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ORIGINAL ARTICLE

Salivary bone turnover markers in healthy pre- and postmenopausal women: daily and seasonal rhythm

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Abstract No studies had investigated circadian and circannual rhythms of bone biomarkers in whole saliva. We evaluated the salivary daily and seasonal rhythm of carboxy-terminal telopeptide of type I collagen (CTX) and bone alkaline phosphatase (b-ALP). Forty clinical and oral healthy ambulatory pre- and postmenopausal women from two southern Argentine cities: Comodoro Rivadavia (latitude 45º S) and Ushuaia (latitude 54º S) were included in the study. CTX levels were evaluated in serum, urine, and saliva, and b-ALP levels were measured in serum and saliva. In both groups of women, salivary CTX showed a maximum percentage of change early in the morning (80%) and a minimum in the late afternoon (45%), similarly to the pattern observed in urinary samples. No daily rhythm was observed in serum or salivary b-ALP. 25-Hydroxyvitamin D levels decreased in winter vs. summer ($p<0.01$) without differences between the two studied groups. Conversely, parathormone reached higher levels in winter ($p<0.05$) which induced a slight non-significant increment in salivary CTX and b-ALP levels. The results showed that, as in serum and urinary samples, salivary CTX exhibits daily and a slight seasonal rhythmicity. Whole non-stimulated saliva is a useful tool to detect several oral and systemic diseases because it has important advantages compared to serum and urinary samples. Then, it may also be a promising sample to test changes in bone metabolism contributing to diagnose and to monitor the therapy of several metabolic bone diseases.

Keywords Bone markers · Daily rhythm · Seasonal rhythm · Saliva

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Introduction

Bone remodeling occurs in the entire skeleton at small cellular packets called, basic multicellular unit (BMU) [1] where osteoclasts resorbed old bone and osteoblasts replaced it with new bone. Millions of BMU are constantly remodeling bone, and the total skeleton bone cells activity can be biochemically evaluated by osteoblastic bone formation and osteoclastic bone resorption markers.

All increment in the rate of bone remodeling is associated with loss of bone mass and architectural

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deteriorations of bone tissue because bone resorption exceeds bone formation. The most important factor contributing to increase bone remodeling is loss of gonadal function. Indeed, bone turnover is increased in postmenopausal compared with premenopausal women as a result of estrogen deficiency; and bone remodeling markers have been clinically used to assess such increment. Normal values of bone markers are those seen in young, healthy premenopausal women and all postmenopausal with a value above the upper limit of the premenopausal reference range should be investigated to rule out a rapid bone loss [2].

The carboxy-terminal telopeptide of type I collagen (CTX) is widely and currently used as a sensitive and specific bone resorption marker to detect increases in bone resorption related to estrogen withdrawal and other metabolic bone diseases [3, 4]. The levels of CTX can be quantified in serum and urine by specific immunoassays [3, 5]. Bone alkaline phosphatase (b-ALP) is the enzyme isoform of total ALP synthesized by the osteoblast. The serum level of b-ALP is a sensitive bone formation marker to evaluate changes in estrogenic status and in monitoring the effect of bisphosphonate (BPs) treatment [6].

Bone remodeling is typically assessed by measuring biochemical bone markers in serum and/or urine samples [7]; however, whole saliva has recently gained significant recognition as a biologic sample for the detection of several oral and systemic diseases [8]. Salivary b-ALP and CTX can be evaluated in both human and rats [9]. Both markers showed a similar pattern of change to those evaluated in serum under normal, increased, or diminished conditions of systemic bone turnover [9, 10].

Bone remodeling has daily and seasonal rhythm. The 24-h rhythmicity of bone formation markers is dependent on serum cortisol daily fluctuations [11, 12] and although the cyclical change of bone resorption markers has not been defined yet, the serum parathormone (PTH) levels may regulate its amplitude [13]. Seasonal rhythm of bone turnover in southern regions is dependent on vitamin D status [14, 15].

