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Chronic lead poisoning magnifies bone detrimental effects in an ovariectomized rat model of postmenopausal osteoporosis

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ABSTRACT

Lead (Pb) is a persistent environmental contaminant that is mainly stored in bones being an important source of endogenous lead exposure during periods of increased bone resorption as occurs in menopause. As no evidence exists of which bone biomechanical properties are impaired in those elderly women who had been exposed to Pb during their lifetime, the aim of the present study is to discern whether chronic lead poisoning magnifies the deterioration of bone biology that occurs in later stages of life. We investigated the effect of Pb in the femora of ovariectomized (OVX) female Wistar rats who had been intoxicated with 1000 ppm of Pb acetate in drinking water for 8 months. Structural properties were determined using a three-point bending mechanical test, and geometrical and material properties were evaluated after obtaining the load/deformation curve. Areal Bone Mineral Density (BMD) was estimated using a bone densitometer. Femoral histomorphometry was carried out on slices dyed with H&E (Hematoxylin and Eosin). Pb and OVX decreased all structural properties with a higher effect when both treatments were applied together. Medullar and cortical area of femurs under OVX increased, allowing the bone to accommodate its architecture, which was not observed under Pb intoxication. Pb and OVX significantly decreased BMD, showing lead treated ovariectomized rats (PbOVX) animals the lowest BMD levels. Trabecular bone volume per total volume (BV/TV%) was decreased in OVX and PbOVX animals in 54% compared to the control animals ($p < 0.001$). Pb femurs also showed 28% less trabeculae than the control ($p < 0.05$). We demonstrated that Pb intoxication magnifies the impairment in bone biomechanics of OVX rats with a consequent enhancement of the risk of fracture. These results enable the discussion of the detrimental effects of lead intoxication in bone biology in elderly women.

1. Introduction

Lead (Pb) is a persistent air pollutant which can be released into the environment via numerous routes, mainly by industrial activities. During infancy and childhood, lead is deposited in trabecular bone because it is the most active site of remodeling; whereas, in adulthood lead is deposited in both trabecular and cortical bone (Aufderheide and Wittmers, 1992). We previously reported that chronic intoxication with Pb impaired growth parameters and induced negative effects on bone structural properties (Conti et al., 2012). Monir et al. (2010) demonstrated that lead exposure in adult bone of female mice decreased bone mineral density and collagen maturity, altered mineral/matrix ratios and increased bone marrow area and bone turnover, resulting in a trend toward weaker bones. As Pb in bone has a half-life in the order of years to decades, skeletal stores may be an important source of endogenous lead exposure during periods of increased bone resorption, as occurs in menopause. Changes in hormonal status related to menopause cause bone tissue loss and a decrease in bone mineral density (Meema and Meema, 1976), affecting bone tissue material properties at multiple scales, resulting in low bone mass and micro-architectural deterioration (Ettinger et al., 1998). Previously reported studies have shown a significant association between older women with higher blood lead levels and an increased risk of osteoporosis with a consequent susceptibility to bone fractures (Khalil et al., 2008). Nash et al. (2004) demonstrated that lead stored in bone could significantly increase blood lead levels in perimenopausal women due to

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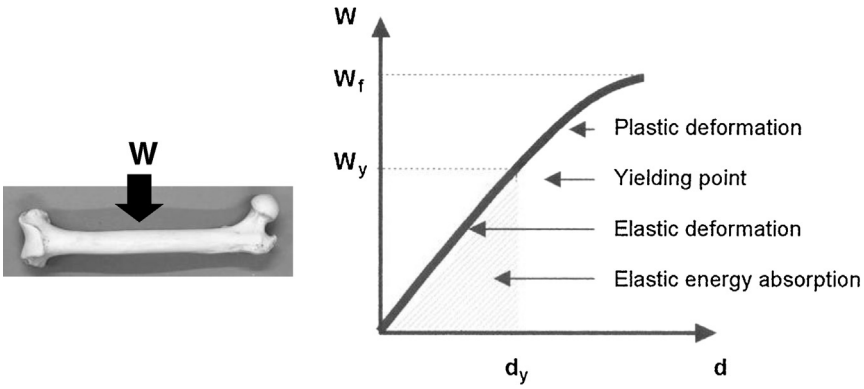

Fig. 1. Schematic representation of femur (LEFT) showing the load (W) applied to perform the three-point bending mechanical test on an Instron Universal Testing Machine Model 4442. Right: diagram of a load (W)/deformation (d) curve showing the elastic (Hookean behaviour) and plastic (non-Hookean behaviour) phases, separated by the yielding point.

