Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 593–598

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2012.01471.x

Relationship between salivary antioxidant capacity and phases of the menstrual cycle

A. Kawamoto[1], N. Sugano[2,3], M. Motohashi[1], S. Matsumoto[1], K. Ito[2,3]

[1]Dental Hygienist Section, Nihon University School of Dentistry Dental Hospital, Tokyo, Japan, [2]Department of Periodontology, Nihon University School of Dentistry, Tokyo, Japan and [3]Division of Advanced Dental Treatment, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan

Kawamoto A, Sugano N, Motohashi M, Matsumoto S, Ito K. Relationship between salivary antioxidant capacity and phases of the menstrual cycle. J Periodont Res 2012; 47: 593–598. © 2012 John Wiley & Sons A/S

Background and Objective: Reactive oxygen species and free radicals are involved in the pathogenesis of periodontal disease. Previous studies have shown that the stage of the menstrual cycle is associated with the levels of gingival inflammation and discomfort. This study examined changes in salivary antioxidant activities, clinical parameters and bacterial levels during the menstrual cycle.

Material and Methods: The study group consisted of 16 women with periodontitis and 12 healthy women. Clinical and bacterial measurements were performed for all subjects during the ovulatory and follicular phases.

Results: Salivary antioxidant activity during the ovulatory phase was significantly lower than during the follicular phase in the women with periodontitis. The antioxidant activity in all subjects during the ovulatory phase was negatively correlated with *Prevotella intermedia* ($r = -0.430$; $p = 0.023$) and total bacterial counts ($r = -0.496$; $p = 0.007$); however, these correlations were not significant for subjects in the follicular phase.

Conclusion: This study showed that salivary antioxidant capacity decreased, while bleeding on probing and *P. intermedia* increased, over the course of the menstrual cycle in women with periodontitis. Antioxidant capacity could be involved in the pathogenesis of periodontitis.

Aki Kawamoto, Nihon University School of Dentistry Dental Hospital, 1-8-13, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
Tel: +81 3 3219 8081
Fax: +81 3 3219 8349
e-mail: imanaga@dent.nihon-u.ac.jp

Key words: bleeding on probing; menstrual cycle; *Prevotella intermedia*; salivary antioxidant capacity

Accepted for publication January 30, 2012

Reactive oxygen species (ROS) and free radicals (FRs) are involved in the pathogenesis of several medical conditions, including rheumatoid arthritis, acute respiratory distress syndrome, cancer, aging, acquired immunodeficiency syndrome (1) and periodontal disease (2–5). Periodontal disease is an inflammatory disorder in which tissue damage occurs via complex interactions between periodontal pathogens and components of the host defense mechanism (6–9). When stimulated by bacterial pathogens, polymorphonuclear leukocytes constitute the first line of cellular host defense against bacteria in the gingival sulcus (8–10). The production of ROS/FRs, such as superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the highly toxic hydroxyl radical (·OH), by polymorphonuclear leukocytes is the primary means of killing bacteria; however, the extracellular release of ROS results in destruction of the surrounding tissues (11,12). A previous report showed that the excessive local production of ROS/FRs might play a role in the pathogenesis of periodontitis (13).

Antioxidants are present in all body fluids and tissues, and protect against endogenously formed ROS/FRs (14,15). Antioxidant defense systems have the following three main components: (i) preventive antioxidants, which suppress the formation of free radicals; (ii) radical-scavenging antioxidants, which scavenge radicals to inhibit chain reactions; and (iii) repair and *de novo* enzymes, which repair damage and reconstitute membranes (16). A deficiency or decrease in antioxidant capacity manifests itself

largely via increased susceptibility to oxidative stress, and the resulting damage is thought to be involved in disease (17). Previous studies have reported that periodontal disease was associated with a reduced salivary antioxidant capacity and increased oxidative damage within the oral cavity (16,18–23). Therefore, the balance between ROS/FRs and antioxidant capacity is likely to be involved in the pathogenesis of periodontitis.