To date, according to our knowledge, there are no studies that investigated the physiological circadian and circannual rhythms of biomarkers of bone remodeling in saliva. On these bases, the aim of the present study was to evaluate the daily and the seasonal rhythm of salivary CTX and b-ALP from pre- and postmenopausal women.

Material and methods

Subjects

Forty healthy volunteers, 20 premenopausal women (aged 33± 10 years) and 20 postmenopausal women (58±5 years) from two southern Argentinian cities: Comodoro Rivadavia (latitude 45º S) and Ushuaia (latitude 54º S) were studied. The selected subjects did not travel outside their city of residence within the year prior to enrollment and during the study period.

Studied women were caucasic, healthy, and had no history of bone fractures. No subject suffered from any diseases or disorders known to influence calcium metabolism, they did not take dietary supplements or medications known to affect bone homeostasis (i.e., oral corticosteroids, fluoride, vitamin D, calcitonin, or bisphosphonates). In order to avoid saliva samples contamination, women did not have history of intraoral surgery within the 6 months prior to the study; they did not undergo dental prophylaxis or any other dental procedure within the past 4 weeks and did not present blood disorders associated with enhanced bleeding or gingival disease.

Premenopausal women had regular menses, and none of them were taking contraceptive pills. Postmenopausal status was defined by the absence of menstrual period for 1 year and confirmed by measuring the circulating levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2). Women presenting FSH and LH levels above 30 IU/l, and E2 values below 10 ng/ml were considered postmenopausal at the time of the study. All subjects underwent initial interviews in which food frequency questionnaires were filled out to ensure adequate intake of calcium and vitamin D. An oral examination was carried on to ensure the absence of periodontal disease.

This study was made in accordance with the current revision of the Helsinki Declaration, and the protocol was approved by the Ethics Committee of the Clinical Hospital School of Medicine, Buenos Aires University. Written informed consent was obtained from all participants before the study.

Protocols design

Daily rhythm

All subjects were ambulatory maintaining their routine activities. According to the known daily variations of bone markers, a peak between 0300 and 0800 hours and a nadir between 1400 and 2000 hours; and to confirm daily change, blood was taken at three different times of the day: fasting between 0800 and 0830 hours, 1500 and 1530 hours, and 1700 and 1730 hours [16]. Conversely, urine and non-stimulated saliva samples were obtained during a 24-h period every 4 h, starting at 0800 hours, recording time of collection. Non-stimulated saliva was obtained under a standardized procedure. Subjects were instructed to avoid the intake of foods and beverages, lipstick, and brushing their teeth. The whole saliva was collected after rinsing their mouth with distillate water during a period of 5 min allowing it to drain out between lips into a test plastic tube which was fitted with a funnel placed near the mouth.

Levels of b-ALP were measured in serum and saliva and levels of CTX were evaluated in every biological sample.

Seasonal rhythm

The biological samples were obtained from each pre- and postmenopausal woman in summer, between the 15th of February and 15th of March when sunshine prevails; and in winter, between the end of August and the 15th of September when sunshine is minimal in the two studied cities. Fasting blood, non-stimulated whole saliva, and the 2nd void fasting urine samples were taken between 0830 and 0930 hours. Biochemical determination included serum calcium (Ca), phosphorus (P), 25-hydroxyvitamin D (25OHD), PTH, b-ALP, and CTX; salivary b-ALP, and CTX and urinary CTX.

Laboratory measurements

Blood was centrifuged to obtain serum samples. Saliva was centrifuged at 25,000×*g* for 5 min to separate cells and large macromolecules and the supernatant was retained for analysis. All saliva samples were assayed for blood contamination using a transferring enzyme immunoassay kit (Wiener SA., Argentine). Serum, saliva, and urine were stored at −20°C till analysis was performed. All biochemical determinations were done in duplicate, in the same assay to minimize interassay variation.

