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Dose effects of propranolol on cancellous and cortical bone in ovariectomized adult rats

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Running title: Prevention of gonadectomy by β-blockers a dose effect study

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List of abbreviations: **Sympathetic nervous system (SNS) ; β : Beta ; Bone Mineral Content (BMC), Bone Mineral Density (BMD) ; ovariectomized (OVX) ; Coefficients of Variation (CV) ; Standard Deviation (SD)**

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ABSTRACT

Animal studies suggest that bone remodeling is under β–adrenergic control via the sympathetic nervous system. The purpose of this study was to examine the preventive effect of different doses of non specific β blockers (propranolol) on trabecular and cortical bone envelopes in ovariectomized rats. Six-month old female Wistar rats were ovariectomized (OVX, n=60) or sham-operated (n=15). Then OVX rats were subcutaneously injected with propranolol 0.1mg.kg-1 (n=15), 5mg.kg-1 (n=15), 20mg.kg-1 (n=15) or vehicle (n=15) for 10 weeks. Tibial and femoral BMD were analyzed longitudinally by dual-energy x-ray absorptiometry (DEXA). At death, the left tibial metaphysis and L4 vertebrae were removed and microcomputed tomography (Skyscan 1072) was performed for trabecular bone structure investigation. Histomorphometry analysis was performed on the right proximal tibia to assess bone cell activities. After 10 weeks, OVX rats had decreased BMD, trabecular parameters and increased bone turnover, as well as cortical porosity compared to the Sham group ($p < 0.001$). Bone architecture alteration was preserved by propranolol 0.1mg.kg-1, due to higher trabecular number and thickness, (respectively +50.35%, +6.81% than OVX $p < 0.001$), and lower cortical pore number (-52.38% than OVX; $p < 0.001$). Animals treated by propranolol 0.1mg.kg-1 had a lower osteoclast surface, and a higher osteoblast activity compared to OVX. Animals treated by propranolol 20mg did not significantly differ from OVX rats. Animals treated by propranolol 5mg have been partially preserved from the ovariectomy.

These results showed a dose effect of β blockers. The lower the dose of propranolol breeding, the better the preventive effect against ovariectomy.

INTRODUCTION

Sympathetic nervous system (SNS) is involved in the mechano-transduction pathway in tail suspended rats (Levasseur et al., 2003) as well as in the negative effect induced by intracerebral injection of leptin on bone (Takeda et al., 2002). By antagonizing the SNS using propranolol, the bone symptoms due to intracerebral leptin and suspension are relieved (Takeda et al., 2002). In addition, the involvement of the SNS in the regulation of bone mass has been demonstrated both pharmacologically and genetically by an increase in osteoblast number and activity and a subsequent increase in bone mass in mice characterized by low sympathetic tone. For instance, some studies are considered mice treated with the β-blocker propranolol, mice deficient for dopamine β-hydroxylase (the step limiting enzyme responsible of catecholamine synthesis) and leptin-deficient Ob/Ob mice (Elefteriou et al., 2005a) (Elefteriou, 2005b). On the other hand, mice or rats treated with β-agonist isoproterenol or clenbuterol displayed a marked decrease in osteoblast number, activity, trabecular bone microarchitecture parameters and biomechanical properties (Takeda et al., 2002) (Bonnet et al., 2005). Therefore, β-blockers has been proposed to overcome the loss of bone mass occurring in postmenopausal women. Two recent epidemiological studies showed that the use of β-blockers was associated with a 30% decrease in fracture risk (Pasco et al., 2004) (Schlienger et al., 2004). Conversely, one study showed that the use of β-blockers did not present any link to bone mineral density (BMD) (Reid et al., 2005) indicating that the relationship between beta-blocker use and fracture risk needs further prospective studies.

It is estimated that up to 45% of adult and ageing people suffer from cardiovascular disease and osteoporosis. Therefore, the interest of a dual benefit effect of only one treatment on both heart and skeletal systems seems important.

However, serious issues have been raised regarding the prescription of propranolol for bone treatment. As the absence of leptin in Ob/Ob mice, the effects of altered sympathetic nervous system signaling on bone, vary throughout the skeleton according to local factors (Warden et al., 2005) (Hamrick et al., 2004). A decrease of muscle tissue mass has been observed in leptin knockout mice (Warmington et al., 2000) where noradrenalin (NA) showed low values, suggesting a decrease in muscle mass when the sympathetic nervous activity impairs or when the effect of NA is inhibited. As muscle mass correlates positively with bone mass (Banu et al., 2003), this observation suggests a negative effect of an alteration of the sympathetic nervous activity on bone mass.

Furthermore, different studies moderate the beneficial effect of the sympathetic nervous system inhibition on bone. Dhillon et al. did not observe any protection from ovariectomy induced bone loss in β1β2-adrenergic receptor KO mice (Dhillon et al., 2004). Preliminary data from Pierroz et al. described a decrease of cortical bone mass in β1β2-adrenergic receptor KO mice (Pierroz et al., 2005). Whether data from mouse models are relevant to humans remain to be addressed, as phenotypes of mouse and human are quite different.

