Pyridinoline and Deoxypyridinoline in Oral Fluids of Menopause Women as Predictor Alveolar Bone Resorption

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ABSTRACT
Menopause presents menstrual cessation that occurs physiologically and is associated with osteoporosis and periodontitis. Osteoporosis and periodontitis are silent diseases that present clinically in the late stage, which alveolar bone resorption causing tooth loss represents both of these disorders. Recently, being developed oral fluids used as predictor diagnostic tools. Pyridinoline and deoxypyridinoline is pyridinium crosslink in collagen arranging bone. Pyridinium crosslink is not re-metabolized in the body and is not influenced by food intake, so it might be a specific predictor of alveolar bone resorption. This study aimed to identify pyridinoline and deoxypyridinoline levels in oral fluids of menopausal women so that they might be used as a specific predictor of alveolar bone resorption. This research was an observational analytic study in which the subjects were menopause women who visited the dental hospital in Universitas Jember. The subjects were assessed their periodontal status and taken the oral fluids (saliva and gingival crevicular fluid). After that, LC-MS/MS analyzed the collected oral fluids. The result showed that although pyridinoline and deoxypyridinoline presented in oral fluids, the pyridinoline level in gingiva crevicular fluid (19.23±9.05) ppm and saliva (30.77±12.82) ppm was lower significantly than the deoxypyridinoline level in gingiva crevicular fluid (70.12 ± 31.01) ppm and saliva (57.48 ± 30.97) ppm. Moreover, there was a relationship between pyridinium crosslink and alveolar bone resorption, except pyridinoline in saliva to alveolar bone loss (r=-0.040 dan p=0.820). Briefly, pyridinoline and deoxypyridinoline presented in oral fluids of menopausal women and might be as the predictor of alveolar bone resorption in menopausal women.

Keywords: *Alveolar bone resorption, Deoxypyridinoline, Menopause, Oral fluid, Pyridinoline.*

1. INTRODUCTION

Menopause is one phase in women signed by the permanent cessation of ovarian function and the transition from the reproductive phase to the nonreproductive phase of life. Menopause is a critical phase for women, in which there are extraordinary physiological and psychosocial changes due to the alteration of hormonal and menstrual patterns. The hormonal alteration, estrogen deficiency, induces disturbances systemically and locally, such as in the oral cavity. These disturbances subsequently cause a decrease in the women's quality of life [1].

The elderlies frequently experience osteoporosis and periodontitis. Both of these disorders frequently impact decreasing the menopause women's quality of life. Menopause women complain about the impact of these disorders at an advanced stage because they are a silent disease without symptoms and obvious signs until fractures and tooth loss occur [2]. Our previous studies showed that menopausal women manifested poor oral health, such as poor oral hygiene, severe periodontal

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disease, bone resorption, and tooth loss. Poor oral health might cause a decrease in nutritional intake [3,4].

Early diagnosis of bone loss in menopausal women is vital to improving the quality of life due to bone fractures related to osteoporosis and mastication disturbance related to tooth loss. By early detection and diagnosis, the dentists and physicians might directly determine care management, such as prevention and treatment efforts. Although the primary standards for assessing bone quality and the condition of the tooth-supporting tissues in menopausal women necessitate DXA and x-ray, these examinations only provide partial information about bone strength and bone height. Moreover, these examinations cannot detect bone microarchitecture changes and bone molecular activities [2].

In the last decade, several studies investigated biological markers of bone turnover. The bone biological markers are substances of resulting bone remodeling processes, including bone formation, bone resorption, and bone turnover. One of the biological substances used as a marker of the bone remodeling process is the pyridinium crosslink. This crosslink is widely used as a bone resorption marker. The pyridinium crosslinks consist of N-telopeptide, C-telopeptide, pyridinoline, and deoxypyridinoline. These posttranslational covalent crosslinks create inter-chain bonds that stabilize fibril collagen molecules, especially the collagen arranging bone [5]. Some reviews explained that the concentration of pyridinoline and deoxypyridinoline in biological fluids originates primarily from the bone [2]. The bone matrix during the osteoclastic bone resorption process releases pyridinoline and deoxypyridinoline. Our previous studies revealed that C-telopeptides and deoxypyridinoline in gingival fluid increased with periodontal disease severity, and deoxypyridinoline presented higher in menopausal women with periodontitis than gingivitis [4,6]. Besides it, pyridinoline and deoxypyridinoline were identified in the saliva of menopausal women. However, these studies unclearly revealed the relationship between pyridinoline and deoxypyridinoline in oral fluids to alveolar bone loss in menopausal women.