Serum 25OHD levels were assessed using the I^{125} radioimmunometric method (Diasorin Inc., Minnesota, USA). Mid-molecular PTH was measured by RIA, employing an antiserum that recognizes intact hormone as well as mid-molecular and carboxy-terminal fragments [17].

The levels of b-ALP were measured using a colorimetric method as previously described after bone isoenzyme precipitation with wheat-germ lectin [17]. Intra-assay CV was 6%. Levels of CTX were measured by an enzyme-linked immune-absorbent assay (ELISA; Nordic Bioscience Diagnostics A/S) with an intra-assay CV of 6%.

Statistical analysis

Data were expressed as mean±SEM. The results between the two cities were compared using unpaired *t* test and between the two season using a paired *t* test for both pre- and postmenopausal women. Statistical analysis of daily variation and differences between pre- and postmenopausal women were performed by a two-way factorial analysis of variance (ANOVA). A one-way ANOVA followed by Bonferroni's multiple comparisons tests was employed to assess the significance among time points. The validity of the linear model was assessed by residual analysis and Shapiro Wilk's and Bartlett's tests. The statistical analysis was performed with SPSS (2006, SPSS Inc., Chicago, IL, USA), and $p<0.05$ was considered statistically significant.

Results

Daily rhythm of bone markers

The biochemical data of bone formation and bone resorption markers showed no statistical differences in the daily rhythm between the two studied groups of women living in Ushuaia and Cro. Rivadavia. Thus, the data were presented as a combined sample.

As expected, there was a significant difference among the mean absolute serum levels of CTX at the three studied times: 0930, 1330, and 1730 hours ($p<0.05$; Fig. 1). In both groups of women, the lowest serum CTX levels were observed between 1330 and 1730 hours. In addition, CTX levels at each time were significantly lower in pre than in postmenopausal women ($p<0.01$).

In order to compare the relative changes in the 24-h profile of urinary or salivary CTX excretion, results were expressed as % of change mean 24 h values (mensor; Figs. 2 and 3). As expected both, the levels of urinary CTX of premenopausal and postmenopausal women showed a maximum percentage of change in the early morning (80%) and a minimum at late afternoon (45%). However, no significant differences were found in the daily rhythm of urinary excretion of CTX between the two groups of women. The factorial ANOVA indicated significant effects of time of day ($F=36.83$, $p<0.00001$); however, there were no significant interaction "time of day × group" ($F=1.84$, pns.) or "between groups" ($F=0.201$, pns.; Fig. 2). CTX levels daily rhythm in saliva showed a similar pattern to those observed in urine in both groups of women (Fig. 3). The factorial ANOVA indicated significant effects of time of day ($F=13.47$, $p<0.00001$); however, there were no

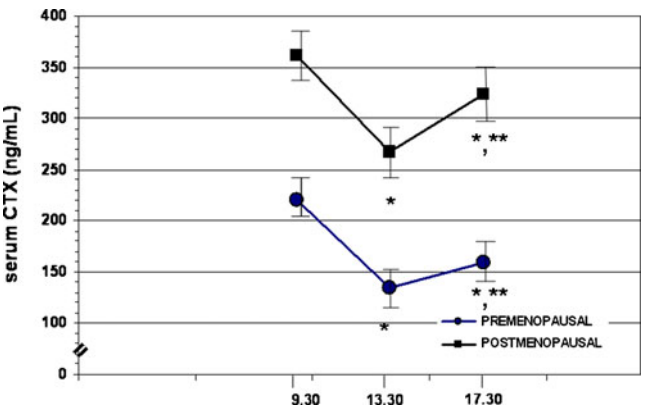

Fig. 1 Pre- and postmenopausal mean levels of serum CTX (ng/dl) at the three studied times. *$p<0.05$ compared to 0930 hours; **$p<0.05$ compared to 1330 hours

significant interaction "time of day × group" (F=1.28, pns.) or "between groups" (F=0.031, pns.). It is important to point out that there was a significant reduction in 24-h mean salivary CTX levels and rhythm amplitude compared to urine CTX sample. In this regard, the mean salivary CTX levels were four times lower than the mean urinary CTX levels, both in pre- and postmenopausal women. In addition, salivary CTX maximum value presented a variation of approximately 40% and 20% and a minimum variation of 22% and 15% for pre- and postmenopausal women, respectively (Fig. 3), while the maximum value of urinary CTX level was 80% for both women and the minimum variation was 40% and 50% for pre- and postmenopausal women, respectively (Fig. 2).

