Austin Journal of Dentistry

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Austin Publishing Group

Research Article

Levels of Bone Turnover in Saliva Correlated with Antirresortive Treatment: Experimental Study

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Received: October 29, 2020; **Accepted:** November 25, 2020; **Published:** December 02, 2020

Abstract

Background: Bone turnover markers are useful to evaluate bone loss and monitor anticatabolic treatments in a short period. The effectiveness of saliva to reflect changes in bone turnover markers during the early administration of different doses of olpadronate (POD) (4 mg or 8 mg/100 g body weight), immediately after Ovariectomy (OVX), was evaluated.

Material and Methods: Adult rats (n=10/group) were divided and treated as follows for 45 days: SHAM+Vh; OVX+Vh; OVX+4OPD and OVX+8OPD. To validate the model, we measure Proximal Tibia Bone Mineral Density (PTBMD) change between the beginning and the end of the study. Blood and saliva were collected post-surgery (t=0; t=10; t=20; t=45).

Results: PTBMD decrease in OVX+Vh was partially prevented by 4OPD and avoided by 8OPD. Salivary and serum β-CrossLaps (CTX) in SHAM+Vh remained without changes and increased in OVX+Vh ($p<0.05$); Bone-Specific Alkaline Phosphatase (B-ALP) increased in OVX+Vh *versus* SHAM+Vh reaching signicance at t=20 ($p< 0.05$). A dose-dependent response was observed in salivary and serum CTX and B-ALP by OPD treatment ($p<0.05$). CTX was lower in OVX+8OPD *versus* OVX+4OPD from t=10 in serum samples and from t=20 in salivary samples ($p<0.05$) while B-ALP reached lower levels at t=45 ($p<0.05$).

Conclusion: Although further clinical studies are needed, our results suggest that CTX and B-ALP in saliva decreased by Bisphosphonate (BP) therapy in a dose dependent manner. Therefore, saliva would be a suitable tool for evaluating changes in bone turnover due to BP therapy in the early stage of estrogen withdrawal.

Keywords: Bone Turnover Markers; Osteoporosis; Saliva; Estrogen Withdrawal; Bisphosphonates

Abbreviations

BMUs: Bone Remodeling Units; BPs: Bisphosphonates; OPD: Alpadronate; OVX: Ovariectomy; Vh: Vehicle; DXA: Dual Energy X-Ray Absorptiometry; BMD: Bone Mineral Density; ROI: Region of Interest; VC: Variation Coefficient; PT: Proximal Tibia; CTX: β-CrossLaps; B-ALP: Bone-Specific Alkaline Phosphatase; SD: Standard Deviation; ANOVA: One-Way Analysis of Variance; NF: Nuclear Factor; RANKL: NF-κB Ligand; RANK: Receptor Activator of NF- κB; OPG: Osteoprotegerin; amino-BPs: nitrogen-conteining BPs.

Introduction

The decline in estradiol levels that follow menopausal transition and early menopause bone is a dynamic and metabolically active tissue that is continuously remodeling by the coordinated actions of osteoclasts and osteoblasts in cellular packages called Bone Remodeling Units (BMUs) [1]. The whole process of bone remodeling is a cycle, which begins with the resorption of old bone by the action of the osteoclasts, followed by the formation of new bone tissue by osteoblasts. The resorption phase lasts approximately 12 days; however, the formation phase is a slower process lasting 3 months. As bone resorption is faster than bone formation, every increase in bone turnover is characterized by bone loss [2].

The decline in estradiol levels that follow menopausal transition and early menopause state induces a dramatic increase in bone remodeling that leads to an accelerated bone loss increasing the risk of osteopenia/osteoporosis [3].

The most commonly experimental model used to evaluate the loss of bone link to estrogen deficiency is the ovariectomized rat [4]. In this sense, it has been shown that this rats exhibits an accelerated bone turnover with a higher increase in bone resorption than bone formation, as well as, trabecular bone loss [5].

Bisphosphonates (BPs) are anticatabolic drugs widely used as the first treatment of choice to reduce the fast bone loss caused by osteopenia/osteoporosis due to their potency to suppress bone remodeling [6].

