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Research Report

Deoxypyridinoline and mineral levels in gingival crevicular fluid as disorder indicators of menopausal women with periodontal disease

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ABSTRACT

Background: *Menopause is a phase of a woman's life marked by menstruation cycle cessation and an increased risk of periodontal disease. It can be caused by estrogen deficiency which alters the microenvironment in the sulcular gingival area and influences the composition and flow of gingival crevicular fluid (GCF). GCF has been widely studied as a non-invasive diagnostic and predictive tool for periodontal diseases. However, insufficient reports exist that explore its role as a predictive or diagnostic tool for bone loss detection in menopausal women.* **Purpose:** *This study aimed was to investigate deoxypyridinoline (DPD) and mineral levels that could be utilized as disorder indicators in menopausal women with periodontal disease.* **Methods:** *This study represents a form of analytical observation. Eighty-four patients of the Dental Hospital, University of Jember who fulfilled certain criteria were recruited. The subjects were divided into two main groups based on the presence of periodontal disease, (gingivitis=26; periodontitis=58) which were subsequently divided into three sub-groups based on their menopausal phase (pre-menopausal=26; perimenopausal=40; post-menopausal=18). GCF was collected using paper points from the buccal site of a posterior maxillary tooth with each subject having their GCF taken on only one occasion. DPD analysis was conducted by means of an ELISA test. The analysis of calcium, magnesium and sodium incorporated the use of an Atomic Absorption Spectroscope (AAS), while that of phosphor was by means of a spectrophotometer. Statistical analyses were performed using a comparison and correlation test (p<0.05).* **Results:** *There were significant differences in DPD and the mineral level of GCF in menopausal women with periodontal diseases (p<0.05). DPD and mineral levels showed significant correlation to those of menopausal women with periodontal diseases and a pH of GCF.* **Conclusion:** *DPD and mineral level in GCF could be used as disorder indicators in menopausal women with periodontal diseases*

Key words: menopause; periodontal disease; deoxypyridinoline; mineral; gingival crevicular fliud

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INTRODUCTION

The menopause is a phase in a woman's life marked by menstruation cycle cessation and changes in sex hormone production, the latter of which increases the risk of periodontal disease in menopausal females. Periodontal disease constitutes tooth support tissue inflammation caused by periopathogens ultimately resulting in tooth loss.[1] A previous study showed that the incidence of periodontal disease in menopausal women was higher than in sexually productive women. This might be caused by a sex hormone deficiency, especially estrogen, which plays a pivotal role in periodontal tissue remodeling and repair. A number of researchers inferred that there was a relationship between periodontitis and the menopause. However, further study is required to explain that relationship more fully.[1–6]

Estrogen deficiency is regarded as affecting the microenvironment in the sulcular gingival area, thereby

influencing the composition and flow of gingival crevicular fluid (GCF). GCF is a physiological fluid, as well as an inflammatory exudate, originating in the gingival plexus of blood vessels in the gingival corium, subjacent to the epithelium lining of the dentogingival space. GCF is composed of serum and locally generated components including: tissue breakdown products, inflammatory mediators, leukocytes, bacteria, and mineral electrolytes. Recently, GCF has been explored and extensively investigated because of its many biochemical components offering several advantages, particularly that of detecting exchanges which occur both locally or systemically in the periodontal tissue.[7,8] GCF has been widely studied as a non-invasive diagnostic and predictive tool in combating periodontal diseases. However, reports investigating the protein and mineral component of GCF as a predictive or diagnostic tool for bone loss detection in menopausal women remain insufficient in number.[1,7,8]

In this recent study, certain parameters potentially applicable as disorder indicators in menopausal women with periodontal disease were investigated. From the authors' perspective, they offer encouraging potential as an early menopause diagnostic tool in the drive to improve women's health. The parameters included deoxypyridinoline (DPD) representing a specific bone tissue breakdown product and mineral components such as: calcium, phosphor, magnesium, and sodium, constituting minerals or electrolytes active in inflammation and bone metabolism.