No daily rhythm was observed in b-ALP levels in both serum and saliva of pre- and postmenopausal women.

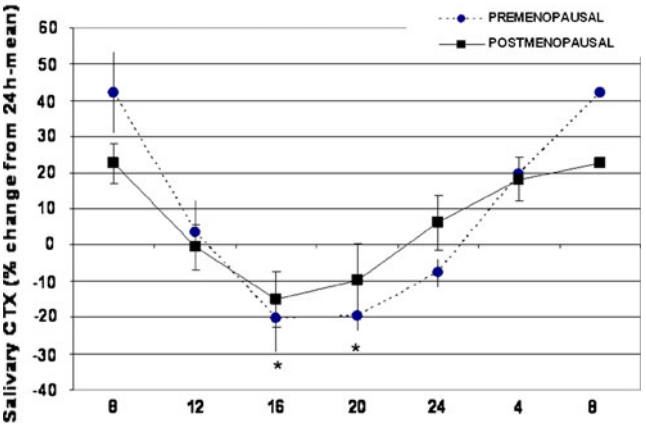

Fig. 3 Pre- and postmenopausal relative changes in the 24-h profile of salivary CTX excretion. Data were expressed as % of change mean 24 h values (mensor). *p<0.05 compared to 0800 hours

CTX seasonal rhythm

The seasonal data of the studied parameters are shown in Table 1. Both groups of women showed a significant decreased of 25OHD levels in winter as compared to summer levels (p<0.01) without differences between pre- and postmenopausal subjects. The levels of 25OHD at the end of winter time were, independently of estrogenic status, lower than 30 ng/ml in all studied women and only four postmenopausal and six premenopausal women reached levels of 25OHD above 30 ng/ml at the end of the summer period. In addition, five pre- and five postmenopausal had 25OHD levels lower than 10 ng/ml during winter time and one postmenopausal woman during summer.

Serum PTH levels showed significant differences between the two seasons with higher levels in winter than in summer for the two groups of women (p<0.05). The PTH levels during summer were higher in postmenopausal than in premenopausal women without reaching significance (p=0.061). Serum Ca levels did not present seasonal or groups differences. Conversely, serum P levels tended to be higher in both groups of women in summer (p=0.058) in agreement with the decrease in PTH levels, without reaching differences between groups.

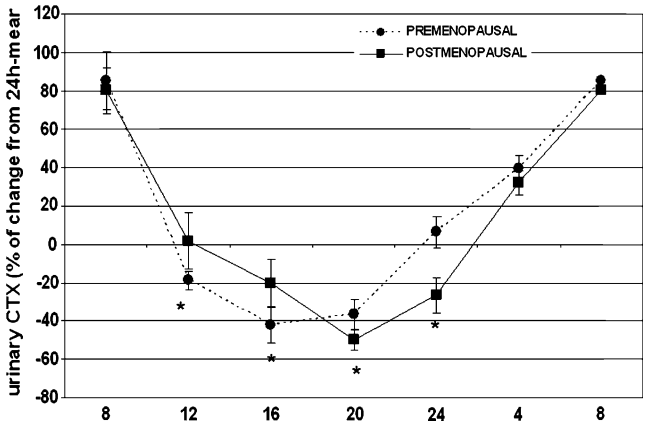

Fig. 2 Pre- and postmenopausal relative changes in the 24-h profile of urinary CTX excretion. Data were expressed as % of change mean 24 h values (mensor). *p<0.05 compared to 0800 hours

The CTX levels in the three biological samples were significantly higher in post than in premenopausal women in both seasons (p<0.05). A slight no significant diminution in CTX levels measured in the three biological samples was found in summer compared to winter in both groups of women without reaching significance. A similar pattern was found for serum and salivary b-ALP levels.

