. gingivalis* ATCC33277. Bacterial levels were expressed as the percentage of total bacteria.

Statistical analyses

Differences in VSC levels, clinical parameters and bacterial numbers between ovulation and follicular phases were analyzed using Wilcoxon's signed-ranks test. The Mann–Whitney U-test was used to determine differences between subjects with periodontal disease and healthy subjects, and p-values of < 0.05 were deemed to indicate statistical significance. The Spearman rank correlation test was used to determine possible associations between VSC and bacterial levels. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Variations in periodontal indices are shown in Table 2. Despite the similar-

Table 3. Bacterial measurements during the menstrual cycle

	Follicular phase	Ovulation phase	p-value
Total bacteria			
Periodontitis subjects	$2.5 \times 10^9 \pm 3.2 \times 10^9$	$2.8 \times 10^9 \pm 3.7 \times 10^9$	NS
Healthy subjects	$1.4 \times 10^8 \pm 2.0 \times 10^8$	$2.7 \times 10^8 \pm 6.1 \times 10^8$	NS
P. intermedia			
Periodontitis subjects	$1.4 \times 10^6 \pm 1.8 \times 10^6$	$3.8 \times 10^6 \pm 3.1 \times 10^6$ ††	0.002**
Healthy subjects	$3.6 \times 10^5 \pm 7.6 \times 10^5$	$1.2 \times 10^5 \pm 2.2 \times 10^5$	NS
P. gingivalis			
Periodontitis subjects	$1.9 \times 10^4 \pm 5.0 \times 10^4$	$5.9 \times 10^4 \pm 1.3 \times 10^5$	NS
Healthy subjects	$3.3 \times 10^3 \pm 6.2 \times 10^3$	$3.9 \times 10^3 \pm 7.3 \times 10^3$	NS
P. gingivalis/total bacteria			
Periodontitis subjects	$9.9 \times 10^{-3} \pm 2.8 \times 10^{-2}$	$5.4 \times 10^{-2} \pm 1.0 \times 10^{-1}$ †	0.004**
Healthy subjects	$3.3 \times 10^{-3} \pm 4.3 \times 10^{-3}$	$1.3 \times 10^{-3} \pm 1.8 \times 10^{-3}$	NS
P. gingivalis/total bacteria			
Periodontitis subjects	$8.9 \times 10^{-4} \pm 2.2 \times 10^{-3}$	$1.4 \times 10^{-3} \pm 3.9 \times 10^{-3}$	NS
Healthy subjects	$1.7 \times 10^{-4} \pm 2.9 \times 10^{-4}$	$3.1 \times 10^{-4} \pm 7.8 \times 10^{-4}$	NS

All values are expressed as mean ± standard deviation.
The Wilcoxon's signed-ranks test was used to determine statistically significant differences between the follicular phase and ovulation phase, $^{**}p < 0.01$, NS, not significant.
The Mann–Whitney U-test was used to determine statistically significant differences between the follicular phase and ovulation phase, $^{†}p < 0.01$, $^{††}p < 0.05$.
P. gingivalis, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*.

Table 4. Spearman correlation coefficient values between parameters of all subjects in the follicular phase

	Organoleptic malodor score	BOP (%)	Mean PD (mm)	Maximum PD (mm)	PCR (%)	Saliva flow rate (mL)	*P. gingivalis* (%)	*P. intermedia* (%)	*P. gingivalis*	*P. intermedia*	Total bacteria
VSC (p.p.b.)	−0.623**	−0.331	0.072	0.337	−0.391	−0.128	−0.222	0.077	0.218	0.534*	0.530*
Total bacteria	−0.095	−0.229	0.165	0.334	−0.134	0.242	−0.645**	−0.258	0.050	0.580**	
P. intermedia	0.065	−0.572	0.141	0.227	−0.358	−0.023	−0.152	0.452*	0.355		
P. gingivalis	−0.120	−0.363	0.201	0.128	0.090	−0.034	0.674**	0.266			
P. intermedia (%)	0.124	−0.509*	−0.203	−0.224	−0.115	−0.464*	0.371				
P. gingivalis (%)	−0.033	−0.085	0.052	−0.040	0.312	−0.188					
Saliva flow rate (mL)	−0.467*	−0.036	0.076	−0.009	−0.084						
PCR (%)	−0.174	0.541**	−0.048	0.339							
Maximum PD (mm)	0.229	0.290	0.455*								
Mean PD (mm)	−0.002	0.093									
BOP	−0.060										

*$p < 0.05$ was considered statistically significant.
**$p < 0.01$ was considered statistically significant.
BOP, bleeding on probing; PD, probing depth; p.p.b., parts per billion; VSC, volatile sulfur compounds.
P. gingivalis, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*.