Bone remodeling levels can be assessed by measuring biochemical bone turnover markers of bone formation and resorption. Bone turnover markers are useful tool to evaluate the rate of bone loss in postmenopausal women as occur in osteoporosis and other metabolic bone diseases [7,8]. In addition, they are suitable to monitor the efficacy of an anticatabolic treatment in a short period of time. Even though bone turnover markers are usually evaluated in blood and

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Citation: Pellegrini GG, Gonzales Chaves MMS, Zeni Coronel ME, Avendaño ME, Bonanno MS and Zeni SN. Levels of Bone Turnover in Saliva Correlated with Antirresortive Treatment: Experimental Study. Austin J Dent. 2020; 7(3): 1145.

urine samples, it has been shown that they can also be measured in saliva samples [7-10].

The use of saliva as a biological sample presents many advantages due to its fast, easy and inexpensive collection. In addition, as the collection of saliva is not invasive, samples are appropriate for home use since it does not require the presence of medical personnel [11].

The present longitudinal experimental study aims to evaluate the effectiveness of saliva as compared to serum samples to reflect changes in bone turnover markers induced by the treatment with two different doses of Olpadronate (OPD) immediately after estrogen withdrawal induced by Ovariectomy (OVX).

Material and Methods

Drugs

The aminobisphosphonate OPD was kindly provided by GADOR (S.A, Argentina) as two saline solutions (3.2 or 6.4 mg/100 mL). The low and high doses of OPD were in keeping with previous experimental dose-response studies [12]. Ketamine hydrochloride (Holliday-Scott, Buenos Aires, Argentina) and acepromazine maleate (0.1 mg each/100 g body weight), were used as light anesthesia. Salivation was stimulated by intraperitoneal injection of pilocarpine 1% (Alcon Argentine laboratories) in a dose of 0.2 mg pilocarpine hydrochloride/100 g body weight. All drugs were administered intraperitoneally.

Animals and experimental design

Forty female, virgin, adult Wistar rats (250 to 300 g) were housed at room temperature (21°C ± 1°C) with 55% ± 10% humidity under 12-hour light/dark cycles; they were fed standard rodent diet (Granave, Argentina) and deionized water ad libitum. Body weight was recorded weekly. The rats were maintained in keeping with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [13].

After 1 week of acclimatization, 10 animals were SHAM surgery, and the remaining 30 rats were subjected to bilateral OVX. Two days post-surgery, the animals were divided into four groups and treated as follows for 45 days:

SHAM+Vh = SHAM+ vehicle (saline solution);

OVX+Vh = OVX+ vehicle (saline solution);

OVX+4OPD= OVX+ 4 mg OPD/100 g body weight;

OVX+8OPD = OVX+ 8 mg OPD/100 g body weight;

The study was approved by the Buenos Aires University Institutional Review Board.

Bone mineral density

To validate the experimental model, all animals were subjected to a densitometric analysis "*in vivo*" by dual energy X-Ray Absorptiometry (DXA) under light anesthesia (0.1 mg ketamine hydrochloride/100 g body weight and 0.1 mg acetopromazine maleate/100 g weight). A whole body scanner and software designed specifically for small animals (DPX Alpha, Small Animal Software, Lunar Radiation Corp, Madison WI) were used as described in a previous report [14]. Briefly, all rats were scanned using an identical scanning procedure. Precision was assessed by measuring one rat five times, repositioning between scans, on the same day and on different days [15]. To evaluate the outcomes of the treatment, Proximal Tibia (PT) Bone Mineral Density (BMD) area was analyzed on the image of the animal on the screen using a Region of Interest (ROI) for the segment. The Variation Coefficient (VC) was 3.5% for proximal tibia. The changes in The changes in PTBMD between the end (t=45) and the beginning of the experience were calculated as a percentage of the baseline (t=0) PTBMD value.

Biochemical determinations

Fasting blood and saliva samples were collected at the onset of treatment and after each 10 days until the end of the experience [t=0 (baseline); t=10; t=20 post surgery and at t=45]. Blood was obtained from the tail under ethyl ether anesthesia; the serum was separated and kept frozen at -20°C until the analyses were performed. Collection of saliva was done 5 minutes post-stimulation for 1-minute period by using a sterile syringe. Saliva was centrifuged at 3.000xg for 10 minutes to separate cells and large macromolecules. The supernatants were collected and frozen at -20°C until the studies were performed.

All biochemical determinations were performed at the same time to avoid inter-assay variations. β-Cross Laps (CTX) levels (ng/mL) were measured using an enzyme-linked immunosorbent assay (Rat Laps, Osteometer BioTech, Herlev, Denmark) with a 6% intra-assay VC and a detection limit of 2.5 ng/mL [15]. Bone-Specific Alkaline Phosphatase (B-ALP) was measured using a colorimetric method (Boehringer Mannheim, Germany) after bone enzyme isoform precipitation with wheat germ lectin [15].