MATERIALS AND METHODS

This study constitutes an analytical observation approved by the Ethical Commission of the Faculty of Dentistry, Universitas Gadjah Mada. Having been recruited by the Dental Hospital, Universitas Jember, all 84 patients signed an informed concern agreement giving them the legal status of research subjects.

The participants were interviewed before completing a questionnaire regarding their menstruation cycles and menopausal symptoms. The criteria applied for participants to be accepted as study subjects included the following: female, 45 to 70 years old and systemic disease-free. Subjects were excluded from the study if they were pregnant or nursing, were smokers, regularly consumed alcohol, and had received periodontal treatment during the previous 6 months, had taken antibiotics during the previous year, regularly used mouthwash, received hormone therapy or received calcium and/or mineral therapy. The study subjects (n=84) were divided into two main periodontal disease-based groups determined according to a periodontal index (Russel's modification). The criteria of the index included the following factors: where gingivitis showed no loss attachment and pocket, but there was bleeding on probing (n=26); periodontitis showed loss attachment more than equal to 3 mm, a periodontal pocket more than 33 mm existed

and bleeding on probing occurred (n=58).[1]Thereafter, the main groups were divided into three sub-groups based on their menopausal phase: premenopausal (regular menstrual cycles in the past 3 months) or <50 years old (n=26), perimenopausal (experienced a menstrual period within the past 12 months but a missed period or irregular cycles during the past 3 months) or 50-55 years old (n=40), postmenopausal (no menstrual period during the previous 12 months) or >55 years old (n=18).[9,10]

GCF was collected between 08.00 and 13.00 because levels of DPD peak during the morning hours. GCF was taken from the buccal site of the posterior tooth infected with gingivitis or periodontitis. GCF was only taken once from each subject. Prior to GCF sampling, the tooth element had to be cleaned of saliva, blood, plaque, debris, and calculus supragingiva. GCF was absorbed using three absorbent paper points number 20. Each paper point was inserted in the buccal sulcus in a parallel position for approximately 60 seconds. It was then inserted into a 0.5 ml Eppendorf tube and sealed with paraffin tape before being placed in a deep-freezer at -300C for DPD and mineral analysis.[1]

The paper point was centrifuged at 2200 rpms for 20 minutes. The tube lip of the Eppendorf (1.5 ml) was subsequently perforated with a 30G needle and closed. After the paper point had been inserted into the tube, 50 ml 0.02 M 30G needle PBS pH 7.0-7.2 was added and incubated for five minutes. It was then centrifuged at 2200 rpms for 20 minutes, the resulting solution being collected in a 1.5 ml Eppendorf tube. Thereafter, 10 ml 0.02 M PBS pH 7.0-7.2 was added and centrifuged at 2200 rpms for 20 minutes. DPD analysis was conducted by means of an enzyme linked immunosorbent assay (ELISA) kit (Elabioscience, China). The procedures of ELISA test followed those contained in the manual booklet.[1]

For the purposes of mineral analysis, this study used two paper points, one of which was subjected to calcium, magnesium and sodium analysis by an atomic absorbance spectrophotometer (AAS), the other being subjected to phosphor analysis by a spectrophotometer. The sample preparation of GCF was the same as that for a DPD assay. However, the solution collected was added to100μL distilled water and 100 μL 0.02 M PBS pH 7.0-7.2, before being centrifuged at 2200 rpms for 20 minutes.[1,11]

All variables compared the DPD and mineral level of GCF in menopausal women with periodontal diseases using a one-way analysis of variance (ANOVA) ($p<0.05$) test. Then, the variables were analyzed by means of least significant differences (LSD) ($p<0.05$) multiple comparison. All variables were subsequently analyzed by multiple regressions and Pearson's correlation ($p<0.05$). This analysis was conducted to establish the association or correlation between variables.

RESULTS

The characteristics of 84 middle-aged women who participated as subjects in this study are shown in Table 1.