Table 5. Spearman correlation coefficient values between parameters in all subjects in the ovulation phase

	Organoleptic malodor score	BOP (%)	Mean PD (mm)	Maximum PD (mm)	PCR (%)	Saliva flow rate (mL)	*P. gingivalis* (%)	*P. intermedia* (%)	*P. gingivalis*	*P. intermedia*	Total bacteria
VSC (p.p.b.)	0.777**	0.184	0.592**	0.733**	−0.158	−0.221	0.119	0.466*	0.305	0.649**	0.230
Total bacteria	0.162	−0.105	0.074	0.268	−0.279	0.363	−0.611**	−0.398	−0.169	0.473*	
P. intermedia	0.360	0.133	0.567**	0.580**	−0.221	−0.208	−0.092	0.528*	0.306		
P. gingivalis	−0.003	0.107	0.402	0.268	0.001	−0.102	0.844**	0.321			
P. intermedia (%)	0.250	0.180	0.464*	0.398	−0.080	−0.634*	0.364				
P. gingivalis (%)	−0.033	0.141	0.208	0.092	0.116	−0.221					
Saliva flow rate (mL)	−0.390	−0.156	−0.180	0.062	0.054						
PCR (%)	−0.334	0.556**	0.159	−0.070							
Maximum PD (mm)	0.408	0.423*	0.608**								
Mean PD (mm)	0.226	0.464*									
BOP	0.018										

*$p < 0.05$ was considered statistically significant.
**$p < 0.01$ was considered statistically significant.
BOP, bleeding on probing; PD, probing depth; p.p.b., parts per billion; VSC, volatile sulfur compounds.
P. gingivalis, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*.

ity in PCR results, BOP was significantly increased in the ovulation phase in subjects with periodontitis (from 13.3% to 23.3%). There was a slight increase in BOP in healthy subjects (from 8.8% to 11.3%), but this was not statistically significant.

The VSC levels in the periodontitis subjects increased by 2.2-fold in the ovulation phase ($p < 0.001$) compared with the follicular phase, whereas the VSC levels of healthy subjects increased by 1.6-fold in the ovulation phase ($p = 0.017$) compared with the follicular phase. In the ovulation phase, VSC and BOP in subjects with periodontitis were significantly higher than in healthy subjects. Similar increases were observed in organoleptic malodor scores. The salivary levels of *P. intermedia* and *P. gingivalis* were assessed

using real-time PCR. *P. intermedia* was detected in 100% of the subjects with periodontitis and in 83.3% of the healthy subjects in the ovulation phase. *P. gingivalis* was detected in 70% of the subjects with periodontitis and in 66.7% of the healthy subjects. The numbers and salivary levels of *P. intermedia* in periodontitis subjects were significantly higher in the ovulation phase than in the follicular phase (Table 3). However, no significant difference between the follicular and ovulation phases was observed in healthy subjects. No significant difference was observed in total bacterial numbers or in *P. gingivalis* in either group. There were several correlations between parameters of all subjects in the follicular phase (Table 4) and the ovulation phase (Table 5). Significant relationships were found in all subjects in the follicular phase ($r = 0.623$; $p = 0.002$, Table 4) and in the ovulation phase ($r = 0.777$; $p \leq 0.001$, Table 5) between VSC and organoleptic malodor scores. A significant relationship was found between VSC and organoleptic malodor scores of all subjects in both phases ($r = 0.781$; $p < 0.001$; Fig. 1). Significant relationships were also observed in all subjects in the follicular phase ($r = 0.534$; $p = 0.011$, Table 4) and in the ovulation phase ($r = 0.649$; $p = 0.001$, Table 5) between VSC and the numbers of *P. intermedia*. A significant relationship was found in all subjects of both phases between *P. intermedia* and VSC ($r = 0.548$; $p < 0.001$; Fig. 2). A significant correlation was also observed between *P. gingivalis* and VSC ($r = 0.308$; $p < 0.05$).

