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Investigation of mineral distribution in bone by synchrotron X-ray fluorescence microscopy after tibolone therapy

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Summary. Tibolone is a synthetic steroid with estrogenic, androgenic, and progestagenic properties used for the prevention of postmenopausal osteoporosis and treatment of climacteric symptoms. Tibolone shows almost no action on breast and endometrium, which are target-organs for estrogens and progesterone activity. The aim of this work was to investigate the spatial distribution of calcium and zinc minerals in the femoral head of ovariectomized rat in order to evaluate the effects of the long-term administration of tibolone. For that purpose X-ray microfluorescence was used with synchrotron radiation imaging technique which was performed at Brazilian Light Synchrotron Laboratory, Campinas, SP. Minerals were not homogeneously distributed in trabecular bone areas; a higher concentration of calcium in the trabecular regions at femoral heads was found in ovariectomized and tibolone-treated rats compared to ovariectomized and control groups.

1. Introduction

In the postmenopausal period, women have a great risk of skeletal fractures by losing bone content because of their estrogen deficiency. In order to combat the symptoms of menopause, women have nowadays several possibilities of treatment. The most common treatment is the hormone replacement therapy (HRT).

HRT gives artificial supply of estrogen and progesterone allowing the body to function with fewer menopausal symptoms. However, this long-term therapeutic administration has demonstrated disadvantages such as an increase of cancer incidence on breast and endometrium, as well as heart diseases. Nevertheless, other alternative appears, such as tibolone treatment.

Tibolone was first produced by Organon (West Orange, NJ, USA) as a synthetic steroid with estrogenic, androgenic, and progestagenic properties. It has been used primarily for the prevention of postmenopausal osteoporosis and treatment of climacteric symptoms [1–3]. The great advantage against classical HRT is its specific tissue action which performs differently in each part of the organism. In this way, tibolone has almost no action on breast and endometrium, which are target-organs for estrogens and progesterone activity [4].

Some short-time investigations have demonstrated that tibolone has an estrogenic effect on bone [1, 5, 6]. Most of the studies involving this drug and bone sites are performed generally for a period from four days to sixteen weeks [7–10]. However, few studies of the effects of long administration of tibolone on bone can be found. This issue is particularly important in order to know possible adverse and/or collateral effects proceeding from this therapy which can interfere with the balance of the bone metabolism.

Some non invasive techniques are used in the evaluation of bone loss [11]. Minute bone microarchitecture can be useful to prevent the occurrence of bone breakings which is not only dependent on the bone mineral density (BMD) value but also by factors such as age and used therapy [12]. It is well known that BMD alone does not predict bone health. Other factors must be taken into account since bone quality includes information such as mineral content, bone mass distribution and microstructures connections. X-ray microfluorescence (μXRF) imaging technique has been used as a significant tool in order to investigate minerals contents and their spatial distributions in several kinds of materials.

μXRF is an analytical technique that provides chemical composition information. When the X-ray impinges on the sample it interacts *via* photoelectric effect with the surface of the material which emits radiation that is characteristic of the atoms present in the sample. This emitted radiation is called X-ray fluorescent radiation and it is specific of each material. The fluorescent X-rays emitted by the material are collected by a solid state detector and when associated with a multichannel analyzer it produces a spectrum that involves the X-ray characteristic intensity (number of counts per second) of each chemical element and its energy. When X-ray fluorescence is associated with synchrotron radiation (SR) source, many advantages can be achieved in respect to a usual X-ray source, such as high energy, tunability and polarization of the X-rays. Besides the chemical composition quantification it is even possible to perform bidimensional

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images of each mineral constituent which lead to the knowledge of the spatial distribution on the sample surface. In this case it is also important to take into account the sensitivity of the experimental system and the samples absorption effects [13].

The aim of this work was to investigate the bidimensional distribution of Ca and Zn contents in femoral bone heads in order to study the effects of a long-term administration of tibolone in rats.