Table 1
Lead and calcium content.

	C	COVX	Pb	PbOVX
μg Pb/g bone ashes	0.07 ± 0.02^{a}	0.08 ± 0.02^{a}	10.76 ± 1.11^{b}	8.17 ± 0.06^{c}
mg Ca/g bone ashes	316.85 ± 59.86^{a}	272.07 ± 16.14^{a}	285.72 ± 28.42^{a}	306.55 ± 77.05^{a}

Values are mean ± SD of 10 rats. Equal letters indicate no significant differences. Different letters indicate a significant difference between groups was ($p < 0.01$ determined by ANOVA followed by Student–Newman–Keuls Multiple Comparison Test). C: control rats, COVX: ovariectomized control rats, Pb: lead-treated rats, PbOVX: lead-treated ovariectomized rats.

postmenopausal bone mineral resorption reinforcing the possibility that bone lead stores represents an endogenous source of lead exposure. Authors suggested that lead released from bone could be more toxicologically relevant than lead entering the bloodstream from environmental sources. However, no evidence exists of which bone biomechanical properties are impaired in those elderly women who had been exposed to Pb during their lifetime. The ovariectomized (OVX) rat is widely adopted in studies to mimic the estrogen-deficiency-induced bone loss (Liu et al., 2015). Therefore, the aim of the present study is to discern whether chronic lead poisoning magnifies the deterioration of bone biology that occurs in later stages of life by evaluating bone biomechanical properties in an ovariectomized rat model of postmenopausal osteoporosis.

2. Materials and methods

2.1. Experimental design

Forty female Wistar growing rats from our own colony, aged 21 days, were randomly divided into 2 groups: C (control, sodium acetate in tap water) and Pb (intoxicated with 1000 ppm of lead acetate in drinking water during 8 months (Hamilton and O'Flaherty, 1994). After 3 months, half of the animals of each group were assigned to undergo bilateral OVX by a dorsal approach and the remaining rats were subjected to a sham operation. Surgery was performed under general anesthesia with a mixture of 2% xylazine hydrochloride (5 mg/kg; i.p. König Laboratories,