Discussion

According to our knowledge, the present study is the first to evaluate daily and seasonal rhythmicity of bone markers in whole unstimulated saliva in healthy pre- and postmenopausal women. Indeed, the bone resorption marker CTX evaluated in saliva sample presented a similar pattern of daily rhythm than that observed in serum and urine samples. It has been shown that there was a peak in the second half of the night (between 0300 and 0700 hours) and a nadir in the late afternoon [18, 19]. Conversely, the bone formation marker b-ALP did not present daily rhythmicity. Both biomarkers of bone remodeling presented a slight seasonal variation. Of interest is also the very low 25OHD levels detected in the two seasons in all the studied women.

Changes in bone remodeling are typically assessed by measuring the levels of markers of bone turnover traditionally analyzed in serum and urinary samples. Saliva is a biofluid readily available and easily collected without specialized equipment or personnel. Measurement of several bone turnover markers in the whole non-stimulated human saliva is possible by the use of

Table 1 Pre- and postmenopausal mean values of serum, urine, and saliva biochemical determinations (mean±SD) in both seasons

	Premenopausal		Postmenopausal	
	Winter	Summer	Winter	Summer
Ca (mg/dl)	9.6±0.1a	9.5±0.1a	9.1±0.2a	9.6±0.1a
P (mg/dl)	3.3±0.31a	3.8±0.2a	3.5±0.2a	3.9±0.2a
250HD (ng/ml)	13.8±1.3a	23.9±2.5b	14.0±1.8a	25.4±2.4b
PTH (pg/ml)	55.4±5.4a	39.4±5.1b	62.1±7.5a	47.5±7.1b
Serum CTX (ng/ml)	223.1±16.8a	217.8±18.7a	332.1±31.8b	298.8.5±27.6b
Urine CTX (ug/l)	188.7±20.3a	166±14.7a	254.8±31.2b	242.5±27.8b
Salivary CTX (ug/l)	52.5±5.1a	49.3±5.5a	76.9±13.1b	69.6±6.1b
Serum b-ALP (IU/l)	61.0±0.4a	57.6±2.5a	73.4±4.8b	68.0±3.7±6.1b
Salivary b-ALP (IU/l)	54.4±2.8a	48.2±5.1a	76.2±4.5b	68.8±3.7±5.0b

Different letters indicate significant differences, $p<0.05$

commercially available ELISA tests or colorimetrical methods. Previous studies from our group [9, 10] and other authors [20] have indicated a significant correlation between salivary and urinary/serum concentrations of the biomarkers of bone turnover. Salivary and serum levels of bone formation markers have the same order of magnitude; however, in relationship with bone resorption markers in saliva presented several times lower levels than serum/urine samples [10, 21].

The C-telopeptide, CTX, is a small peptide composed by 26 amino acids which is generated when type I collagen is cleaved by cathepsin K, an osteoclastic proteinase, at the C-telopeptide region [22, 23]. It is released into blood circulation and excreted in urine without being modified. Due to its very small molecular weight, CTX can be filtered by the serum and appears in saliva where it can be measured [9, 10]. This molecule is considered the most sensitive and specific indicator of a high resorption activity. During the active resorption of bone, CTX is released into the periodontal tissues, gathers into the gingivo-crevicular fluid, and transferred to whole saliva, where it could be evaluated to assess disease activity and severity and to monitor periodontal therapy [10]. The b-ALP is an intracellular enzyme released by osteoblastic and fibroblastic activity, and its increment in salivary is probably the result of increased formation processes.