2. Materials and methods

2.1 Animals

Seventeen Wistar rats (8 to 12 weeks old, mean weight of 250 g) were used. All the animals were obtained from the Animal Facility of the Experimental Nutrition Laboratory (LABNE) at the Fluminense Federal University (UFF) in Niterói (RJ, Brazil). Rats were housed in individual cages, in a temperature controlled (24 ± 2 °C) facility with 12-h light-dark cycle. Commercial food (FRI-LAB RATOS II, FRI-RIBE S.A.©) and tap water were supplied *ad libitum* throughout the experiment. The research was in agreement with the determinations of the Brazilian Association for Laboratory Animal Science (SBCAL) and was approved by the Committee of Ethics in Animal Research of the Research and Postgraduate Pro-Rectory/UFF.

2.2 Protocol

The animals were randomly divided in three groups. The first one was the control group (C, $n = 5$) used as a parameter of normal bone density. The second one was the ovariectomized group (OVX, $n = 4$) and the last one was the ovariectomized submitted to tibolone treatment (OVX + T, $n = 8$). All the surgical procedures were performed under xylazine (20 mg/kg) and ketamine (100 mg/kg) anesthesia. The hormonal treatment began thirty days after the ovariectomy and was performed for five months. Every day 1 mg of tibolone was administered *per os* in the vehicle (0.5% carboxymethylcellulose solution) in a volume of 0.5 ml. Every 30 d the evolution of the hormonal status was checked by vaginal cytology. After the treatment, all animals were killed by spinal cord dislocation after anesthesia. Complete skeletons were fixed in 10% buffered formalin and kept in 70% alcohol at 4 °C. The femurs were dissected and cleaned carefully. In order to perform the μXRF-SR technique, femoral bones were embedded in poly (methyl methacrylate) resin (PMMA) and thin sections (about 400 μm thick) were cut with a diamond-coated saw in Buehler Isomet® motorized microtome.

2.3 Synchrotron radiation X-ray microfluorescence

μXRF-SR was carried out on beam line D09-XRF at Brazilian Synchrotron Light Laboratory (LNLS), Campinas, SP, Brazil. A white beam (4–23 keV) with a capillary optic (20 μm diameter) was used for sample excitation and the incident beam was monitored with an ionization chamber placed before the sample. The samples were placed at 45° to the incident beam and at this same angle of degree a Si (Li) solid state detector with 8 μm of beryllium window was used to collect the X-ray fluorescent radiation.

It is well know that bone samples have a lot of calcium as they are composed mainly by hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Therefore, this mineral has a great contribution in the XRF spectra. In order to avoid saturation the beam was attenuated using three Al foils (0.25 μm each) placed in front of the detector collimator.

The measurements were performed in a computed controlled XYZ table which allowed the choice of the region of interest (ROI) in each bone sample. The choice of the ROI was made based on the trabecular bone area of femoral head. It must be taken into account a commitment among the area of the ROI, the beam life time and the number of samples. The life time of the beam at LNLS is around 14 h, although there is a reinjection of the beam after each 7 h passed. Therefore, the optimization among these factors was made of choosing a scan area of 1.0–1.2 mm^2 (9–10 s/point, ∼ 4 h/sample) for 17 samples (C = 5, OVX = 4, OVX + T = 8). The ROI produced approximately 1681 XRF spectrum and all of them were analyzed using QXAS/AXIL software which is freely provided by IAEA. As a result, it was produced X-ray fluorescence mapping scans of each bone sample with an image resolution of 30 μm. The concentration values were calculated based on fundamental parameter method using QXAS/AXIL software.

2.4 Statistical analysis

ANOVA one-way and pos-hoc Bonferroni were performed for each mineral concentration. The significance level was set as $p < 0.05$. Statistical analysis was processed by the software Prism 5 for Windows.