One of the modifying factors that may influence the pathogenesis of periodontal disease may be the sex hormones (24–26). Increasing concentrations of sex hormones at the time of ovulation and during pregnancy may cause the proliferation of specific periodontal micro-organisms (27–31) and change the host immunological response (32–37). The salivary concentration of estradiol rises immediately before the ovulatory phase (28,38), and the gingival inflammation index increased without significantly changing the plaque index (25,26). Our previous study (39) showed the same result, and detected increasing numbers and salivary levels of *Prevotella intermedia* during the ovulatory phase. The present study examined changes in salivary antioxidant capacity, clinical parameters and bacterial levels during the menstrual cycle.

Material and methods

Subject selection

The study group consisted of 16 women with periodontitis (mean age 29.7 years, range 22–38 years), who visited the Nihon University School of Dentistry Dental Hospital for an oral examination, periodontal treatment or conservative dental treatment. The clinical criteria for periodontitis were standard measurements of clinical probing depth. The periodontitis patients had at least two sites with probing depth ≥ 4 mm during the follicular phase of the menstrual cycle. Twelve female dental staff members from Nihon University School of Dentistry Dental Hospital with a clinically healthy periodontium served as control subjects (mean age 25.8 years, range 20–36 years). All subjects had a history of normal, regular menstrual cycles and no evidence of systemic pelvic pathology, such as pelvic inflammatory disease. No subject had received professional dental 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 (39).

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

The following measurements were performed in all subjects during the ovulatory and follicular phases. On the day of the examination, the subjects were requested to refrain from oral activities, including drinking, eating, chewing, mouth rinsing and brushing for 2 h before data collection. All subjects were measured during the same time period (39).

Clinical parameters and saliva sampling

The clinical parameters were probing depth, percentage of sites with bleeding on probing and O'Leary's plaque control record. These parameters were measured at six sites around six reference teeth (11, 16, 24, 31, 36 and 44) using a PCPUNC15 probe (Hu-Friedy, Chicago, IL, USA). After the clinical examination, paraffin wax-stimulated whole saliva was collected, and the samples were stored at −80°C until use (39).

Quantitative analysis with real-time PCR

To determine the numbers of total bacteria and two anaerobic bacteria, *Prevotella intermedia* and *Porphyromonas gingivalis*, in saliva, real-time PCR was used with an ABI PRISM 7700 Sequence Detection System (ABI, Foster City, CA, USA). The saliva samples were vortexed for 3 min, boiled for 10 min, and immediately placed on ice. The saliva samples were then centrifuged (12,000*g*, 5 min) to remove large debris, and aliquots of supernatant were harvested for PCR processing (39,40). The PCR amplification protocol consisted of 5 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C (39,41).

The primer and probe sets used for the two bacterial species were the same as those used in our previous study (39,42–44). To quantify total bacteria, conserved sequences in the 16S genes were selected. Simultaneously, total bacterial ribosomal RNA was amplified in a separate reaction in the same conditions as those used for the 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 analysed 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. A universal primer and probe set was used for quantifying the total proportion of bacterial cells in specimens (39,44). Bacterial levels were expressed as the percentage of total bacteria.

Antioxidant capacity

The antioxidant capacity was measured using an antioxidant potential measurement kit (Radical Catch®; Aloka, Tokyo, Japan) and a chemiluminescence reader (AccuFLEX Lumi400®; Aloka) based on the Fenton reaction, in which antioxidants are trapped by luminol, resulting in light emission (45). This is a procedure for assessing the OH^- scavenging activity using ferrous iron-induced luminol chemiluminescence (46). The saliva samples were centrifuged (12,000*g*, 5 min). After centrifugation to remove large debris, aliquots of supernatant were harvested for measuring the antioxidant capacity. First, 50 μL of cobalt solution and 50 μL of luminol solution were mixed with 20 μL of sample solution and incubated for 5 min at 37°C. The generation of hydroxyl radicals was started by the

addition of 50 μL of H_2O_2 solution. Light emission at 430 nm was measured for 120 s immediately after initiation. Distilled water was used as a control. The light emissions from 80 to 120 s were integrated. The rate of decrease in light emission compared with the control was expressed as the antioxidant capacity (47).