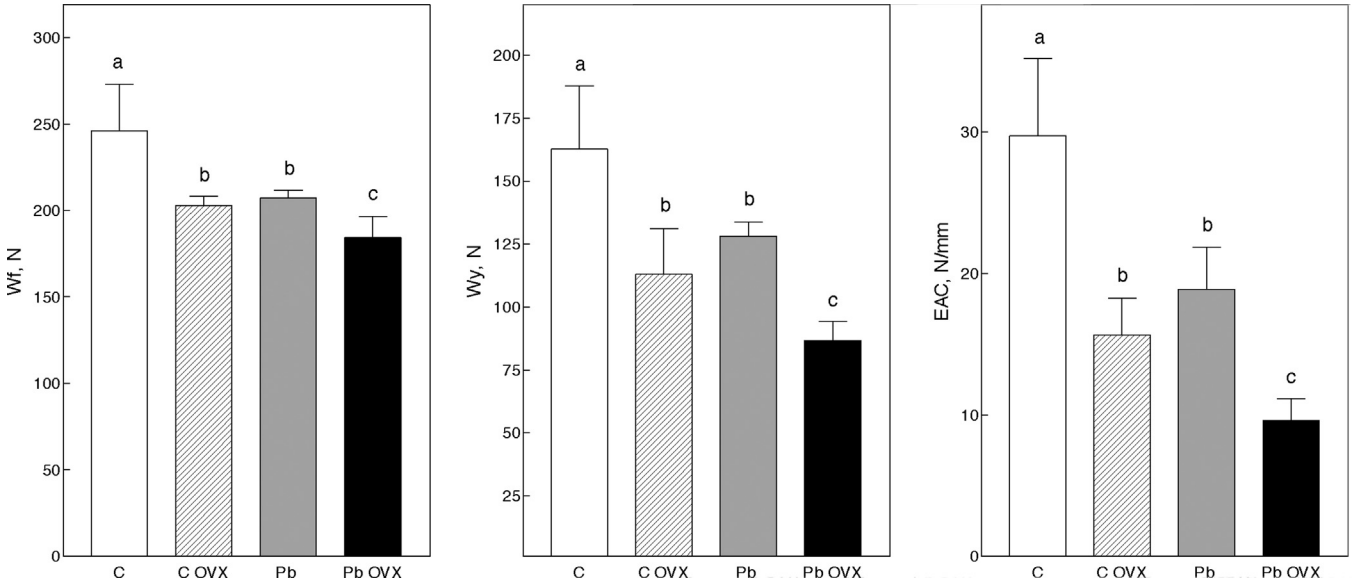

Fig. 2. Changes in femoral mechanical properties. Equal letters indicate no significant differences. Different letters indicate $p < 0.05$.