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Table 1. Descriptions of characteristic of subjects (n=84)

Variable	Gingivitis			Periodontitis			P value*
	Pre-menopause (n=6)	Peri-menopause (n=14)	Post-menopause (n=6)	Pre-menopause (n=20)	Peri-menopause (n=26)	Post-menopause (n=12)	
Age (years)	46.33 (2.73)	52.13 (2.28)	62.50 (3.73)	45.95 (2.26)	53.03 (2.58)	60 (2.27)	-
Teeth	26.83 (2.40)	26.79 (3.56)	23.33 (2.66)	26.60 (3.35)	24.38 (2.33)	21.75 (1.36)	-
PI	0.43 (0.20)	0.52 (0.16)	0.45 (0.19)	2.81 (1.29)	2.86 (0.51)	3.04 (1.67)	-
pH	6.92 (0.08)	6.82 (0.13)	6.92 (0.08)	7.21 (0.17)	7.18 (0.14)	7.24 (0.14)	0.000*
DPD (nmol/l)	61.91 (1.74)	77,28 (9,26)	72.41 (2.14)	92,17 (9,21)	144,25 (25,91)	107.31 (10,00)	0.000*
Ca (ppm)	2.42 (0.39)	4.50 (1.07)	5.51 (0.89)	3.25 (0.68)	5.32 (0.84)	5.28 (0.99)	0.000*
P (ppm)	63.83 (6.79)	67.43 (6.97)	64.17 (1.17)	69.55 (4.68)	71.81 (6.26)	67.00 (2.22)	0.003*
Mg (ppm)	2.67 (2.21)	1.68 (0.16)	1.22 (0.08)	1.64 (0.23)	0.99 (0.25)	0.96 (0.21)	0.000*
Na (ppm)	572.17 (12.35)	619.71 (14.41)	621.33 (6.65)	609.40 (16.30)	642.73 (20.42)	644.83 (24.55)	0.000*

Data was expressed as a mean (SD, standard deviation) for all variables.
P value was derived from one-way analysis of variance
* significantly different among the groups (p<0.05)
n, number of study subjects; PI, periodontal index based on Russel's modification; pH, acid base degree of GCF; DPD, deoxypyridinoline; Ca, calcium; P, phosphor; Mg, magnesium; Na, sodium.

The majority of participants who experienced periodontitis were aged approximately 49 years old. Based on intra-oral status, they had approximately 26 remaining teeth and a periodontal index of around 2.8. Moreover, the pH level of GCF participants suffering from periodontitis was just above 7 (slightly alkaline).

Table 1 show that there were significant differences between the DPD and mineral levels in the GCF of menopausal women with periodontal diseases (p<0.05). The pre-menopausal women with gingivitis registered the lowest DPD and mineral level in their GCF, with the exception of magnesium (2.67±2.21, mean±SD). The lowest magnesium level was recorded in postmenopausal women with periodontitis (0.96±0.21, mean±SD). The highest level could be seen among those women then experiencing the menopause. Premenopausal women presented the highest magnesium level (gingivitis subjects=2.67±2.21; periodontitis subjects=1.64±0.23). In contrast, perimenopausal women presented the highest level of DPD (gingivitis subjects=77.28±9.26; periodontitis subjects=144±25.91) and phosphorous (gingivitis subjects=67.43±6.47; periodontitis subjects=71.81±6.26), while postmenopausal individuals presented the highest level of calcium (gingivitis subjects=5.51±0.89; periodontitis subjects=5.28±0.11) and sodium (gingivitis subjects=621.33±6.65; periodontitis subjects=644.83±24.55).