3. Results and discussion

Fig. 1 shows typical μXRF-SR spectra for the analyzed samples, C (control), OVX (ovariectomized) and OVX + T (ovariectomized submitted to tibolone treatment). It can be notice that bone samples presented P, Ca, Fe, Zn, Cu and Sr

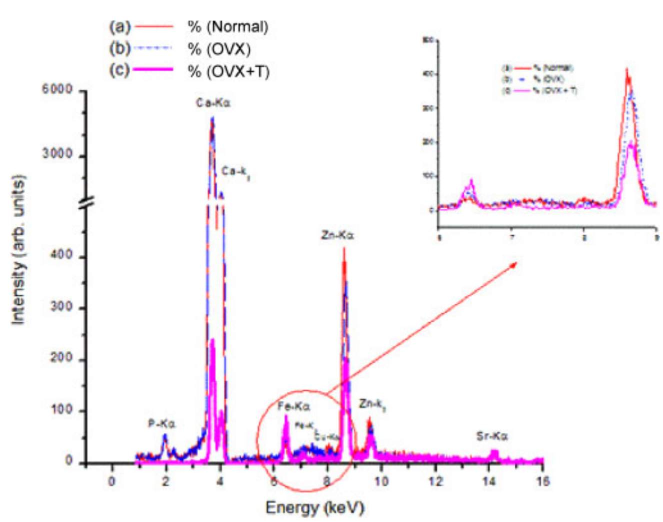

Fig. 1. Example of a typical μXRF-SR spectra: (a) normal bone, (b) OVX and (c) OVX + T.

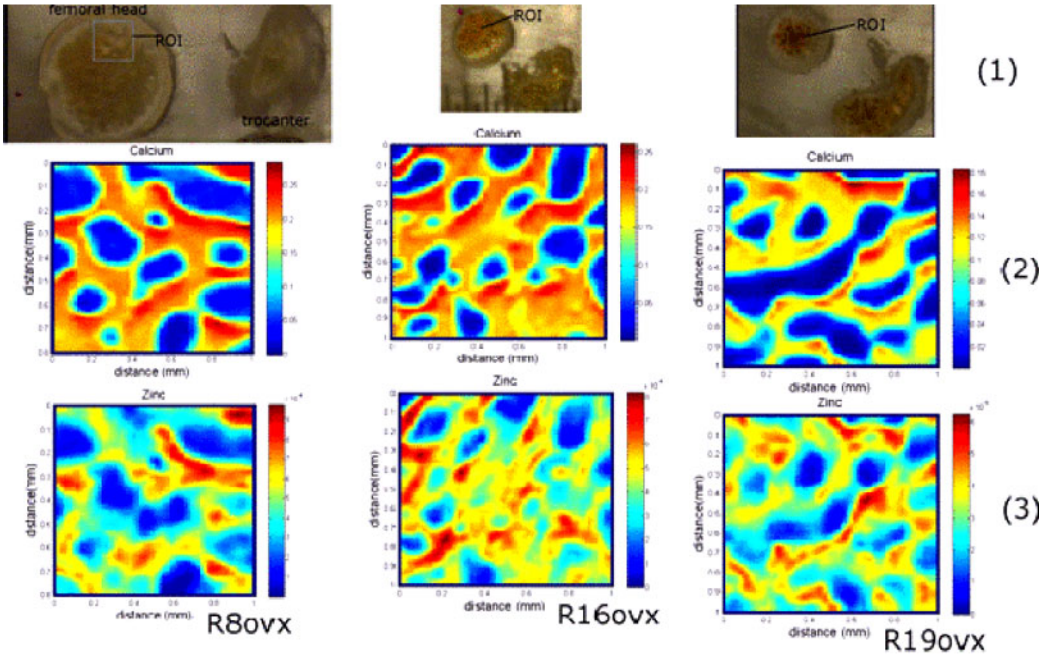

Fig. 2. Ca and Zn distribution in C femoral head by μXRF-SR. The image scale is in $\mu g\,g^{-1}$: (1) sample photography. (2) Ca and (3) Zn mapping.