Statistical analyses

Differences in the salivary antioxidant capacities, clinical parameters and bacterial levels between the ovulatory and follicular phases were analysed using Wilcoxon's signed-ranks test. The Mann–Whitney *U*-test was used to determine the differences between subjects with periodontal disease and healthy subjects. *p*-Values < 0.05 were deemed to indicate statistical significance. The Spearman rank correlation test was used to determine possible associations among the antioxidant capacities, clinical parameters, and bacterial levels. Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

In this study, six subjects with periodontitis were added to the data set from our previous study (39). Bleeding on probing in subjects with periodontitis was significantly increased during the ovulatory phase, which is the same result as in our previous study, despite the similarity in O'Leary's plaque control record results (Table 1). The bleeding on probing in subjects with periodontitis was significantly higher than in the healthy subjects during the ovulatory phase ($p = 0.014$). The bleeding on probing was increased in 87.5% of subjects with periodontitis and 58.3% of healthy subjects at ovulation. The mean and maximum probing depth differed significantly between the healthy subjects and those with periodontitis during the follicular and ovulatory phases.

P. intermedia was detected in 100% of the subjects with periodontitis and 83.3% of the healthy subjects during the ovulatory phase, while *P. gingivalis* was detected in 81.3% of the subjects with periodontitis and 66.7% of the healthy subjects. The numbers and salivary levels of *P. intermedia* in the subjects with periodontitis were significantly higher during the ovulatory phase than in the follicular phase ($p = 0.025$ and $p = 0.001$, respectively; Table 2). However, no significant difference between the follicular and ovulatory phases was observed in the healthy subjects. The numbers and salivary levels of *P. intermedia* in the subjects with periodontitis were significantly higher than those of the healthy subjects during ovulation ($p = 0.004$ and $p = 0.017$, respectively). No significant difference was observed in the total bacterial number or in *P. gingivalis* in either group.

The salivary antioxidant capacity during the ovulatory phase was significantly lower than that of the follicular phase in the subjects with periodontitis ($p = 0.037$; Fig. 1). The antioxidant capacity in all subjects during the ovulatory phase was negatively correlated with *P. intermedia* ($r = -0.430$, $p = 0.023$) and total bacteria ($r = -0.496$, $p = 0.007$), but not during the follicular phase (Table 3).

Discussion

In this study, the subjects with periodontitis had significantly lower salivary antioxidant capacities compared with the healthy subjects during the ovulatory phase ($p = 0.037$). The salivary antioxidant capacity was lower in 75.0% of the subjects with periodontitis and 50.0% of the healthy subjects during the ovulatory phase compared with the follicular phase. In contrast, we could not find a difference in the salivary antioxidant capacities of subjects with periodontitis and healthy subjects during the follicular phase (Fig. 1). However, Chapple *et al*. (48) found that the antioxidant capacity in saliva was lower in subjects with periodontitis compared with healthy subjects. Moreover, studies have reported that periodontal disease is associated with a reduced

Table 1. Changes in clinical measurements during the menstrual cycle

	Periodontitis ($n = 16$)			Healthy ($n = 12$)		
Variable	Follicular phase	Ovulatory phase	*p*-Value	Follicular phase	Ovulatory phase	*p*-Value
Bleeding on probing (%)	16.3 ± 14.3	27.3 ± 19.3	0.001	8.8 ± 10.5	11.3 ± 9.0	n.s.
Mean probing depth (mm)	2.7 ± 1.1	2.8 ± 1.0	n.s.	2.0 ± 0.4	1.9 ± 0.3	n.s.
Maximum probing depth (mm)	5.4 ± 2.2	5.3 ± 2.0	n.s.	2.8 ± 0.4	3.1 ± 0.3	n.s.
O'Leary's plaque control record (%)	45.6 ± 19.5	38.5 ± 15.7	n.s.	34.4 ± 16.9	35.1 ± 17.3	n.s.
Salivary flow rate (mL/5 min)	7.2 ± 2.5	6.7 ± 2.4	n.s.	8.0 ± 2.9	7.8 ± 3.6	n.s.