Whether β blocker may improve bone quantity and quality in ovariectomized models has still to be investigated. Drug doses and forms of administration described in the literature are different. To our knowledge there is no specific information on low, middle and high dose effects of propranolol on axial and appendicular bone characteristics.

The aim of this work was to investigate the dose-effect of propranolol on bone of ovariectomized rats and to further elucidate its role on trabecular and cortical bone compartments.

MATERIALS AND METHODS

Animals and treatment

Ninety female Wistar rats (animal production center, Olivet, France) were acclimatized during two weeks and maintained under constant temperature (21 ± 2°C) and under 12h/12h light-dark cycles during the experience. The rats were housed by three in standard cages and provided with a commercial standard diet. At 34 weeks of age, animals were either ovariectomized (OVX, n = 60) or sham-operated (SHAM, n = 30). Bilateral ovariectomies were performed under anesthesia with pentobarbital. Sham operations were performed by exteriorizing the ovaries. One group of 15 rats (sham-operated at 34 weeks), chosen at random, was sacrificed at a 36 weeks age for baseline histomorphometry and microarchitecture evaluation. In the remaining rats, the whole body bone mineral density (BMD) was determined by dual-energy X-ray absorptiometry (DXA). These animals were then divided into five groups of 15 rats matched for the whole body BMD. At 36 weeks of age, treatment on3 OVX groups (OVX PRO) was initiated with propranolol (Sigma-Aldrich chimie, St. Quentin Fallavier) at doses of 0.1, 5 and 20 mg.kg-1, the last group of OVX being treated with sterile saline, injected subcutaneously 5 days per week, during 10 weeks. The SHAM group received saline injections at an identical dosage regimen. Dose and treatment protocol were based upon those described by Kondo et al. and Minkowitz B. et al.: therapeutic regime (range 0.1mg.kg-1 to 5mg.kg-1) and doping doses (20mg.kg-1) (Kondo and Togari, 2003) (Minkowitz et al., 1991). Propranolol 20 mg.kg-1 have been previously used for doping purpose in rats to study the effect on muscle, it corresponds to a 10-fold higher dose than those typically used to treat hypertension in humans (Kondo and Togari, 2003). Food consumption was recorded weekly for the SHAM group and this amount was then fed to the OVX rats over the following week.

Bone labeling of rats by an intraperitoneal injection of tetracycline (30 mg.kg-1 body mass) was performed 14 and 4 days before death. At the end of the study, all groups were sacrified

by an overdose of Pentobarbital. Soon after death, the weights of hindlimb muscle (soleus, gastrocnemius and extensor digitorum longus), uterus and the heart were recorded. In all rats, femurs, tibiae and lumbar vertebrae (L2, L3 and L4) were excised, cleared of fat and connective tissues. The right tibia and L2-L3 vertebrae were immediately fixed in 10% formaldehyde for 48h at +4°C. The other bones were placed in plastic tubes and frozen at –20°C for the microarchitectural and biomechanical tests. The procedure for care and killing of the animals was in accordance with the European Community standards on the care and use of the laboratory animals (Ministère de l'Agriculture, France, Authorization INSERM45-001).

Body mass, fat mass and lean mass

Body mass was recorded at weekly intervals throughout the study. At baseline, 3 , 6 and 9 weeks, lean and fat masses were measured by DXA using a specific rat body composition mode (line spacing 1.5 mm, and resolution 0.7 mm). As muscle mass represents 94-96% of lean mass, it is generally accepted to extrapolate from lean to muscle mass. The coefficients of variation (CV = SD/mean) were determined for these parameters from seven repeated measures with repositioning on one cadaverous animal. The CV was 4.76% and 1.64% respectively for fat and muscle masses.

Bone Mineral Content (BMC), area and Bone Mineral Density measurements

In vivo BMC and BMD of the left tibia and femur were measured at baseline, 3 , 6 and 9 weeks by DXA using a Hologic QDR-1000W apparatus adapted for small animals. An ultra-high-resolution mode (line spacing: 0.254 mm, resolution: 0.127 mm) was used with a 0.9mm-diameter collimator.

Ex-vivo, the left femur, left tibia and L4 vertebrae was bathed in saline water during DXA measurements (2.5cm height for all experiments). BMC and BMD of the total femur and total

tibia and two sub-regions were determined ex-vivo as previously described by Pastoureau et al. (Pastoureau et al., 1995). The first sub-region corresponds to the femoral distal metaphysis and to the tibia proximal metaphysis, which is rich in cancellous bone. The second region is the diaphysis, mainly represented by cortical bone. The coefficients of variation were determined by seven repeated measures on one femur, one tibia and one vertebrae over several days, with repositioning for each scan. The CV for BMC and BMD measurements ranged from 0.33% to 4.64%, depending on the bone site.