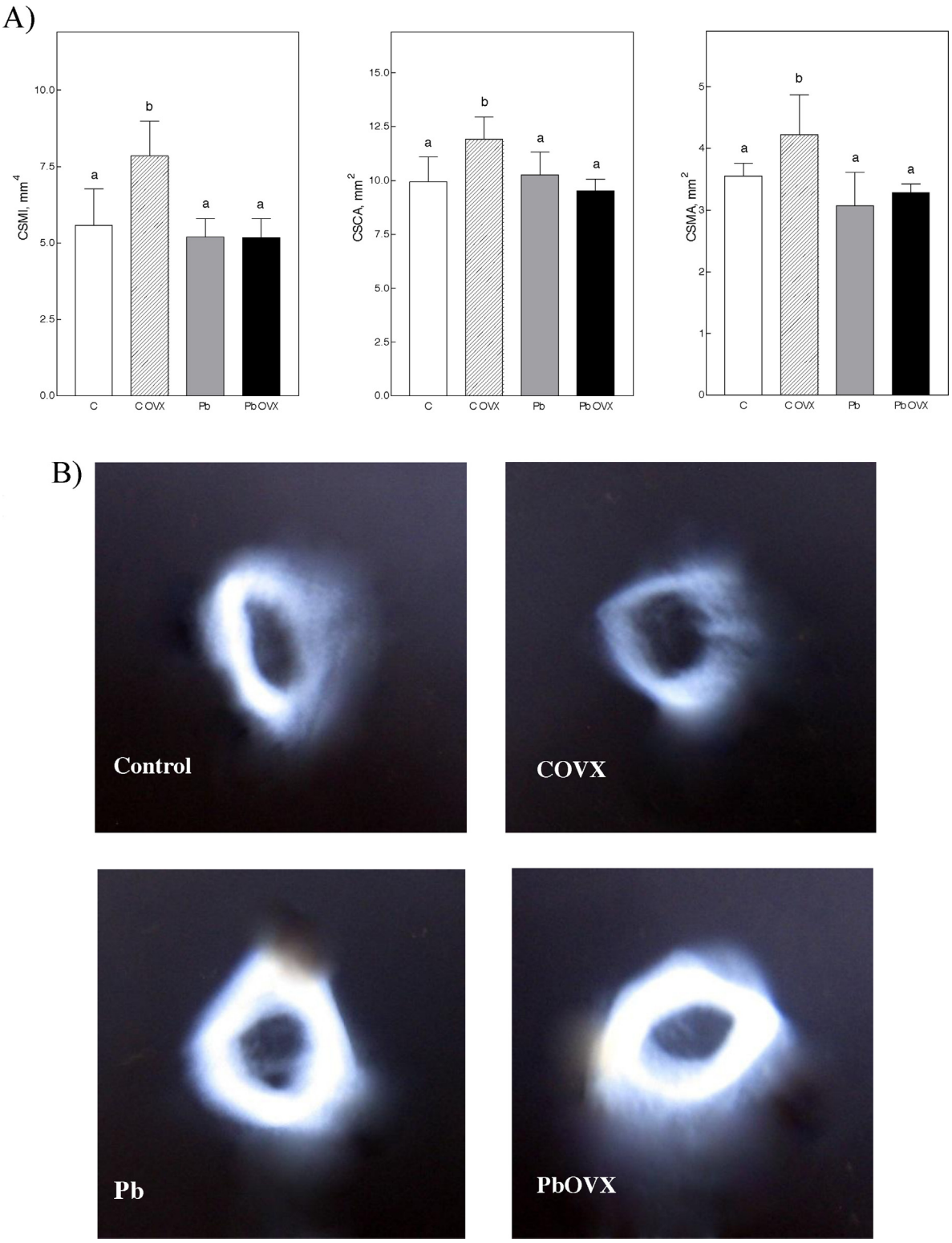

Fig. 3. (A) Changes in femoral geometrical properties. Equal letters indicate no significant differences. Different letters indicate $p < 0.05$. (B) Photographs of Rx of transverse slices of the longitudinal sections of femur of one animal per group selected randomly.

Buenos Aires, Argentina) and 5% ketamine hydrochloride (50 mg/kg; i.p. Holliday-Scott SA, Buenos Aires, Argentina). Since then, animals were divided into 4 groups: C=control rats sham operated, COVX: bilateral ovariectomized rats, Pb= lead treated rats sham operated and PbOVX: lead treated ovariectomized rats). All animals were housed in stainless-steel cages and maintained under local vivarium conditions (temperature 22–23 °C, 12-h on/off light cycle) and were allowed free access to water and a standard pelleted chow diet. At the end of the experimental period (8 months) all animals were euthanized, weighed and measured. Femurs were properly dissected to perform anthropometric, mechanical and histological studies. All animals were treated in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals (NIH 8th edition, 2011) and

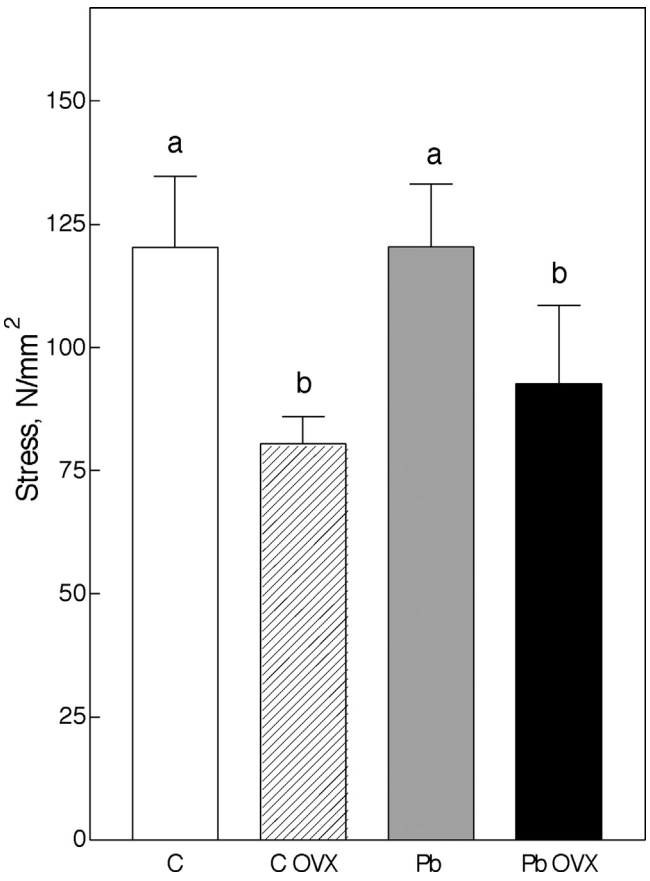

Fig. 4. Changes in femoral material property. Equal letters indicate no significant differences. Different letters indicate $p < 0.05$.