All values are expressed as the means ± SD. Wilcoxon's signed ranks test, statistically significant difference between follicular and ovulatory phase; n.s., not significant. Mann–Whitney *U*-test, statistically significant difference between follicular and ovulatory phase; $*p < 0.05$, $**p < 0.01$.

Table 2. Bacterial measurements during the menstrual cycle

Bacterial species	Follicular phase	Ovulatory phase	*p*-Value
Total bacteria			
Periodontitis ($n = 16$)	$1.7 \times 10^9 \pm 2.7 \times 10^9$	$1.9 \times 10^9 \pm 3.2 \times 10^9$	n.s.
Healthy ($n = 12$)	$1.4 \times 10^8 \pm 2.0 \times 10^8$	$2.7 \times 10^8 \pm 6.1 \times 10^8$	n.s.
Prevotella intermedia			
Periodontitis	$1.5 \times 10^6 \pm 2.4 \times 10^6$	$2.7 \times 10^6 \pm 3.0 \times 10^6$ ††	0.004
Healthy	$3.6 \times 10^5 \pm 7.6 \times 10^5$	$1.2 \times 10^5 \pm 2.2 \times 10^5$	n.s.
Porphyromonas gingivalis			
Periodontitis	$1.7 \times 10^4 \pm 4.0 \times 10^4$	$4.4 \times 10^4 \pm 1.0 \times 10^5$	n.s.
Healthy	$3.3 \times 10^3 \pm 6.2 \times 10^3$	$4.0 \times 10^3 \pm 7.4 \times 10^3$	n.s.
Prevotella intermedia (%)			
Periodontitis	$6.8 \times 10^{-1} \pm 2.2$	4.1 ± 8.2	0.017
Healthy	$3.3 \times 10^{-1} \pm 4.3 \times 10^{-1}$	$1.3 \times 10^{-1} \pm 1.8 \times 10^{-1}$ †	n.s.
Porphyromonas gingivalis (%)			
Periodontitis	$6.2 \times 10^{-2} \pm 1.7 \times 10^{-1}$	$1.3 \times 10^{-1} \pm 3.2 \times 10^{-1}$	n.s.
Healthy	$1.7 \times 10^{-2} \pm 2.9 \times 10^{-2}$	$3.1 \times 10^{-2} \pm 7.8 \times 10^{-2}$	n.s.

All values are expressed as means ± SD. Wilcoxon's signed ranks test, statistically significant difference between follicular and ovulatory phase; n.s., not significant. Mann–Whitney *U*-test, statistically significant difference between follicular and ovulatory phase; † $p < 0.05$, †† $p < 0.01$

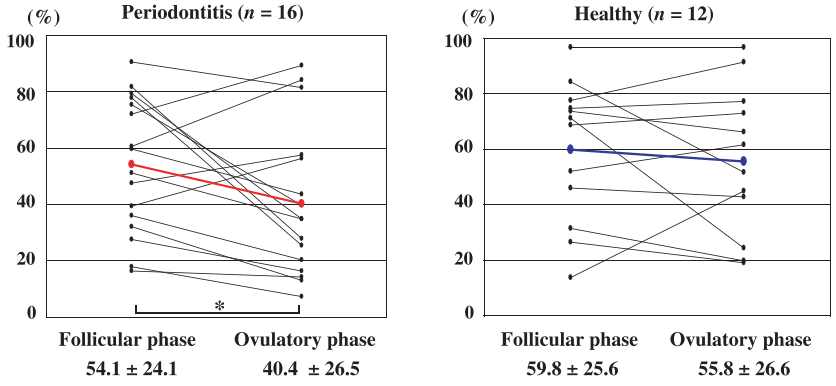

Fig. 1. Changes in salivary antioxidant capacities of healthy women and those with periodontitis during the menstrual cycle.