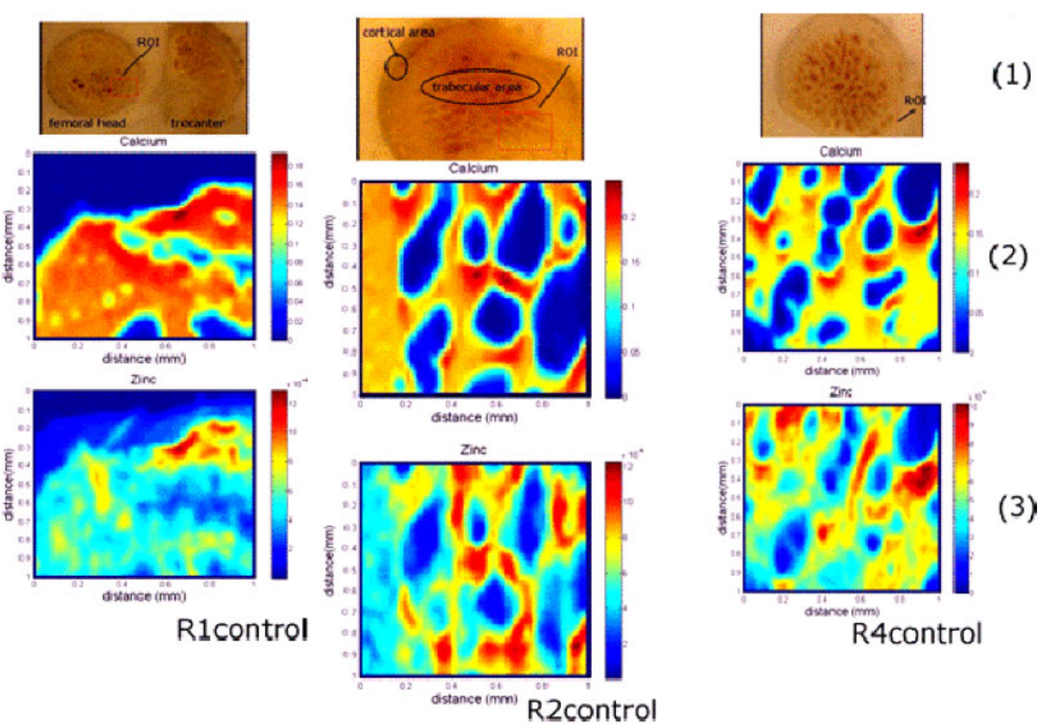

Fig. 3. Ca and Zn distribution in OVX femoral head by μXRF-SR. The image scale is in $\mu g\,g^{-1}$: (1) sample photography. (2) Ca and (3) Zn mapping.

elements also the most important elements in bone tissue are P and Ca because bones are mainly composed by hydroxyapatite.

Figs. 2–4 show an example of bone samples X-ray fluorescence imaging scans for Ca and Zn elements and Fig. 5 shows the mean values and standard deviation of those concentrations. Those results show that Ca was more concentrated in the trabecular region of femoral head in OVX + T group compared to C and OVX, whereas Zn was more concentrated in C followed by OVX + T and finally OVX showing the lower concentration. The distribution of Ca and Zn were not homogeneous all over the trabecular area. It can be notice that the minerals were more pronounced at the edges of the trabecular, mainly for Ca.

Elderly women are at higher risk for hip fracture because of additional and relatively rapid bone loss due to estrogen deficiency by loss of the ovarian function and a longer average life span than men [14, 15]. The incidence of osteoporosis has even increased in the last decades, because of a higher life expectance. As osteoporosis fractures cause significant morbidity, mortality and costs to the public health system, the early detection is very important for establishing effective therapeutic strategies to prevent bone fractures [1, 16, 17]. To predict bone health, some factors that must be taken into account include Bone Mineral Density (BMD), mineral content and microstructures connections.

Bone is an active tissue that undergoes continuous remodeling, during which active osteoclasts promote bone