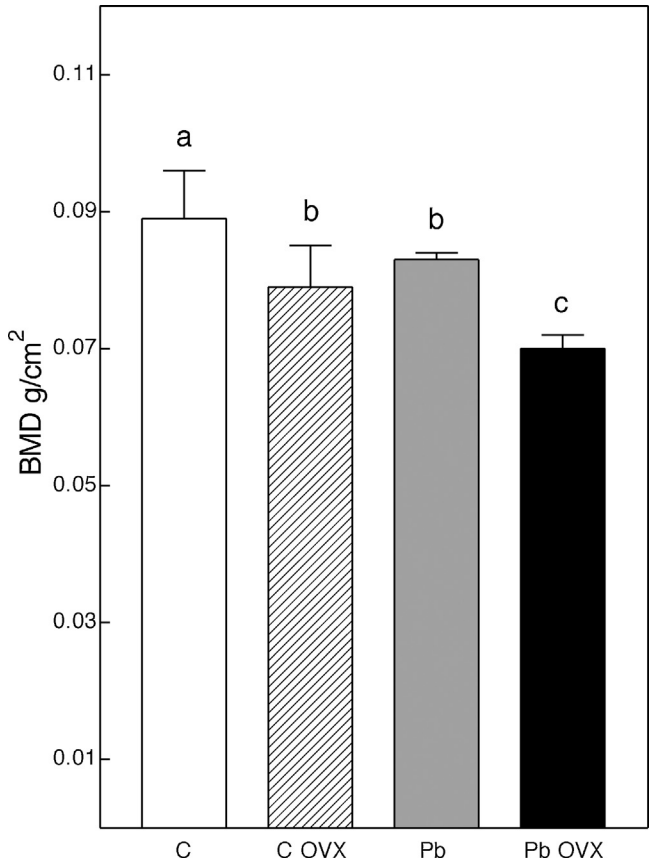

Fig. 5. Changes in femoral areal bone mineral density. Equal letters indicate no significant differences. Different letters indicate $p < 0.05$.

protocols were approved by the Ethical Commission of the Faculty of Dentistry, University of Buenos Aires (No. 11/06/2012-23).

2.2. Whole-bone mechanical testing

Mechanical properties of the left femur of each animal were determined by a three-point bending test on an Instron Universal Testing Machine Model 4442; Canton, MA, USA. The load/deflection (W/d) curves showed, successively, the linearly elastic (Hookean) behaviour followed by the non-linear (non-Hookean) behavior, separated by the yield point (y), until fracture (Fig. 1). From the load/deflection curves, the following whole-bone (structural) properties were measured: (a) limit elastic load, Wy; (b) energy absorption capacity by the whole bone during the elastic period, EAC: Wy.dy/2; and (c) maximal load supported (ultimate strength), Wf. Evaluation of geometrical properties (bone architecture) was performed by using an Isomet low-speed diamond saw (Buehler, Lake Bluff, IL, USA). A 2-mm cross-section slide was cut from the regularized fracture section in order to perform micromorphometrical determinations of the vertical and horizontal outer and inner diameters of the elliptic-shaped fracture sections (B: vertical outer diameter, H: horizontal outer diameter, b: vertical inner diameter, h: horizontal inner diameter) using a stereomicroscope (Stemi DV4 Stereomicroscope, Carl Zeiss Micro Imaging, Göttingen, Germany) and a digital caliper (Digimess, Geneva, Switzerland). This procedure enabled calculation of the following geometric properties: (a) the cross-sectional medullar area, CSMA, mm^2; (b) the cross-sectional cortical area, CSCA, mm^2 and (c) the second moment of inertia of the cross section in relation to the horizontal axis, CSMI, mm^4 as a measure of the architectural efficiency of the cortical design in relation with the distance at which the cortical tissue is distributed from the bending axis in the cross-section, concerning the kind of deformation. The CSMI values (given in mm^4) increase linearly with bone mass, but are also proportional to the squared distance from the bone cortex to the reference axis. The more peripheral the disposition of the cortical tissue with respect to the reference axis, the higher the corresponding moments of inertia and the bending or torsion stiffness or strength of the bone in the assayed conditions, independently of the bone mass and material properties (Ferretti, 1997).

Expression of structural properties in relation to geometric properties indirectly allowed calculation of the following material or intrinsic properties of the bone, which are independent of its size and shape: (a) Young's modulus of elasticity of the bone mineralized tissue, E, an estimator of the intrinsic stiffness of the "solid" bone tissue and (b) bone tissue strength, stress, which represents the load supported per unit of cortical bone at the end of the elastic period (Ferretti et al., 2001).

2.3. DXA measurements

Areal bone mineral density (BMD) of the left femur was determined using a bone densitometer (LUNAR DPX-L) and specific software for small animals designed by LUNAR General Electric Medical Systems (Madison, WI, USA). All measurements were carried out with a fine-diameter collimator on the X-ray output. Results are expressed as g/cm^2. The BMD can be considered as an indicator of the degree of concentration of mineral within the whole bone (Ferretti et al., 2001).