Table 3. Spearman correlation coefficients between salivary antioxidant capacity and parameters in all subjects during the follicular and ovulatory phases

	Bleeding on probing	Mean probing depth	Maximum probing depth	O'Leary's plaque control record	Age	Salivary flow rate	*Porphyromonas gingivalis* (%)	*Prevotella intermedia* (%)	*Porphyromonas gingivalis*	*Prevotella intermedia*	Total bacteria
Antioxidant capacity (%) during the follicular phase	0.310	−0.221	−0.098	0.200	−0.205	−0.240	−0.008	−0.097	−0.091	−0.148	−0.194
Antioxidant capacity (%) during the ovulatory phase	0.070	−0.157	−0.230	−0.020	−0.258	0.086	0.116	0.079	−0.177	−0.430*	−0.496**

*$p < 0.05$, **$p < 0.01$ were considered statistically significant.

salivary antioxidant capacity and increased oxidative damage within the oral cavity (16,18–23). The reason for the discrepancy in the present study could be due to the different assay methods used, the mode of saliva stimulation and storage, or the classification of patient groups by disease status.

There were some differences in the salivary antioxidant capacity of periodontitis subjects between the follicular and ovulatory phases. One possible reason for the decreased antioxidant capacity may be increasing salivary estrogen levels during the ovulatory phase. Some studies have indicated that the immunosuppressive actions of estrogen vary with the reproductive age of the organs (49,50). The effects of estrogen on periodontal tissues, which may relate to reducing salivary antioxidant capacity, include stimulation of phagocytosis by polymorphonuclear leukocytes and reduction of T-cell mediated inflammation (51). Moreover, Akalin *et al*. (52) found that the antioxidant capacity in gingival crevicular fluid was lower in pregnant than in nonpregnant periodontitis groups, and was lower in the periodontitis groups than in the healthy groups.

Another reason for the reduction in salivary antioxidant capacity may be that it is mediated via an increase in ROS/FRs induced by the host response to periodontal bacteria. Previous studies reported that the antioxidant capacity decreases as probing depth increases (53), and that efficient radical scavengers appear to be reduced in periodontitis (13,18). In the present study, the antioxidant capacity in all subjects during the ovulatory phase was negatively correlated with *P. intermedia* and total bacteria. We detected increasing numbers and salivary levels of *P. intermedia* during the ovulatory phase, which may be caused by rising salivary estradiol. In addition, the salivary levels of *P. intermedia* were positively correlated with those of *P. gingivalis* in all subjects (ovulatory phase, $r = 0.573$, $p = 0.002$; and follicular phase, $r = 0.477$, $p = 0.01$), although there was no correlation between the antioxidant capacity and *P. gingivalis* in all subjects. Some studies reported that periodontal bacteria would be associated with a reduced salivary antioxidant capacity (18,19). M. Takane and N. Sugano (unpublished data) found that the salivary antioxidant capacity of periodontitis patients was significantly correlated with the numbers of *P. gingivalis* ($r = -0.50$, $p < 0.05$) using the same methods as those of the present study.

Changes in the antioxidant capacity and ROS/FRs have been reported in major human disease (1). Although in many cases these changes are likely to occur as a result of the underlying disease progression, in other cases these changes are closely linked to the onset or development of the disease. The antioxidant capacity deficiency or decrease manifests itself largely through an increased susceptibility to oxidative stress, and the resulting damage is thought to be involved in diseases such as cancer, Parkinson's disease and Alzheimer's disease (17). Moreover, several studies found that a significant reduction in the salivary antioxidant capacity was related to a higher prevalence of oral cancer (54–56). In our study, it is possible that the decreased salivary antioxidant capacity during the ovulatory phase might contribute to increased damage to the gingiva and surrounding structures, although it is unclear whether the reduced antioxidant capacity is a cause or an effect of periodontal disease. To our knowledge, this was the first clinical study to assess the relationship between salivary antioxidant capacity and periodontal conditions during the menstrual cycle and to show that antioxidant capacity decreases significantly with the stage of the menstrual cycle in young adult women.