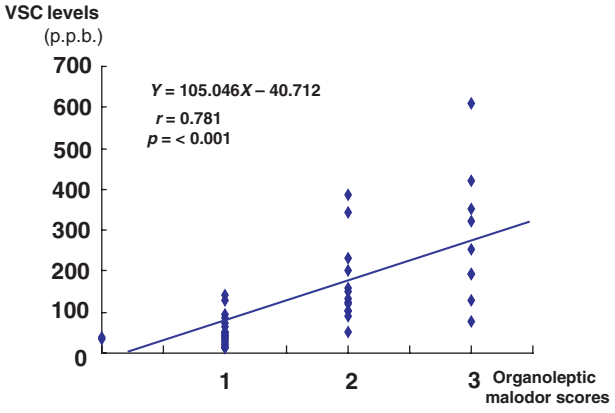

Fig. 1. Correlation between volatile sulfur compounds (VSC) levels and organoleptic malodor scores in all subjects in both the ovulation phase and the follicular phase. The Spearman rank correlation test was used to examine possible associations between VSC levels and organoleptic malodor scores.

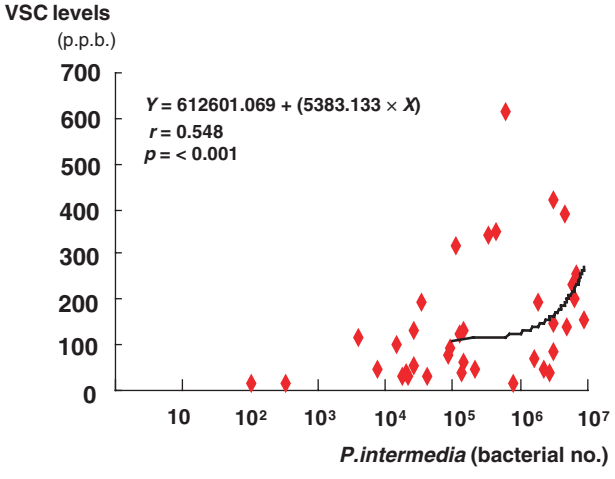

Fig. 2. Correlation between volatile sulfur compounds (VSC) levels and *Prevotella intermedia* in all subjects in both the ovulation phase and the follicular phase. The Spearman rank correlation test was used to examine possible associations between VSC levels and bacterial number.

Discussion

Various studies have shown that increased levels of female sex hormones, such as estrogen and progesterone, correlate with an increased prevalence of gingivitis. The prevalence of gingivitis in pregnant women is also significantly increased (9,10,32,33), and gingivitis has also been related to other systemic situations that affect the levels of sex hormones, such as puberty and menstruation (13,34,35). The present study confirms earlier reports that VSC and gingival inflammation, as assessed by BOP, increased in the ovulation phase (36–39). These reports support a relationship between periodontal bleeding and VSC levels. Blood and cellular elements provide essential substrates for odor production. Additionally, blood provides certain factors that accelerate bacterial growth and stimulates proteolysis and odor production of putrescent saliva.

Volatile sulfur compounds have been shown to result from the bacterial putrefaction of proteins with sulfur-containing amino acids. Several studies have reported that periodontal disease-associated bacteria, such as *P. gingivalis* and *P. intermedia*, produce VSC (20–23). Awano *et al.* (21) reported that the presence of *P. intermedia*, *P. gingivalis* and *Tannerella forsythia* affected the production of VSC and that the presence of *P. intermedia* and *T. forsythia* in saliva was associated with both oral malodor and periodontitis. In the present study, the number and the level of *P. intermedia* and *P. gingivalis* in subjects with periodontitis increased during the ovulation phase, although the number of total bacteria remained at the same level. Thus, increased VSC levels in

subjects with periodontitis may be related to the number, and the level, of *P. intermedia* in saliva. Lu *et al.* (14) found that the levels of estradiol in saliva rose in the ovulation phase. Several reports have indicated that sex hormones induce the proliferation of specific periodontal microorganisms (40–44). Estradiol can substitute for menadione as an essential growth factor in *P. intermedia* (40–42). Serum levels of estradiol showed a positive correlation with the number of *P. intermedia* in plaque (12,40–44). In this study, we confirmed these interactions using real-time PCR. To the best of our knowledge, this is the first study to assess the relationship between oral malodor and bacterial number, using real-time PCR in female subjects with periodontitis, during the menstrual cycle.

In summary, the present study indicates changes in VSC, BOP and *P. intermedia* during the menstrual cycles of women with periodontitis. These changes need to be considered when analyzing data from periodontal examinations and bacteriological testing in premenopausal women. Further studies are required to explore the mechanisms by which this phenomenon occurs and to examine whether these changes have any lasting negative effect on the periodontium.

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