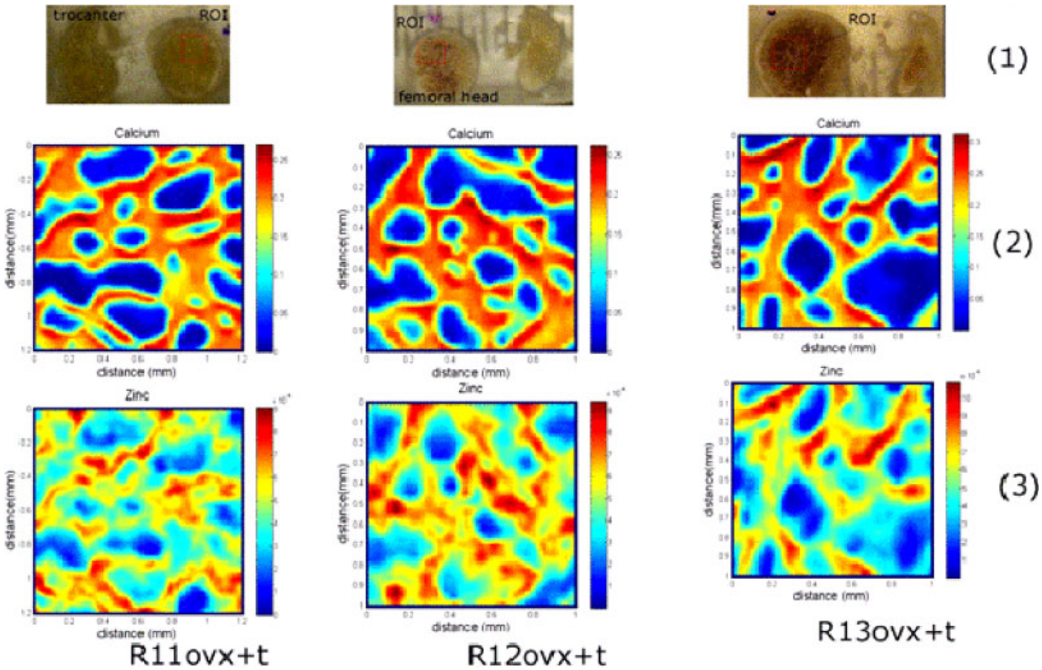

Fig. 4. Ca and Zn distribution in OVX + T femoral head by μXRF-SR. The image scale is in $\mu g\, g^{-1}$: (1) sample photography. (2) Ca and (3) Zn mapping.

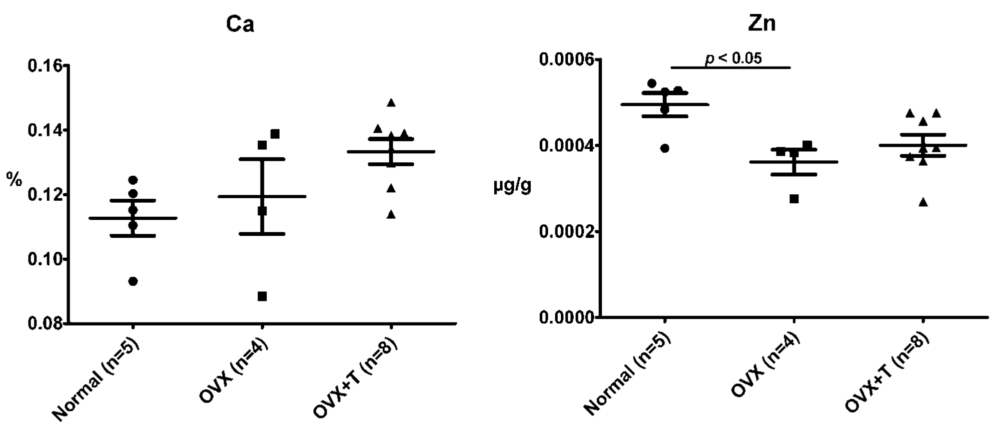

Fig. 5. μXRF-SR Mineral Concentration Values. ANOVA one-way and pos-hoc Bonferroni; Zn Normal > Zn OVX ($p < 0.05$).

resorption and osteoblasts are responsible for new bone formation (collagen synthesis and mineralization) [18]. Elderly women are at higher risk for hip fracture because of relatively rapid bone loss due to estrogen deficiency [5, 15]. Estrogen deficiency induces a negative balance in the bone remodeling process, as bone resorption increases much more than bone formation.