In summary, the salivary antioxidant capacity decreased, while bleeding on probing and *P. intermedia* increased, during the course of the menstrual cycle in women with periodontitis. The evidence implicating inflammatory mediators and cells in the disease process suggests that the local antioxidant status is important in determining the susceptibility to disease and its progression following initial bacterial colonization. It is also important to determine the possible contribution of diet to the salivary antioxidant status during the menstrual cycle. In the future, antioxidant supplementation may be used in the treatment or prevention of periodontal disease in young adult women.

References

1. McCord JM. Human disease, free radicals, and the oxidant/antioxidant balance. *Clin Biochem* 1993;**26:**351–357.
2. Battino M, Bullon P, Wilson M, Newman H. Oxidative injury and inflammatory periodontal disease: the challenge of antioxidants to free radicals and reactive oxygen species. *Crit Rev Oral Biol Med* 1999;**10:**458–476.
3. Chapple IL. Reactive oxygen species and antioxidants in inflammatory diseases. *J Clin Periodontol* 1997;**24:**287–296.
4. Bullon P, Morillo JM, Ramirez-Tortosa MC, Quiles JL, Newman HN, Battino M. Metabolic syndrome and periodontitis: is oxidative stress a common link? *J Dent Res* 2009;**88:**503–518.
5. Chapple IL, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol 2000* 2007;**43:**160–232.
6. Takane M, Sugano N, Ezawa T, Uchiyama T, Ito K. A marker of oxidative stress in saliva: association with periodontally-involved teeth of a hopeless prognosis. *J Oral Sci* 2005;**47:**53–57.
7. Page RC, Offenbacher S, Schroeder HE, Seymour GJ, Kornman KS. Advances in the pathogenesis of implications and future directions. *Periodontol 2000* 1997;**14:**216–248.
8. Page RC, Kornman K. The pathogenesis of human periodontitis: an introduction. *Periodontol 2000* 1997;**14:**9–11.
9. Lamont RJ, Jenkinson LRJ. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Mol Biol Rev* 1998;**62:**1244–1263.
10. Hewitt C, McCormick D. The role of cathepsin C in Papillon–Lefèvre syndrome, prepubertal periodontitis, and aggressive periodontitis. *Hum Mutat* 2004;**23:**222–228.
11. Halliwell B. Oral inflammation and reactive species: a missed opportunity? *Oral Dis* 2000;**6:**136–137.
12. Passo SA, Syed SA, Silva J. Neutrophil chemiluminescence in response to Fusobacterium nucleatum. *J Periodontal Res* 1982;**17:**604–613.
13. Canaci CF, Cicek Y, Canakci V. Reactive oxygen species and human inflammatory periodontal diseases. *Biochemisty* 2005;**70:**619–628.
14. Sculley DV, Langley-Evans SC. Salivary antioxidants and periodontal disease status. *Proc Nutr Soc* 2002;**6:**137–143.