Statistical analysis

Results are expressed as mean ± Standard Deviation (SD). Data was analyzed using One-Way Analysis Of Variance (ANOVA). The Bonferroni multiple comparisons test was performed when significant differences were encountered. Statistical analyses were performed using a software package. $p < 0.05$ was considered significant.

Results

Model validation

As expected, SHAM+Vh group exhibited a slight change in PTBMD, whereas animals in the OVX+Vh group lost approximately 20% of their PTBMD (Figure 1). OPD treatment decreased the loss of PTBMD in a dose-dependent manner. Indeed, treatment with 4 mg of OPD partially prevented the decrease in PTBMD while treatment with 8 mg of OPD avoided the decrease (Figure 1).

Bone turnover markers levels evaluated in saliva and serum presented a similar pattern of changes

No differences in baseline values of CTX and B-ALP in both, saliva and serum samples were observed. (Figures 2A,2B) showed the longitudinal changes in salivary and serum CTX levels. A similar pattern of change for CTX was observed in salivary and serum samples. In both biological samples, CTX levels evaluated in the SHAM+Vh group remain without changes throughout the study. However, CTX levels increased significantly in the OVX+Vh group as compared to SHAM+Vh as a function of time from t=10 ($p<0.05$). Figures 3A,3B showed the longitudinal variations in salivary and serum B-ALP levels. Both samples showed a similar pattern of change. Although a

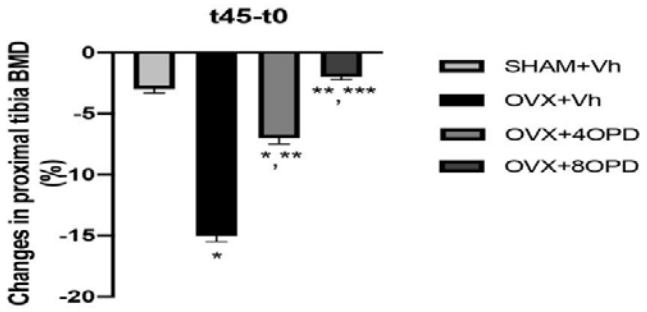

Figure 1: Changes in Proximal Tibia Bone Mineral Density (PTBMD). The percent change was calculated as PTBMD values at the end of the experiment (t=45) minus PTBMD values at the beginning of treatment (t=0). Bars represent mean ± SD (n=10 rats/group). *p<0.05 *versus* SHAM+Vh group, ** p<0.05 *versus* OVX+Vh group, ***p <0.05 *versus* OVX+4 OPD group, by one-way ANOVA and Bonferroni as a post hoc test.

trend towards higher levels in B-ALP was observed in the OVX+Vh *versus* SHAM+Vh group since the first week after the OVX surgery, these levels did not reach statistical signicance until t=20 (p< 0.05).

In agreement with the changes in PTBMD, a dose-dependent response was observed in CTX and B-ALP levels in salivary and serum samples by treatment with OPD (Figures 2,3). In both samples, CTX reached lower significant levels in OVX+4OPD, as compared to OVX+Vh group since t=20. Moreover, CTX levels in SHAM and OVX+4OPD group did not show statistical changes during the studied period. Levels of CTX in both samples were significantly lower in OVX+8OPD *versus* OVX+Vh since t=10 and *versus* SHAM group since t=20. When the two dosis of OPD were compared, the CTX levels in OVX+8OPD were significantly lower than in OVX+4OPD from t=10 in serum samples and from t=20 in salivary samples (p<0.05). Levels of B-ALP in saliva and serum decreased in OVX+4OPD group as compared to OVX+Vh group, reaching statistical significance at t=45 (p<0.05), while as compared to SHAM+Vh group these levels did not show differences througut the study (Figure 3). Salivary and serum B-ALP levels decrease in OVX+8 OPD as compared to both OVX+Vh and SHAM+Vh group since t=20 (p<0.05). When the two doses of OPD were compared, the salivary and serum B-ALP levels in OVX+8OPD reached higher significant levels than in OVX+4OPD at t=45 (p<0.05).