Figure 1 illustrates multiple comparisons based on the mean difference in DPD and the mineral level of GCF between groups. With regard to the mean of the DPD level, there were significant differences between groups (p<0.05), except for menopausal women with gingivitis (p>0.05). Within the mean of the mineral level, there were varying significance values. With regard to the calcium level, there were significant differences between the groups (p<0.05), except between the postmenopausal with gingivitis to peri- and postmenopausal with periodontitis, and the perimenopausal to postmenopausal with periodontitis (p>0.05). Phosphor levels between groups showed almost no significant differences (p>0.05), except pre- and postmenopausal with periodontitis to pre- and postmenopausal with gingivitis, and gingivitis to periodontitis in perimenopausal (p<0.05). Turning to magnesium levels, almost all groups presented significant differences (p<0.05), except perimenopausal with gingivitis to premenopausal with periodontitis, and perimenopausal to postmenopausal with periodontitis (p>0.05). For sodium levels, there were significant differences between the groups (p<0.05), except perimenopausal to postmenopausal with gingivitis, premenopausal with periodontitis to peri- and postmenopausal with gingivitis, and perimenopousal to postmenopausal with peridontitis (p>0.05).

Table 2. Association between periodontal disease in menopausal women and pH to DPD level and minerals in GCF

Variable	Periodontal diseases in menopausal women[a]			pH[b]		
	β coefficient	R square	P value*	β coefficient	R square	P value*
DPD (nmol/l)	24.399	0.569[‡]	0.000*	-481.999	0.521[‡]	0.000*
Ca (ppm)	1.479	0.698[‡]	0.039*	1.042	0.077	0.242
P (ppm)	71.543	0.332[†]	0.035*	21.591	0.236[#]	0.015*
Mg (ppm)	3.174	0.634[‡]	0.000*	7.300	0.337[†]	0.001*
Na (ppm)	541.929	0.512[‡]	0.000*	381.217	0.267[#]	0.007*

[a] Multiple regression test
[b] Pearson's correlation test
β coefficient was adjusted for periodontal disease, menopause phase, pH of GCF
* significant correlation between variables (p<0.05)
[#]significant but weak correlation
[†]significant and moderate correlation
[‡]significant and strong correlation
pH, acid base degree of GCF; DPD, deoxypyridinoline; Ca, calcium; P, phosphor; Mg, magnesium; Na, sodium.

According to a correlation analysis (Table 2), a correlation existed in most of the variables between the periodontal disease of menopausal women and pH. Although the correlation power varied for each variable, the levels of DPD, calcium, magnesium, and sodium demonstrated a significant and strong correlation with menopausal women suffering from periodontal diseases (p<0.05, R2>0.5), except for phosphor which had a moderate correlation with menopausal women presenting periodontal diseases (p<0.05, R2=0.3). Moreover, DPD, phosphor, magnesium, and sodium levels demonstrated a significant correlation (p<0.05) and varying correlation power with the pH of GCF, a weak to strong correlation. However, there was correlation between the calcium level and pH of GCF (p>0.05, R2<0.1) (Table 2).

DISCUSSION

This study confirmed gingival sulcus microenvironment changes indicated by significant pH shifts in the GCF. The pH of GCF in menopausal women with periodontitis was more alkaline than that of counterparts suffering from gingivitis (Table 1). This could be due to initial inflammation in gingival triggered by acid-sensitive proteolytic bacteria colonization, such as *Porphyromonas gingivalis*, in the gingival sulcus area where the bacteria induced protein metabolism and increased the pH of the GCF. Saccharolytic bacteria, *Fusobacterium nucleatum* and *Prevotella intermedia*, are initially colonized on the surface of periodontal pocket and decrease the pH in the gingival sulcus area. The acidity is caused by the bacteria utilizing glucose in that location and producing lactic acid. There is subsequent bacteria change to asaccharolytic bacteria colonization, *Porphyromonas gingivalis*. These bacteria result in the fermentation of amino acids into organic acids generating a high pH.[8]

In the present study, the highest level of DPD was found in perimenopausal subjects with periodontitis. Moreover, the DPD level in such subjects was higher than that in subjects suffering from gingivitis. Statistically, menopausal women with gingivitis demonstrated a similar DPD level. However, menopausal women with periodontitis presented a significant difference in DPD levels. This might provoke alveolar bone loss during the menopause with the periodontitis related to inflammation in periodontal tissue exacerbating estrogen deficiency in menopausal women. Moreover, differences existed in the collagen composition between the gingiva and alveolar bone, where the gingiva is largely composed of type I, III and V, with collagen and alveolar bone and periodontal ligament being formed from type I collagen. In contrast, DPD is a collagen cross-link of bone-specific type I collagen, resulting in high levels of DPD level in menopausal women with periodontitis.[1]