Aforementioned, this study aimed to know the pyridinoline and deoxypyridinoline in oral fluid of menopausal women, which later were used as predictors of alveolar bone resorption. The outlook of this study explored oral fluid as the non-invasive method that contains biological substances which predict health, disease, and progression.

2. MATERIALS AND METHODS

This study was an analytical observational study approved by the Ethics Commission of the Faculty of Dentistry, Gadjah Mada University. The study population was patients who came to the Dental Hospital of Jember University in March 2018. The subjects in this study were female patients who consented to participate in this study and signed the informed consent. Then, the subjects were interviewed about their menstrual cycle status, systemic diseases, and smoking habits. Therefore, the criteria of subjects this study were: female, aged 50-70 years, no systemic disease, not pregnant or breastfeeding, not smoking, not consuming alcohol, not received periodontal treatment for less than six months, not taking medication for less than three the previous month, and not receiving hormone, calcium, or mineral therapy. The screening obtained 36 females as the subject of this study. Then they underwent intra-oral and X-rays examination to determine the periodontal index (Russel's modification) [4,7].

Saliva and gingival fluid were collected between 08.00 and 13.00 due to peak pyridinium crosslink in the morning. Before saliva and gingival fluid collection, subjects had to clean and use dental floss and abstain from eating and drinking for 2 hours. The duration of saliva collection is about 5 minutes and without stimulation [8].

Gingival fluid was taken from the buccal side of a posterior tooth infected with periodontitis. Gingival was only taken once from each subject. Before gingival sampling, the dental elements were cleaned of saliva, blood, plaque, debris, and supragingival calculus. Gingival fluid was absorbed using three paper point absorbers number #20. Each paper point was inserted into the buccal sulcus in a parallel position for approximately 60 seconds. Then put into 0.5 ml of Eppendorf tubes and sealed with paraffin tape before placing in the deep-freezer at -300C for pyridinium crosslink analysis, then centrifuged at 2200 rpm for 20 minutes. The gingival fluid filtrate was put into an Eppendorf tube, added 10 ml of 0.02 M PBS pH 7.0-7.2, and incubated for five minutes. Then centrifuged at 2200 rpm for 20 min, the resulting solution was collected in 1.5 ml Eppendorf tubes [4].

Pyridinium crosslink was measured by Micromass Quattro II tandem quadrupole mass spectrometer (LC-MS/MS) connected by MassLynx version 3.4 operating software. Primary and daughter ions were determined from the direct infusion of compounds to analyze it. The ion transition of pyridinoline and deoxypyridinoline was detected with molecule weights 429 and 413, respectively. The sensitivity of every compound was optimized by using several voltages and energy with multiple-reaction monitoring (MRM) mode [8].

The voltage used to detect was 48 V, 36 eV. Liquid chromatography system (Perkin-Elmer (Norwalk, CT)

Series-200 auto-sample) and pump (flow rate 0.2 ml/min). The mobile phase used acetonitrile 0.1% (E-Merck), acetic acid (10/90, v/v), and aquabidest. The retention time of chromatography was minutes sixth and ninth [8].

Before saliva and gingival were detected pyridinium crosslink level, standard pyridinium crosslink had to be prepared and determined. The standard solution of pyridinium crosslink (Quidel, San Diego, CA92121, USA) contained 6.20 ppm of pyridinoline and 2.28 ppm of deoxypyridinoline. The standard solution was taken 50 µl and eluted serially in 0.5% acetic acid to be 1 mL of 311 ng/mL of pyridinoline and 114 ng/mL deoxypyridinoline equally, as standard solution A. For standard solution B, the standard solution was taken 75 µl and eluted to be 1 mL of 311 ng/mL of pyridinoline and 216 ng/mL deoxypyridinoline. Injection volume was started from 1 µL to 12 µL [8].

500 µL saliva and gingival fluid were eluted aquabidest to be 1 mL; then, the solution was filtered by nylon filter 0.2 µM. All samples were taken into auto-analyzer LC-MS/MS with injection volume 0.1 µL to 10 µL. Pyridinium crosslink was measured following the standard and determined by the crosslink per mol collagen [8]. All of the data was subsequently analyzed by independent t-test and Pearson correlation ($p<0.05$).