15. Halliwell B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *Am J Med* 1991; **91:**14–22.
16. Guentsch A, Preshaw PM, Bremer-Streck S, Klinger G, Glockmann E, Sigusch BW. Lipid peroxidation and antioxidant activity in saliva of periodontitis patients: effect of smoking and periodontal treatment. *Clin Oral Invest* 2008;**12:**345–352.
17. Ballatori N, Krance M, Notenboom S, Shi S, Tieu K, Hammond C. Glutathione dysregulation and the etiology and progression of human diseases. *Biol Chem* 2009;**390:**191–214.
18. Brock GR, Butterworth CJ, Matthews JB, Chapple IL. Local and systemic total antioxidant capacity in periodontitis and health. *J Clin Periodontol* 2004;**31:**515–521.
19. Scully DV, Langley-Evans SC. Periodontal disease is associated with lower antioxidants capacity in whole saliva and evidence of increased protein oxidation. *Clin Sci* 2003;**105:**167–172.
20. Diab-Ladki R, Pellat B, Chahine R. Decrease in the total antioxidant activity of saliva in patients with patients with periodontal diseases. *Clin Oral Invest* 2003;**7:**103–107.
21. Canakci CF, Cicek Y, Yildirim A, Sezer U, Canakci V. Increased levels of 8-hydroxydeoxyguanosine and malondialdehyde and its relationship with antioxidant enzymes in saliva of periodontitis patients. *Eur J Dent* 2009;**3:**100–106.
22. Canakci V, Yildirim A, Canakci CF, Elatas A, Cicek Y. Total antioxidant capacity and antioxidant enzymes in serum, saliva, and gingival crevicular fluid of preeclamptic women with and without periodontal disease. *J Periodontol* 2007;**78:**1602–1611.
23. Baltacioglu E, Akalin FA, Alver A, Balaban F, Unsal M, Karabulut E. Total antioxidant capacity and superoxide dismutase activity levels in serum and gingival crevicular fluid in post-menopausal women with chronic periodontitis. *J Clin Periodontol* 2006;**33:**385–392.
24. Mriotti D. Sex steroid hormones and cell dynamics in the periodontium. *Clit Rev Oral Biol Med* 1994;**5:**27–53.
25. Parker M, Tabona P, Newman H, Olsen I. IL-6 expression by oral fibroblasts is regulated by androgen. *Cytokine* 1998;**10:** 613–619.
26. Hofbauer LC, Heuffelder AE. Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *J Mol Med* 2001;**79:** 243–253.
27. Kornman KS, Loesche WJ. The subgingival microbial flora during pregnancy. *J Periodont Res* 1980;**15:**111–122.
28. Jensen J, Liljemark W, Bloomquist C. The effect of female sex hormones on subgingival plaque. *J Periodontol* 1981;**52:**599–602.
29. Kornman KS, Loesche WJ. Effects of estradiol and progesterone on *Bacteroides melaninogenicus* and *Bacteroides gingivalis*. *Infect Immun* 1982;**35:**256–263.
30. Raber-Durlacher JE, van Steenbergen TJM, van der Velden U, de Graaff J, Abragam-Inpijin L. Experimental gingivalis during pregnancy and postpartum: clinical, endocrinological, and microbiological aspects. *J Clin Periodontol* 1994;**21:**549–558.
31. Yokoyama M, Hinode D, Masuda K, Yoshioka M, Grenier D. Effect of female sex hormones on *Campylobacter rectus* and human gingival fibroblast. *Oral Microbiol Immunol* 2005;**20:**239–243.
32. Lopatin DE, Kornman KS, Loesche WJ. Modulation of immunoreactivity to periodontal disease-associated microorganisms during pregnancy. *Infect Immun* 1980;**28:**713–718.
33. Lundgren D. Influence of estrogen and progesterone in vascularization of granulation tissue in preformed cavities. *Scand J Plast Reconstr Surg* 1973;**7:**1–6.
34. Lundgren D, Magnusson B, Lindhe J. Connective tissue alterations in gingivae of rats treated with estrogen and progesterone. A histologic and autoradiographic study. *Odontol Revy* 1973;**24:**49–58.
35. Ojanotko-Harri AO, Harri M-P, Hurttia HM, Sewon LA. Altered tissue metabolism of progesterone in pregnancy gingivitis and granuloma. *J Clin Periodontol* 1991;**18:**262–266.