With regard to GCF mineral level observation, the study showed that the levels of minerals (calcium, phosphor and sodium) in the GCF of menopausal women with gingivitis were significantly lower than in those of menopausal women with periodontitis (p<0.05). Magnesium level in the GCF of menopausal women with gingivitis was significantly higher than in their counterparts with periodontitis (p<0.05). These variations might be related to the severity of inflammation and hormonal changes in menopausal women.

The highest calcium level occurred in post-menopausal women with gingivitis, the calcium level of periodontitis was higher than gingivitis. Furthermore, the calcium level was statistically similar in perimenopausal and postmenopausal women with periodontitis (p>0.05). The calcium level in GCF in perimenopausal and postmenopausal females was higher than in their premenopausal counterparts with periodontitis and gingivitis. This suggests that the levels of steroid sex hormones and estrogen, in perimenopausal and postmenopausal individuals, are influenced to a greater extent by the calcium level in GCF than by the inflammation
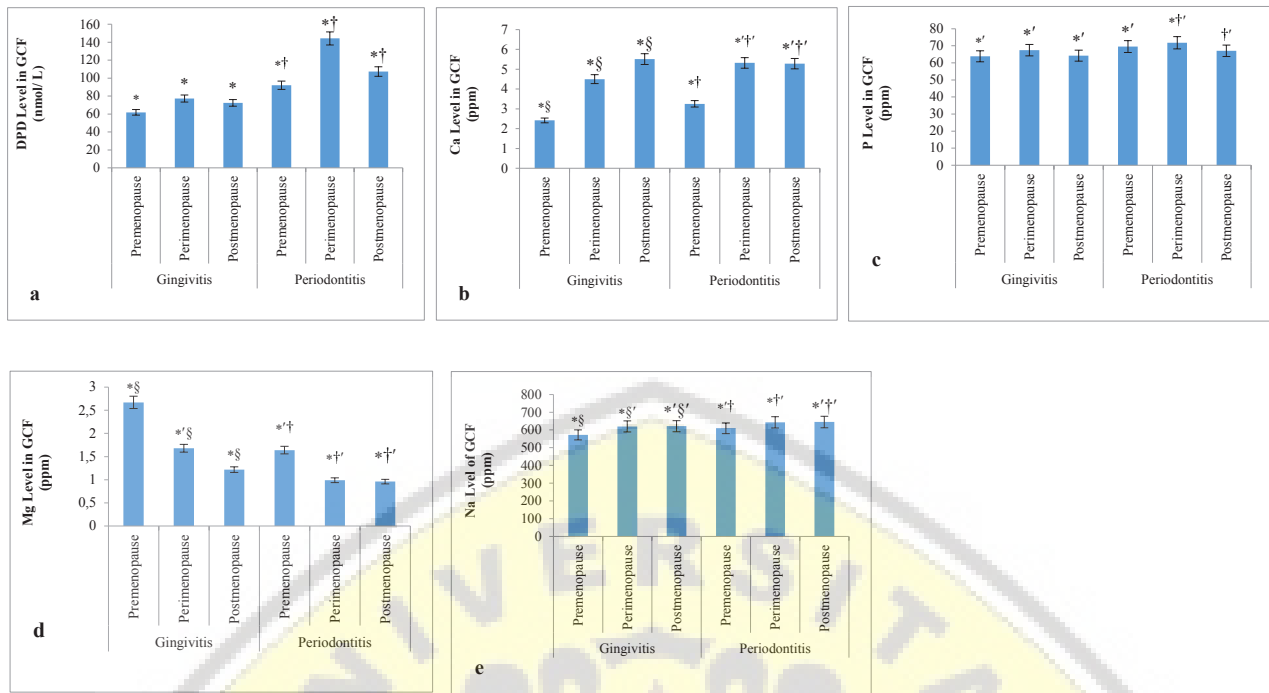
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Figure 1. Bar diagrams showing deoxypyridinoline and mineral levels in GCF and the significant differences in inter-group comparisons of: a) deoxypyridinoline levels b) calcium levels c) phosphor levels d) magnesium levels and e) sodium levels. Data presented includes: mean and standard errors and significant differences of the LSD multiple comparison test. * Significant differences in the gingivitis and periodontitis of menopausal women (p<0.05); † Significant differences in periodontitis (p<0.05) in menopausal women; § Significant differences in gingivitis (p<0.05) in menopause women; * Significant differences in gingivitis and periodontitis in menopausal women (p<0.05) except for gingivitis in perimenopausal individuals and gingivitis and periodontitis in one menopausal phase; † Significant difference in periodontitis of menopausal women (p<0.05) except in one menopausal phase; § Significant difference in the gingivitis of menopausal women (p<0.05) except in one menopausal phase.