3. RESULT

Table 1 showed the characteristics of the subjects who participated in this study. These characteristics described age, periodontal tissues, and biological markers in oral fluids. Women who participated in this study were postmenopausal women aged 55 years. Probing depth and periodontal index represented the status of the periodontal tissues, especially the alveolar bone as tooth-supporting tissue. The status of periodontal tissues indicated that the average probing depth was more than 3 mm (3.57±0.53) and the periodontal index was 2.17. The probing depth and periodontal index indicated periodontal tissue destruction and alveolar bone resorption. The biologic markers level in oral fluids showed that the average biological markers in saliva (pyridinoline=30.77 ± 12.82; deoxypyridinoline=57.48 ± 30.97) were higher than in gingival fluid (pyridinoline=19.23 ± 9.05; deoxypyridinoline=70.12 ± 31.01).

The oral fluid consisting of saliva and gingival fluid (gingiva-mucosal transudate) contains biological substances that can predict health and diseases. This study used pyridinoline and deoxypyridinoline in oral fluids as predictors of alveolar bone resorption in menopausal women. The results showed a relationship between biologic markers in oral fluid and alveolar bone resorption, except pyridinoline in saliva and alveolar bone resorption($r=-0.040$ and $p=0.820$). This recent study revealed a strong and significant relationship between deoxypyridinoline in oral fluid and alveolar bone resorption ($r=0.412$, $p=0.014$ in saliva; $r=0.445$, $p=0.007$ in gingival fluid). Therefore, the levels of pyridinoline in the gingival fluid were weak correlated ($0.2 < r < 0.3$) and not significant to alveolar bone resorption ($p> 0.05$), even the levels in saliva did not correlate with alveolar bone resorption ($r < 0.2$) (Table 2).

Table 1. Description of the subject characteristics (n=36)

Variables	
Age	55.97 ± 3.40[a]
Probing Depth (mm)	3.57 ± 0.53[a]
Periodontal index	2.17 ± 0.71[a]
Salivary indicator	
Pyridinoline level (ppm)	30.77 ± 12.82[a]
Deoxypyridinoline level (ppm)	57.48 ± 30.97[a]
Gingival fluid indicator	
Pyridinoline level (ppm)	19.23 ± 9.05[a]
Deoxypyridinoline level (ppm)	70.12 ± 31.01[a]
Interview Result	
Presence menopause syndrome[b]	
• Yes	9
• No	27
Menstrual cycle cessation (month)	
• Less than 6 months	2
• 6-12 months	9
• 13-18 months	1
• 19-24 months	13
• More than 24 months	11

Pyridinoline and deoxypyridinoline represent mature nonreducible pyridinium crosslink collagen. The table revealed that the pyridinoline level in the oral fluid of menopausal women was significantly lower than the deoxypyridinoline level ($p<0.05$). Based on the levels in oral fluid, salivary pyridinoline levels were significantly higher than in the gingival fluid of postmenopausal women ($p<0.05$). Interestingly, although the deoxypyridinoline level in the gingival fluid of

menopausal women was higher than in the saliva of menopausal women, the level of deoxypyridinoline in saliva was statistically the same as that in the gingival fluid of menopausal women (p>0.05).

Table 2. The relationship between oral fluid and the alveolar bone loss of menopausal women (based on age of respondents)

Variables	Periodontal index	
	Pearson correlation	P value
Salivary indicator		
Pyridinoline level (ppm)	-0.040‖	0.820
Deoxypyridinoline level (ppm)	0.412‡	0.014*
Gingival fluid indicator		
Pyridinoline level (ppm)	0.202§	0.244
Deoxypyridinoline level (ppm)	0.445‡	0.007*

4. DISCUSSION

This recent study revealed a strong and significant relationship between deoxypyridinoline levels in oral fluid and alveolar bone resorption. The periodontal index assessed alveolar bone resorption, which measured the presence of attachment loss and alveolar bone resorption both clinically and radiographically—the greater the periodontal index, the higher the deoxypyridinoline level in the oral fluid. The high level of deoxypyridinoline in oral fluid in menopausal women reflected the high severity of alveolar bone resorption either locally or systemically. Locally, increased deoxypyridinoline levels in oral fluids resulted from increased alveolar bone resorption in periodontitis in menopausal women. Meanwhile, systemically, deoxypyridinoline in oral fluids was due to increased bone loss caused by osteoporosis in menopausal women. We all know that osteoporosis changes bone microarchitecture systemically by the increase in osteoclastogenesis (bone loss) [9,10].

Estrogen deficiency in menopausal women triggers bone loss and causes osteoporosis. Osteoporosis magnifies the risk and worsens the periodontal disease. Clinically, menopausal women experience significant attachment loss and alveolar bone resorption. Moreover, estrogen deficiency in menopausal women causes host susceptibility to periodontal pathogens, thereby increasing the severity of periodontal disease and impacting alveolar bone loss [11,12].