2.4. Histological analyses

For histomorphometric studies femurs were resected and fixed in 10% buffered formaldehyde solution for 48 h, decalcified in ethylendiaminotetracetic acid (EDTA, Sigma) pH 7.4 for 25 days and then embedded in paraffin to perform sections following the longitudinal axis. The sections were stained with Hematoxylin and Eosin (H&E) for histological analysis of subchondral trabecular bone volume, (BV/TV%, Parfitt et al., 1987). The histomorphometric determinations were performed on digital microphotographs (40x) of the sections, using Image Pro Plus 4.5 software (Media Cybernetics, Inc., Warrendale, PA, USA).

2.5. Bone ash determinations

After mechanical testing, femurs were desiccated for bone ash determination in a muffle furnace at 600 °C for 18 h. Pb and calcium (Ca) content in these ashes was determined by a Varian SpectrAA-10 Plus atomic absorption spectrophotometer (Varian®) equipped with a deuterium lamp for background correction and hollow-cathode lamps for each of the elements studied.

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls Multiple Comparison Test. Analyses were performed using the Software package Instat and Prism V.3 (GraphPad Software Inc., San Diego, USA). A p-value less than 0.05 was considered statistical significant.

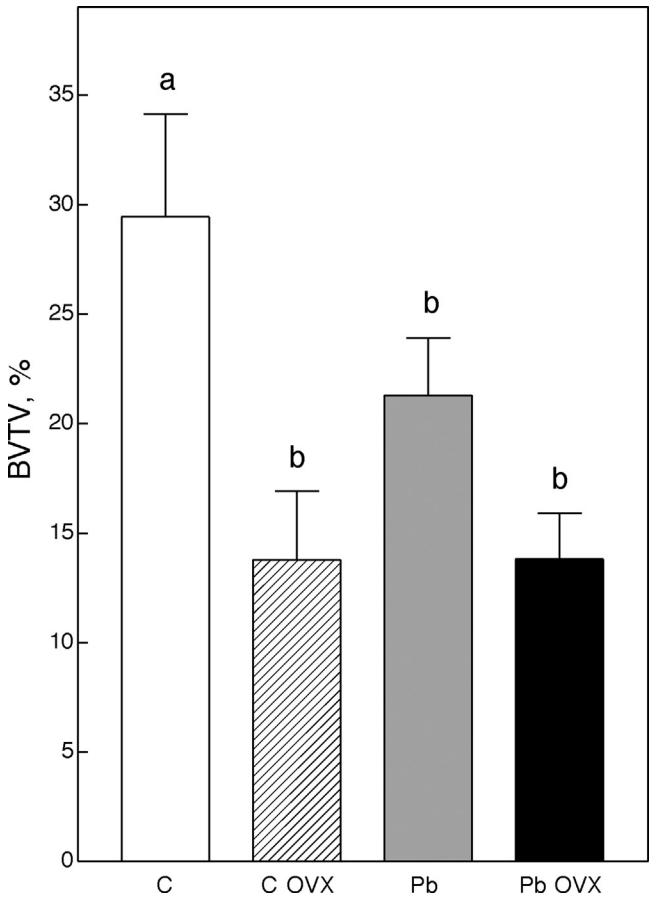

Fig. 6. Static histomorphometry analyses of distal femur. Changes in trabecular bone volume per total volume (BV/TV, %) of the left femur.

3. Results

3.1. Lead and calcium content in ashes

Results showing the changes in lead and calcium content are shown in Table 1. Significantly high-level lead accumulation was observed in femoral ashes from treated animals (Pb and PbOVX groups) compared to the control animals, indicating that the administered Pb was deposited in the skeleton in significant amounts. A significant difference ($p < 0.01$) was found between Pb and PbOVX groups which indicates lead mobilization from OVX rat bones.

Bone ash Ca content did not significantly differ between controls and Pb or PbOVX.

3.2. Whole-bone mechanical testing

3.2.1. Mechanical properties

Pb treatment decreased the maximal load supported (Fig. 2 left), the limit elastic load (Fig. 2 middle) and the elastic absorption of energy up to the yield point (Fig. 2 right).

OVX treatment decreased all the mechanical properties assayed as lead treatment did, with no significant differences between them.

Combined treatments showed additive effects, increasing the risk of fractures of those OVX animals exposed to Pb ($p < 0.001$).