process of periodontal disease. Moreover, estrogen levels tend to fluctuate during the perimenopausal phase before stabilizing fully in the postmenopausal phase. Calcium enhancement was associated with calcium ion mobilization from bone surface to extracellular fluid, thereby inducing bone loss. Several studies have suggested that estrogen deficiency in perimenopausal and postmenopausal women inhibited calcium absorption on bone surfaces and induced alveolar bone destruction. Inhibition of calcium absorption may cause calcium on the bone surface to be released. This calcium may subsequently be discharged into the immediate blood stream and then be excreted through the epithelium of gingival into the sulcus gingiva that is dissolved in GCF.[12,13] The amount of calcium release in GCF depends on the GCF flow during periodontitis which is higher than during gingivitis. The higher the GCF flow, the greater the calcium level in GCF.[14]

Although the study revealed no significant differences between the phosphor level of GCF in the groups (p>0.05), the phosphor level of perimenopausal women with periodontal diseases was higher than that of their pre- and postmenopausal counterparts. This could be related to calcium levels in GCF whose phosphor, in phosphate

form, iss often bound with calcium. If the calcium level in GCF increases, the phosphor level in GCF also rises. Most phosphor is located in bones and teeth, about 85% of the total within the human body, with small amounts bound with calcium, magnesium, and sodium in extracellular fluid.[15] Changes in phosphor levels can often be associated with bone and teeth disorders. However, phosphor levels in the osteoporosis serum of postmenopausal women was lower than that of their counterparts without osteoporosis.[16] Calcium and phosphate homeostasis is related to the biological effects of calcitonin in bone metabolism.[17]

In this study, the lowest magnesium level in GCF was during the post-menopause with periodontitis phase. The level of periodontitis was significantly lower than that of gingivitis in menopausal women. However, perimenopausal women with gingivitis had a statistically similar magnesium level to that of pre- and postmenopausal women with periodontitis (p>0.05). It is suggested that inflammation and estrogen deficiency could decrease the magnesium level in GCF and cause bone loss. Magnesium is one of the minerals involved in bone turnover that plays a role in bone formation.[17] Magnesium deficiency inhibits osteocalcin synthesis and secretion, subsequently inducing disruption of

calcium and electrolyte homeostasis. Estrogen deficiency-related menopause could induce inflammation.[16]Magnesium levels decreased following the inflammation and severity of periodontal disease. [18]Inflammation causes magnesium deficiency which increases the levels of P substances. P substance levels increase pro-inflammatory cytokines that induce osteoclast activation and bone loss.[19]