Interestingly, although pyridinoline is the same as deoxypyridinoline, a pyridinium crosslink that stabilizes collagen molecules, especially collagen in bone, there was a weak relationship in this study even no relationship between pyridinoline levels and alveolar bone relationship. The concentration of pyridinoline in the bone matrix might be insignificant so that the concentration in the oral fluid was also low. This study proved that pyridinoline levels were significantly lower than deoxypyridinoline in the oral fluids of menopausal women. Kline *et al.* stated that the pyridinoline level in bone was insignificant. Moreover, Tsung Rong Kuo stated that pyridinoline was found in many tissues, such as cartilage, ligaments, bones, and blood vessels so that pyridinoline was not specific to identify bone resorption activity [2].

Moreover, the subjects in this study experienced osteoporosis without arthritis systemically, and locally the alveolar bone resorption in subjects might not be followed by other tissue destruction. These two conditions might cause low levels of pyridinoline and did not correlate with bone loss. Arthritis conditions accompanying osteoporosis might destroy cartilage tissue and degrade type I and II collagen fiber, the predominant cartilage composition. Therefore, the alveolar bone loss followed by attachment loss provoked destruction to the gingiva and periodontal ligament structure. Gingiva and periodontal tissue are composed of collagen types I, III, and V [6]. Chao wang stated that the pyridinoline crosslink plays a vital role in regulating the type III collagen fibrils structure and the biomechanics of the meniscus and cartilage predominantly composed of type II collagen [13].

Although pyridinoline was not associated with alveolar bone resorption, it was significantly more in saliva than gingival fluid. The pyridinoline in saliva might have originated from collagen breakdown in the periodontal tissues and other tissues such as the temporomandibular joint or masticatory mucosa. The degradation products might be excreted either in the serum or the saliva. Vandilson concluded that biological substances in saliva were related to serum biomarkers, in