Discussion

Bone homeostasis is maintained by a delicate balance between bone formation and bone resorption processes. On this regard, bone remodeling maintains the health in the skeleton affected by microfractures and fatigue and, by the damage caused by stress. This process occurs at the BMUs by the couple action of osteoclasts, bone resorptive cells and osteoblasts, bone forming cells. Nonetheless, since bone resorption is faster than bone formation, any increase in bone remodeling results in a negative bone balance at each BMU leading to an increase risk of bone fractures [16].

Although the diagnosis of osteoporosis can be made by BMD using DXA, the increased concentrations of specific bone markers are associated with an accelerated bone loss [17]. Indeed, bone turnover markers provide dynamic information related to changes in bone cells activity in a short period of time, while the changes in BMD take longer to occur. For this reason, they are a predictable tool to identify patients at high risk of bone loss and subsequent fractures.

Estrogen is an important regulator of bone homeostasis since it regulates the receptor activator of Nuclear Factor (NF)-kB-ligand (RANKL), receptor activator of NF-kB (RANK), and the soluble decoy receptor Osteoprotegerin (OPG) axis [18]. During the menopausal transition, estradiol levels decline but they show an accelerated fall in the early menopause. Several cross-sectional studies indicate that the highest increase in bone turnover markers is observed during the menopausal transition and remained higher after menopause [19]. This is why menopausal transition and early menopause represent a window of opportunities to prevent the rapid bone loss and micro architectural damage to counteract osteoporosis in later years [20].

BPs are the first drug of choice to initiate an anticatabolic therapy to prevent the accelerated bone loss and preserve the integrity of bone structure [21]. BPs bind with high affinity to hydroxyapatite, predominantly at areas of high bone turnover. In the bone microenviroment nitrogen-containing BPs (amino-BPs) are internalized by the osteoclasts inhibiting the mevalonate pathway and therefore the osteoclast mediated bone resorption [6]. OPD is an amino-BP that shares the therapeutic and pharmacological properties of pamidronate and alendronate, inducing a rapid and prolonged suppression of bone resorption [22].

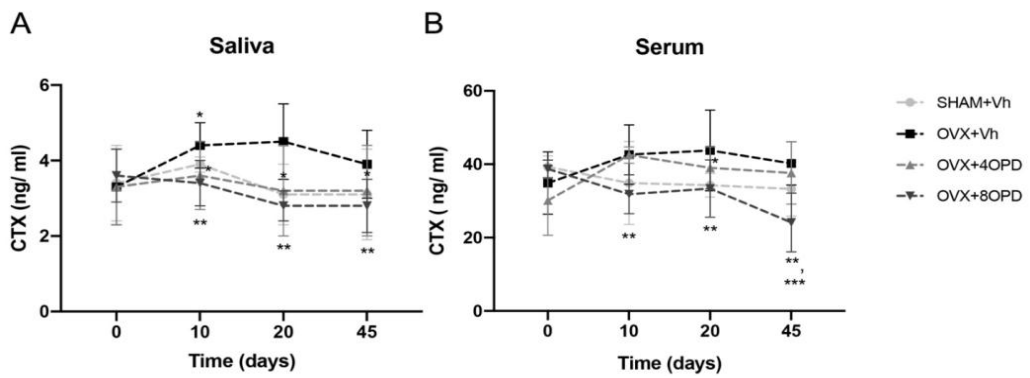

Figure 2: Longitudinal changes of β-CrossLaps (CTX) in saliva (A) and serum (B) samples thought out the study in the four studied groups. SHAM+Vh, OVX+Vh, OVX+4 OPD and OVX+8 OPD groups. n=10 rats/group. Results were expressed as mean ± SD.*p<0.05 *versus* SHAM+Vh group by one-way ANOVA followed by multiple comparisons using Bonferroni post hoc test.