Sodium is contained in bone, extracellular fluid, serum and tissue. The sodium in bone constitutes a reserve source for the body and is utilized when the sodium level in serum is low in order to meet muscle tissue, heart and neural needs. Moreover, in extracellular fluid, sodium is the major cation which plays a role in cellular osmotic gradient regulation. In females, its level is affected by age-related hormone production.[20] The sodium levels in this study increased in postmenopausal women with periodontal diseases. Statistically, there were significant differences in sodium levels between the groups (p<0.05), except for perimenopausal women with gingivitis compared to postmenopausal women with gingivitis, premenopausal women with periodontitis compared to postmenopausal women with gingivitis and perimenopausal women with periodontitis compared to postmenopausal women with gingivitis (p>0.05). It was suggested that menopause-related estrogen deficiency affected sodium levels in GCF. Sodium level in the GCF of periodontitis patients was higher than in that of individuals with gingivitis. It was suggested that the change was related to GCF flow rate which is higher in cases of periodontitis compared to gingivitis. Consequently, the amount of sodium extracted from intracellular and extracellular sources due to GCF in periodontitis-afflicted patients was also higher than in their counterparts suffering from gingivitis.[14] Borras described how the sodium level in GCF correlated with loss attachment in periodontal disease with sodium in GCF passing through damaged connective and alveolar bone tissue.[21] Moreover, sodium served as a transporter of other ions, such as calcium, magnesium, and phosphor, enabling them to pass through cell membranes and the epithelium cell layer.[8]

Based on the results of the correlation test, significant correlation existed between DPD and minerals in the GCF of menopausal women with periodontal disease and the pH of GCF. However, there was no significant correlation between the calcium levels and pH of GCF. This might be explained by certain roles of calcium in GCF. Two major points of view exist regarding calcium levels in GCF. In the first, calcium enhancement is associated with calcium ion mobilization from bone surface to extracellular fluid inducing bone loss. Bone loss also occurs during the menopause because of periodontal disease. Menopause-related estrogen deficiency causes oral microenvironment changes triggering bacteria colonization on dental surface and gingival tissue. It also results in the loss of estrogen receptors on periodontal tissue that play a role in fibroblast proliferation and differentiation. Bacteria colonization and virulence-induced inflammation and host response both activate cytokine pro-inflammation and induce bone matrix degradation and bone destruction. Furthermore, the cytokines cause altered permeability in the junctional and sulcular epithelium with spaces between cells becoming wider, so that intercellular and extracellular components, such as leukocytes, electrolyte, bone degradation products and inorganic bone matrix components pass through the epithelium to the sulcus gingival area.[1,5,8]

It was also revealed that the calcium level increased in GCF owing to plaque genesis.[14] Initially, the colonization of saccharolytic bacteria on dental surfaces altered the acidity of GCF and induced inflammation. This condition promoted bacterial colonization, by saccharolytic and asaccharolytic strains, which resulted in the alkalinity of GCF. Changes in pH to alkaline induced the proliferation of anaerobic bacteria which were acid sensitive and whose products negatively affected calcium absorption resulting in calcium being excreted into GCF. Nevertheless, calcium in GCF promoted protein precipitation on enamel surfaces whose accumulation causes dental plaque and calculus.[8,14]

Furthermore, changes in the mineral component in GCF might be related with the symptoms of menopause that middle-aged women often report. In this study, perimenopausal and postmenopausal women with periodontal disease had higher calcium and phosphate levels than their pre-menopausal counterparts, although the levels found in the serum might be equal. Hypercalcemia and hyperphosphatemia in menopausal women result in cardiovascular and renal diseases.[22] These conditions might be related to estrogen deficiency inducing oxidative stress in some organs. Estrogen therapy in post-menopausal women can, therefore, reduce cardiovascular disease.[23]

While in the study reported here the magnesium level in peri- and post-menopausal women with periodontal diseases was lower than that of pre-menopausal women, it might occur in the serum of peri- and post-menopausal women. Hypomagnesia associated with estrogen deficiency and periodontal inflammation could cause reactive oxidative stress (ROS) accumulation that manifests itself in depressive syndrome.[22] Hirose *et al.*[9] argue that the depressive symptoms of middle-aged women is related to mood disorders and oxidative stress. In conclusion, the DPD and mineral level in GCF could be used as disorder indicators in menopausal women with periodontal diseases. However, this study needs further research in order to investigate the mechanism of DPD and mineral component of GCF as specific and sensitive parameters for menopausal women.

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