36. Pack ARC, Thomson ME. Effects of topical and systemic folic acid supplementation on gingivitis in pregnancy. *J Clin Periodontol* 1980;**7:**402–414.
37. Sooriyamoorthy M, Gower DB. Hormonal influences on the gingival tissue: relationship to periodontal disease. *J Clin Periodontol* 1989;**16:**201–208.
38. Chatterton RT, Mateo ET, Hou N *et al*. Characteristics of salivary profiles of oestradiol and progesterone in premenopausal women. *J Endocrinol* 2005;**186:**77–84.
39. Kawamoto A, Sugano N, Motohashi M, Matsumoto S, Ito K. Relationship between oral malodor and the menstrual cycle. *J Periodont Res* 2010;**45:**681–687.
40. Chan C, Ashimoto A, Sangsurasak S, Jane F, Slots J. Oral food consumption and subgingival microorganisms: subgingival microbiota of gasatrostomy tube-fed children and healthy controls. *J Periodontol* 1997;**68:**1163–1168.
41. Koshi R, Sugano N, Orii H, Fukuda T, Ito K. Microarray analysis of nicotine-induced change in gene expression in a macrophage-like human cell line. *J Periodont Res* 2007;**42:**518–526.
42. Boutaga K, Winkeloff AJV. Periodontal pathogens: a quantitative comparison of anaerobic culture and real-time PCR. *FEMS Immunol Med Microbiol* 2005;**45:**191–199.
43. Suzuki N, Yoshida A, Nakano Y. Quantitative analysis of multi-species oral biofilms by TaqMan Real-Time PCR. *Clin Med Res* 2005;**3:**176–185.
44. Kuboniwa M, Amano A, Kimura KR *et al*. Quantitative detection of periodontal pathogens using real-time polymerase chain reaction with TaqMan probes. *Oral Microbiol Immunol* 2004;**19:**168–176.
45. Parejo I, Petrakis C, Kefalas P. A transition metal enhanced luminol chemiluminescence in the presence of a chelator. *J Pharmacol Texicol Methods* 2000;**43:** 183–190.
46. Yildiz G, Demiryürek AT. Ferrous iron-induced luminol chemiluminescence: a method for hydroxyl radical study. *J Pharmacol Toxicol Methods* 1998;**39:** 179–184.
47. Tsubaki S, Iida H, Sakamoto M, Azuma J. Microwave heating of tea residue yields polysaccharides, polyphenols, and plant biopolyester. *J Agric Food Chem* 2008;**56:** 11293–11299.
48. Chapple IL, Mason GI, Garner I, Matthews JB, Thorpe GH, Maxwell SR, Whitehead TP. Enhanced chemiluminescent assay for measuring the total antioxidant capacity of serum, saliva and crevicular fluid. *Ann Clin Biochem* 1997;**34:**412–421.
49. Nordell VL, Scarborough MM, Buchanan AK, Sohrabji F. Differential effects of estrogen in the injured forebrain of young adult and reproductive senescent animals. *Neurobiol Aging* 2003;**24:**733–743.
50. Wilson R, Spiers A, Ewan J, Johnson P, Jenkins C, Carr S. Effects of high dose oestrogen therapy on circulating inflammatory markers. *Maturitas* 2009;**62:**281–286.
51. Güncü GN, Tözüm TF, Cağlayan F. Effect of endogenous sex hormones in the periodontium – review of literature. *Aust Dent J* 2005;**50:**138–145.
52. Akalin FA, Baltacioglu E, Alver A, Krdem K. Total antioxidant capacity and superoxide disumutase activity levels fluid in pregnant women with chronic periodontitis. *J Periodontol* 2009;**80:**457–467.
53. Ellis SD, Tucci MA, Serio FG, Johnson RB. Factors for progression of periodontal disease. *J Oral Path Med* 1998;**27:**101–105.
54. Hershkovich O, Shafat I, Nagler RM. Age-related changes in salivary antioxidant profile: possible implications for oral cancer. *J Gerontol A Biol Sci* 2007;**62:**361–366.
55. Nagler RM. Altered salivary profile in heavy smokers and its possible connection to oral cancer. *Int J Biol Markers* 2007; **22:**274–280.
56. Reznick AZ, Hershkovich O, Nagler RM. Saliva – a pivotal player in the pathogenesis of oropharyngeal cancer. *Br J Cancer* 2004;**91:**111–118.