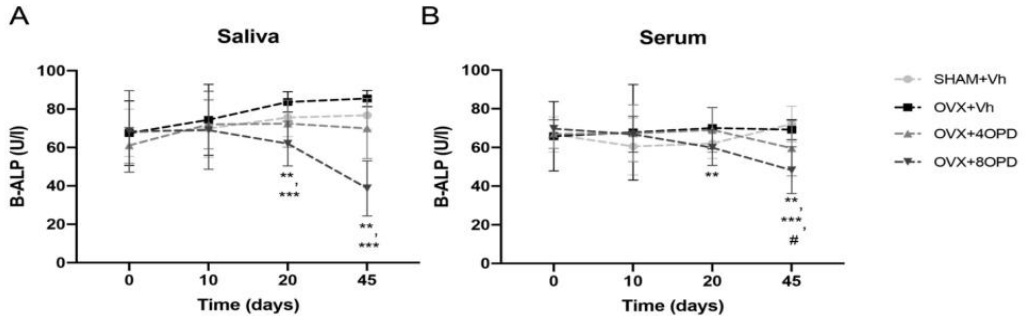

Figure 3: Longitudinal changes of Bone-Specific Alkaline Phosphatase (B-ALP) in saliva (A) and serum (B) samples thought out the study in the four studied groups. SHAM+Vh, OVX+Vh, OVX+4 OPD and OVX+8 OPD groups. n=10 rats/group. Results were expressed as mean ± SD. *p<0.05 *versus* SHAM+Vh group by one-way ANOVA followed by multiple comparisons using Bonferroni post hoc test.

Although BMD is used to diagnose osteoporosis, it has limitations to monitor treatment efficacy and compliance in a short period of time. Conversely, changes of specific and sensitive bone markers in response to antirresortive agents occur as early as 15 days to one month after treatment. In this regard, BPs treatments are associated with a decrease up to 50%-70% in CTX levels depending on the agent, dose and mode of administration [23]. Another clinical utility of bone turnover markers is the compliance for the specific treatment. A significant fall in bone markers after starting a treatment indicates not only efficiency but also a good adherence to therapy [24].

CTX is a very small molecular fragment (2900 daltons), product of the breakdown of type I collagen containing pyridinium cross-link, which is cleaned by the kidney [25]. Currently, CTX is the most sensitive bone resorption marker to assess changes in bone turnover both, in catabolic or anticatabolic conditions such as estrogen withdrawn and BPs treatment. On the other hand, B-ALP is the bone-specific isoform of the serum enzyme alkaline phosphatase, found on the surface of osteoblasts. B-ALP is a glycoprotein that presents a half-life of 1–2 days in the circulation, providing a specific and reliable index of bone formation [26]. Indeed, levels of B-ALP increase significantly in menopause and continue thereafter for more than 20 years, decreasing rapidly after BPs treatment [27]. On this regard, the mechanism by which amino BPs like OPD inhibits B-ALP might be the result of a chelation of divalent cations since this enzyme is strongly activated by Mg^{2+} and has a requirement for Zn^{2+} [28].

Previous studies evaluated bone turnover markers in saliva with different results. Early studies conducted by our group demonstrated that CTX and B-ALP can be evaluated in saliva samples. Both bone turnover markers in saliva samples exhibited a correlation with those evaluated in serum samples during estrogen repletion and depletion conditions, both in women and in osteopenic rats [9,10]. McGehee et al found that other bone markers can be evaluated in saliva. Their results demonstrated that there were a significant correlations between the bone markers deoxypyridinium and osteocalcin with age, body mass index, calcaneus T-scores suggesting that saliva could be used as a fluid sample for assessing bone remodelling [29]. A recent clinical study in postmenopausal osteoporotic and healthy non-osteoporotic females determined osteocalcin and CTX in stimulated saliva. This study failed to find any correlation between these two bone markers in saliva and serum concentrations or BMD [30].

The results of the present study demonstrate that the bone turnover markers CTX and B-ALP, evaluated in saliva samples, responded in the same fashion as serum samples to the decrease in bone turnover that follows anticatabolic treatment with different doses of the potent amino-BP OPD. In addition, like normally seen in serum, salivary bone turnover markers levels evidenced the changes that occurred later in BMD.

Therefore, the results of the present report suggest that the assessment of bone markers in saliva samples would become a valuable alternative to blood-borne samples in a wide variety of clinical settings such as compromised vein access, obesity, hemophilia, elderly people, those with stability problems or in field studies [29]. In these cases the use of saliva samples would be a rapid and less invasive collection method.

In conclusion, although further clinical studies are needed, our results indicate that CTX and B-ALP assessment in saliva samples are able to detect changes in bone turnover due to BPs therapy in a dose depend manner. Therefore, saliva would be a suitable tool to evaluate changes in bone turnover due to BP therapy in the early stage of estrogen withdrawal.

Acknowledgment

The authors thank Mr. Orzuza Ricardo, animal housing technician, Oral and General Biochemistry Department, School of Dentistry, University of Buenos Aires, for his technical assistance and Gardor Company, Buenos Aires, Argentina, for supplying the olpadronate. This study was performed with the support of the University of Buenos Aires (Grant UBACyT M099).

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