Bone turnover has a circadian rhythm in both animals and humans, with bone resorption and, to a lesser extent, bone formation increasing at night [12]. The daily rhythmicity of bone resorption marker determines the importance of standardizing sampling procedures for food intake. In fact, it is known that food intake has a strong effect on bone resorption, especially on serum CTX levels, whereas its effect on bone formation is weak or negligible. Fasting reduced (although did not abolish), the morning decrease in the serum CTX concentration [19]. Our results demonstrated that the bone resorption marker CTX assessed in saliva sample has a similar pattern of variation as those determined in serum and urinary samples. We found that the time of salivary CTX peak is similar for pre- and postmenopausal women whereas the amplitude is higher for premenopausal subject compared to postmenopausal women (maximal: 40% vs. 20%; minimal 25% vs. 15% of the average concentration, respectively). Conversely b-ALP has only slight variations and did not present rhythmicity. This lack of salivary changes during a 24-h period may be partially explained by the 2 days half-life of b-ALP when compared with that of CTX which is in the order of 1 h [24].

It is important to take into account that in the studied latitudes, cutaneous vitamin D synthesis is minimal during winter because people are covered with heavy clothing and because the sun presents a low angle at midday. Vitamin D status is defined according to the serum concentration of 25OHD [15]. Because of scarce ultraviolet-B light, short-term deprivation of vitamin D stores is observed during winter leading circannual variations in 25OHD levels. Seasonality in 25OHD levels has been extensively investigated, with most studies reporting a nadir during winter and a peak during summer and early autumn [25]. It has been also shown that bone turnover follows a circannual rhythm, with highest levels of bone turnover markers during the winter months which seem to be directly related to variations in vitamin D status. Indeed, bone turnover was highest during winter [25], coinciding with a nadir in serum 25OHD levels that lead to a rise in serum PTH [26] which is the main regulator of bone turnover.

The results of the present report demonstrated that in the two studied cities of the southern hemisphere, as in the northern hemisphere, 25OHD levels decrease, in both groups of women at the end of winter. Such changes induced an increase in PTH levels which, in turn, increased CTX and b-ALP levels in the three studied biological samples without reaching significance. However, the mean levels of these two bone turnover markers had a greater value in postmenopausal women than in premenopausal women in the two studied periods and in the three studied samples.

Non-stimulated saliva should be obtained under a standardized procedure, like it is usually done in blood

samples, to avoid the normal physiological variables such as duration of stimulation, nature of the stimulus, the method of saliva collection, and flow rate which can influence the results. As for the latter, non-stimulated salivary flow rate presents circadian and circannual variations [27, 28]. In this regard, salivary flow rate is extremely low during the sleep period and high in the afternoon, possibly influenced by changes in hormone secretion during the 24-h period. In addition, a lower flow rate is observed during the summer months probably by influencing the degree of hydration [28].

A weakness of the present study was the relatively small number of subjects in each group. However, this could not be avoided as so many subjects in the studied regions were not interested in having dental examination or several blood extractions. Also the sample was limited by the fact that subjects who live in the southern region did not always remain all the year in their cities thus were not qualified in the inclusion criteria. Nevertheless, since needle-stick procedures may not always be feasible, the whole non-stimulated saliva represents a promising sample to test changes in bone metabolism contributing to diagnose and to monitor the therapy of several metabolic bone diseases.

In summary, several conclusions can be made as follows: the present results suggest that CTX in saliva, as occurs in serum and urine samples, exhibits daily and a slight seasonal rhythmicity. In these two situations, CTX in saliva followed a similar pattern of change than the observed in serum and urine samples. Saliva could be a diagnostic method to asses bone turnover markers since it presents important advantages such as multiple non-invasive collections for obtaining samples, not needed for sharps (avoiding potential cross contamination), samples could be collected at home or in remote locations, compliance pediatric populations.

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Conflict of interest The authors declare that they have no conflict of interest.

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