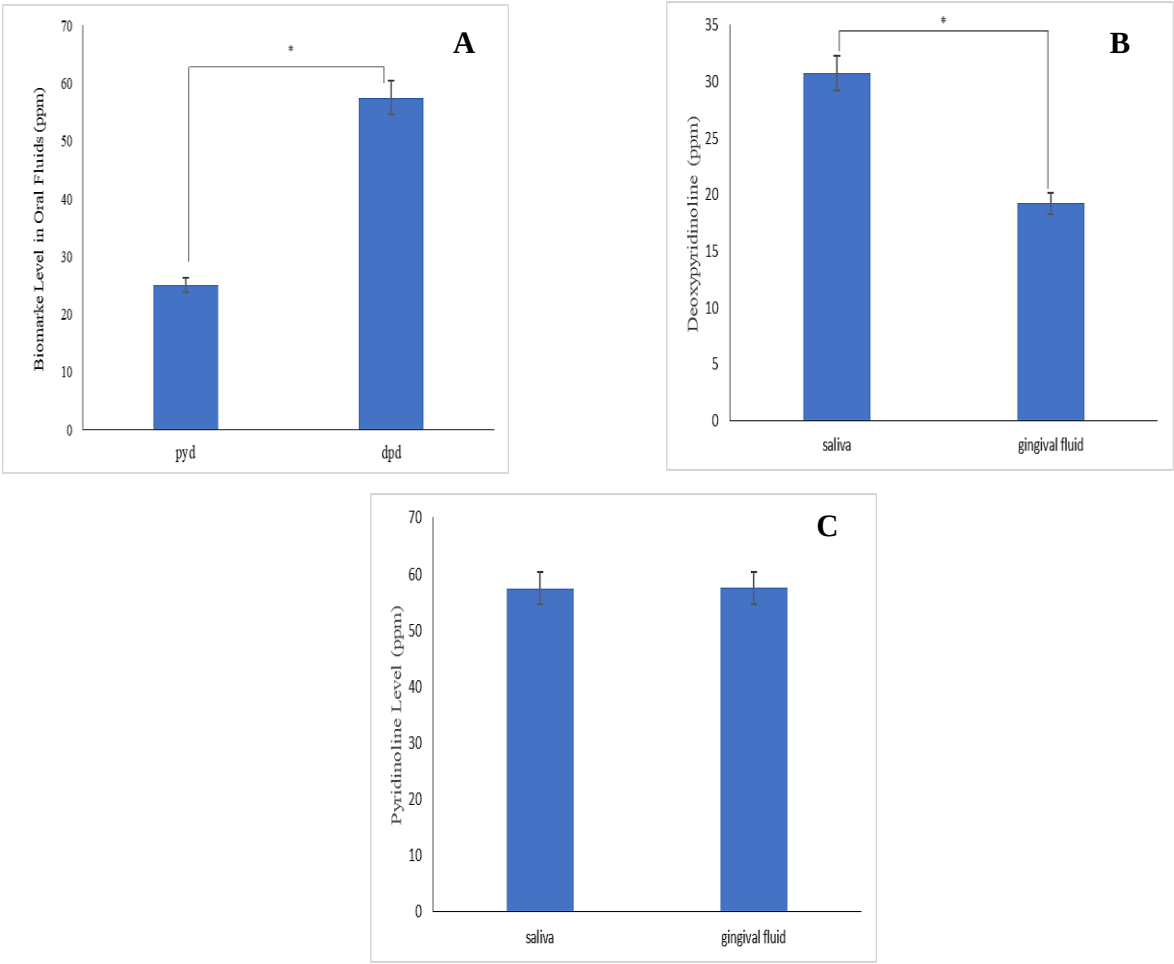

Figure 1. Bar chart of differences in levels of pyridinoline and deoxypyridinoline in postmenopausal women. A. levels of pyridinoline and deoxypyridinoline in oral fluid Menopausal women; B. pyridinoline levels in saliva and gingival fluid Menopausal women; C. levels of deoxypyridinoline in saliva and gingival fluid Menopausal women.The data in the bar chart shows the mean, standard error, and significance of differences which were analyzed using the independent t-test ($p<0.05$)

which their concentrations reflected their concentrations in serum [14]. However, the concentration in saliva was more insignificant than in serum [15]. In gingival fluid, pyridinoline probably only originated from the breakdown of type I collagen, which arranges the alveolar bone, which we know is insignificant in bone [5].

Contradiction to pyridinoline, the deoxypyridinoline level in the gingival fluid was higher than in the saliva of menopausal women, although the level in saliva was statistically the same as in the gingival fluid of menopausal women ($p>0.05$). It might be that menopausal women in the study experienced bone loss due to osteoporosis, periodontitis, or both. These conditions triggered type I collagen fibril degradation, especially deoxypyridinoline. Our previous study showed that deoxypyridinoline levels increased along with the menopause phase and the severity of periodontal disease, in which menopausal women with periodontitis expressed higher deoxypyridinoline levels than premenopausal women. Likewise, menopausal women with gingivitis indicated higher deoxypyridinoline levels than premenopausal women with gingivitis [4].

Menopausal women in this study might experience bone loss systemically due to estrogen deficiency. The estrogen deficiency disturbs bone remodeling and the immune system. Bone remodeling disturbance stimulates bone loss and impacts bone microarchitecture alteration. The bone loss and bone microarchitecture alteration affect osteoporosis in the long bones and the mandibular bone, including the alveolar bone (the bone that supports the teeth). At the same time, the disturbance of the

immune system in menopausal women causes susceptibility host to periodontal pathogens and triggers periodontal disease. The susceptibility and osteoporosis due to estrogen deficiency enhance osteoclastogenesis. Then, osteoclastogenesis triggers bone loss, either marked by a decrease in the alveolar bone height or alveolar bone loss [5,10]. An inflammatory process initiates both alveolar bone height reduction and bone loss, which activates cytokines pro-inflammatory and the MMP enzyme, triggering collagen degradation, particularly type 1 collagen as the primary collagen that makes up bones [16]. Type 1 collagen fibrils are bound by pyridinium crosslinks, one of which is pyridinoline and deoxypyridinoline. These collagen fibril crosslinks will also be degraded simultaneously with collagen degradation. The degradation by-products are excreted in the free form (not bound to other proteins) into the serum, then excreted through the urine. Although excreted in the urine, these degradation products are also found in oral fluid, both in saliva and gingival fluid [17,18].

However, this study presented limitations, in which this study was cross-sectional, so we could not generalize the results to the global population of menopausal women. Then, this study could not explore the cause-and-effect relationship. Moreover, we did not track other factors such as oral habits and the other confounding factors. However, our research analysis has the strength of being population-based and using standard data collection methods.

Briefly, only deoxypyridinoline presented in oral fluids of menopausal women and might be used as the predictor of alveolar bone resorption in menopausal women. However, this study needed further studies to explore the advantages this marker and the sensitivity.

AUTHORS' CONTRIBUTIONS

Agustin Wulan Suci Dharmayanti conducted the study and wrote the paper. Hendy Hendarto supervised the study, wrote the paper and reviewed the paper.

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