As in humans, ovarian hormone deficiency in rats also accelerates bone remodeling. Both (rats and humans) exhibit similar changes in bone mass and structure at numerous skeletal locations following estrogen deficiency [1, 19, 20]. Our approach using a high dose of tibolone for a long period represents a unique proceeding to investigate effects on the bone, taking into account that human hormonal replacement therapy is actually used for many years.

The life expectancy of most strains of rats is around two and a half to three years [21]. Compared to the human body, the metabolism of rats is very fast. When rats reach the age of 24 to 28 months, they are considered old and they correspond in age to humans in the sixth to seventh decades of life [22]; others refer 18-month-old rats as aged [23]. This means that each day of rat's life is equivalent to approximately 30 d of life in humans. The current experiment lasted for 20 weeks, representing approximately 11.5 years of women lifetime. Furthermore, by comparing the current research to the experiments analyzing rat bone, it can be said that the period of treatment is prolonged, since the works are generally made for a period ranging from 4 weeks [7, 8] to 16 weeks [10].

The dose of tibolone used in this experiment was considered high based on comparison with the usual dose of 2.5 mg used in humans, which corresponds to 0.041 mg/kg for a 60-kg woman. The equivalent dose for the rat calculated using the allometric system [24] was 0.161 mg/kg. Therefore, the dose of 1 mg/day per animal weighing 0.250 kg, as used in this work, corresponds to 4 mg/kg and represents 24 times the normal dose in humans [25]. This dose is referred in one study on the effect of 4 weeks of tibolone treatment in rat bone [8].

Estrogen deficiency in humans causes both trabecular bone loss and architectural deterioration, giving rise of an increased incidence of osteoporotic fracture [1, 15]. Trabecular bone is much more responsive to hormonal as well as pharmacological intervention than cortical bone [20, 26]. Metabolically, it is about 20 times more active and turns over more quickly than the cortical bone [27]. This prompted us to choose the femoral proximal regions as ROIs for studying the trabecular bone resorption using the μXRF-SR.

Minerals play an important role in bones in regard to the dynamic equilibrium between the intake of elements and their availability locally and systemically [18]. Although the major mineral components in bone are Ca and P, other element such as Zn and Sr can be found in bones. Their role is still not well established and continues to be researched. However, some considerations can be made about the studied elements, *i.e.*, Ca is considered an essential mineral in the composition of bones. Zn content is related to bone formation and Zn promotes the release of growth hormone and participates in the structure of bones [13, 18]. According to our results, it can be said that Zn concentration was visibly affected with castration and increased with tibolone replacement, probably due to the estrogenic effect of this drug.

The skeleton is considered the target organ for many heavy metals, and when absorbed they affect the risk and course of many diseases [18]. For example, the deficiency of Ca increases bone fractures and the development of osteoporosis. A deficiency of minerals like Fe, Mn, Cu, and Zn are associated with bone lesions in animals but in humans this aspect still remains to be elucidated [28]. In our study, the use of high doses of tibolone administrated in a long period of time seems to increase Ca concentration on the selected ROI. Several studies have demonstrated that estrogen replacement therapy can effectively prevent trabecular as well as cortical bone loss by reducing bone resorption and the risk of skeletal fractures [1, 15, 20]. The present study confirms the estrogenic effect of tibolone at the femoral head by the higher concentrations of some chemical elements like Ca in the trabecular regions, when compared to both other groups, although statistical significance was not found.

μXRF-SR becomes a very important tool to elucidate the interaction of the major mineral components in bone, demonstrating that it can be an effective alternative technique for characterizing bone structures by 2D chemical element maps. Besides being a non-destructive method, it makes possible to see the distribution of chemical elements with greater resolution than in the usual clinical imaging techniques. Thereby, μXRF-SR becomes an important method for aid the analysis and consequent development of treatments, prevention and improvement of new drugs against diseases.

4. Conclusions

The present study show the calcium and zinc spatial distribution in femoral bone heads of Wistar rats are not homogeneous all over the trabecular area. Bones of rats submitted to ovariectomy and tibolone treatment have higher concentrations of calcium in the trabecular regions, when compared to ovariectomized and control groups which seems to confirm the estrogenic effect of tibolone.

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