3.2.2. Geometrical properties

OVX enhanced CSMI by means of increasing the cortical and medullar area, in an attempt of improving architecture efficiency. This was not observed under Pb intoxication (Fig. 3A). Those changes can be observed in the Rx photographs of transverse slices from longitudinal sections of the proximal femur of one animal per group selected randomly (Fig. 3B).

3.2.3. Material properties

The yield stress of cortical bone tissue, a bone material quality indicator, was significantly reduced only by means of OVX treatment being unaffected by Pb (Fig. 4).

3.2.4. DEXA measurements

Fig. 5 shows that femoral BMD was negatively affected by OVX, being the impairment even greater in the Pb OVX group ($p < 0.05$).

3.2.5. Histological analyses

The histomorphometric results shown in Fig. 6 indicate that OVX decreased trabecular bone volume (measured as bone volume/total volume; BV/TV%) in 54% compared to the control animals ($p < 0{,}001$). Pb femurs also showed 28% less trabeculae than the control ($p < 0.05$). Histological sections of the femur corresponding to the different groups in Fig. 7 show a diminution of trabecular volume as well as disconnection of trabeculae among each other in every experimental group, without evidence of any other pathological alteration. No modifications in the cartilage plate thickness were observed in any of the experimental groups. Physiological deposition of adipose tissue in the bone marrow of the animals can be observed.

4. Discussion

In the present investigation we demonstrated that ultimate strength, limit elastic load and energy absorption capacity were negatively affected in OVX rats exposed to Pb for 8 months, suggesting that the impairment of bone structure due to OVX was exacerbated by means of Pb. The significant difference found in Pb content from femoral ashes between Pb and PbOVX groups is in

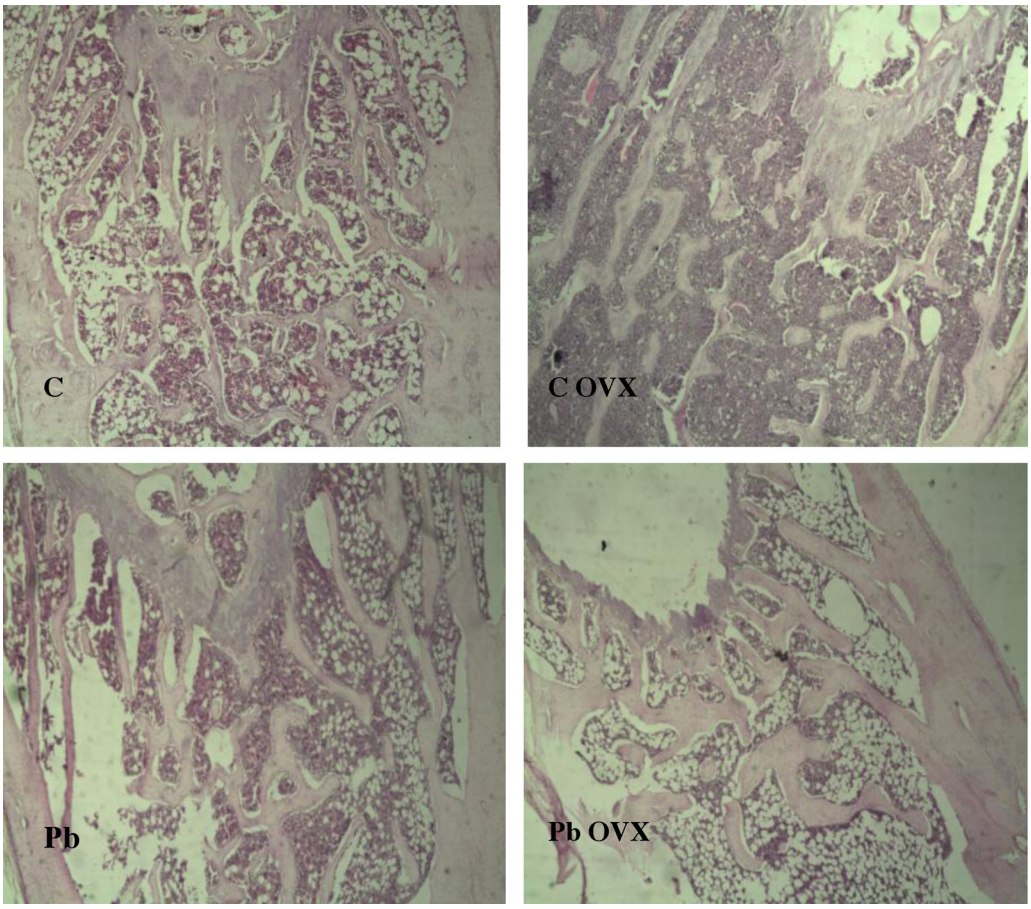

Fig. 7. Femur histomorphometric analyses showing the effects of chronic lead poisoning in an ovariectomized rat model of postmenopausal osteoporosis. (C) Photograph of transverse slice of the longitudinal section of femur (40×) of one randomly selected animal per control group. *A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01012.* C OVX: Photograph of transverse slice of the longitudinal section of femur (40×) of one randomly selected animal per control ovariectomized group (bilateral ovariectomy after 3 months from the beginning of the experimental period). *A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01013.* Pb: photograph of transverse slice of the longitudinal section of femur (40×) of one randomly selected animal per lead intoxicated group (1000 ppm of lead acetate in drinking water during 8 months). *A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01014.*Pb OVX: Photograph of transverse slice of the longitudinal section of femur (40×) of one randomly selected animal per ovariectomized lead intoxicated group (1000 ppm of lead acetate in drinking water during 8 months and with bilateral ovariectomy after 3 months from the beginning of the experimental period). *A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01015.*

agreement with previously reported studies indicating that lead was mobilized from the skeleton during conditions of high bone turnover, such as menopause (Silbergeld et al., 1988). The mechanical properties of bone strongly depend on the intrinsic mechanical quality of its constitutive substance (material properties) and the amount and spatial distribution of the mineralised tissue (geometrical properties) (Ferretti, 1997). Lead poisoning may affect either of both. When we analysed bone tissue strength (stress), we found that this property decreased by means of OVX. This reduction was not observed in femurs of the lead intoxicated groups. Femurs of OVX animals enhanced its moment of inertia (CSMI) by resorption on the endosteal surface and apposition on the periosteal surface in order to try to compensate the impairment in the material properties. It is known that bones with material distribution further away from the centre are significantly stronger (Cole and Van der Meulen, 2011). Interestingly, those femurs of the PbOVX group could not perform the adaptation mentioned above. It seems that Pb does not allow architectural accommodation which may be due to an altered osteoclastic activity widely reported in the literature (Pounds et al., 1991).

The ability of bone to bear loads does not only depend on material and geometrical properties, but also on bone total mass. In this experiment, BMD was decreased in femurs of the OVX group and an additive effect due to Pb in OVX animals was observed. It has been established that small changes in BMD induce large changes in bone strength and that sites with similar BMD but different architecture, show differences in strength and stiffness (Cole and Van der Meulen, 2011). These findings would partially explain the increased impairment in structural properties of the OVX animals exposed to Pb reported in the present investigation.

The manifestation of lead intoxication in bone is the result of complex interplay between many effects, involving cellular and chemical processes in the bone matrix, and also systemic and endocrine effects. It has been established that Pb decreases calcium absorption and replaces calcium hydroxyapatite to form Pb phosphate, resulting in a decrease of Ca in bone (Hongke et al., 2014). In our study, Ca content in femur ashes did not show changes between experimental groups, in accordance with other studies (Martínez et al., 2011; Monir et al., 2010). It would seem that Ca content is not directly responsible for the impairment in bone structural properties, but also other factors as alteration in collagen fibers or microstructural crystal arrangements may be involved (Yerramshetty and Akkus, 2008). Further analyses of micro and nanoscale characteristics of the bone are necessary to fully elucidate the femoral material properties in this experimental model.

In summary, we demonstrated that lead intoxication magnified the impairment in bone biomechanics of OVX rats with a consequent enhancement of the risk of fracture. What appears to be happening, at least concerning mechanical properties, is that Pb would restrict the physiological architectural accommodation of long bones to compensate changes in material properties. It also seems to enhance the decreased bone mass in those bones already affected by OVX. The above results enable the discussion of the detrimental effects of lead intoxication in bone biology in elderly women.

Conflict of interest

All authors have no conflicts of interest.

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