Morphological and Topological characteristics of the trabecular bone

Microarchitecture of the femoral, tibial and L4 vertebrae trabecular bone was investigated using a microcomputed tomograph (µCT, Skyscan 1072; Skyscan, Aartselaar, Belgium). The characteristics and methods have already been described elsewhere (McLaughlin et al., 2002). The X-ray source was set at 75 kV and 100µA, with a pixel size at 11µm. Four hundred projections were acquired over an angular range of 180° (angular step of 0.45°). The image slices were reconstructed using the cone-beam reconstruction software version 2.6 based on the Feldkamp algorithm. The registered data sets were segmented into binary images. Because of a low noise and the relative good resolution of the data sets, we used simple global thresholding methods. The trabecular bone was extracted by drawing ellipsoid contours with the "CT analyzer" software (Skyscan, Aartselaar, Belgium). Trabecular bone volume (BV/TV, %), trabecular number (Tb.N) and trabecular separation (Tb.Sp, µm) were calculated by the Mean Intercept Length (MIL) method. Trabecular thickness (Tb.Th, µm) was calculated according to the method of Hildebrand & Ruegsegger (Hildebrand and Ruegsegger, 1997). The structure model index (SMI), was measured for the prevalence of plate-like or rod-like trabecular structures, whereby 0 represents "plates" and 3 "rods" (Hildebrand and Ruegsegger, 1997). The degree of anisotropy (DA) was calculated by

superimposing parallel test lines in various directions on the 3D image. DA defines the magnitude of the preferred orientation of the trabeculae. The higher the DA, the more trabeculae are preferentially oriented (Ulrich et al., 1999).

The L4 microarchitecture analysis was performed on the middle region of L4 defined as 35-65% of the total height, which corresponds to two hundred slices. On the femur, two hundred and fifty slices were selected from the distal growth plate to the shaft proximally. On the tibia, two hundred and fifty slices were selected from the proximal growth plate to the shaft distally.

Cortical scanning electron microscopy

A proximal-diaphysis section of one tibia in each group was rendered anorganic by a 5% sodium hypochlorite treatment. The sections were then rinsed in water, dehydrated in acetone and dried. Bones were examined in a scanning electron microscope (Hitachi S-4500) with a 1kv energy. We observed two levels of pores. Small pores characterized by a small diameter (<10µm) present in all groups and large pores characterized by a diameter higher than 40µm. By the spot size of the X-ray source microcomputed tomography allows only large pore analysis higher than 11µm.

Morphological characteristics of the cortical bone

Cortical bone has been described in the femoral and tibial mid diaphysis using a microcomputed tomograph. The characteristics and methods have already been described elsewhere (Lotinun et al., 2004). We used the same acquisition characteristics as for trabecular bone. After reconstruction, the cortical bone was extracted by drawing polygon contours with the “CT analyzer” software. Before inversion of the image we applied simple global thresholding methods, and the algorithms developed for trabecular bone analysis were

used in order to characterize the network of the porosity. The porosity (BV/TV equivalent) was labelled Ct.Po. PoN (pore number, TbN equivalent) was measured by the MIL method. PoDm (pore diameter, TbTh equivalent) and PoSp (pore spacing, TbSp equivalent) were derived from the Hildebrand method and PoS/PoV (pore surface on volume, BS/BV equivalent) from triangulation method (Hildebrand and Ruegsegger, 1997).

Femur: One hundred slices were selected starting 12 mm far from the distal growth plate on the shaft proximally for cortical femur analysis, corresponding to the distal diaphysis region.

One hundred slices were selected starting 12 mm far from the proximal growth plate on the shaft distally for cortical tibia analysis, corresponding to the proximal diaphysis region.

Bone histomorphometry

Scanning electron microscopy and after 48h of fixation, the right tibia was dehydrated in absolute acetone and embedded in methylmethacrylate at low temperature according to the method described by Chappard et al. (Chappard et al., 1987). The central plane of the proximal part of the tibia was sliced frontally with a microtome (Reichert-Jung Polycut, Heidelberg, Germany). Five 8-µm thick sections were stained with Goldern's trichrome. They were used for measurement in secondary spongiosa of several parameters according to the ASBMR histomorphometry nomenclature (Parfitt et al., 1987) using an automatic image analyzer (BIOCOM, Lyon, France): BV/TV, Tb.Th, Tb.N, Tb.Sp, osteoid surface (OS/BS, %) and osteoid thickness (O.Th). Five 8-µm thick sections were stained with tartrate-resistant acid phosphatase activity (TRAcP) to measure active osteoclastic surfaces (Oc.S/BS) and osteoclast number (N.Oc/BS). Histodynamic parameters were determined on five unstained, 12 µm thick sections under UV light: mineral apposition rate (MAR, µm.day-1), single labeled surface (sLS/BS, %), and double-labeled surface (dLS/BS, %). Mineralizing surface per bone surface (MS/BS, %) was calculated by adding dLS/BS and one-half sLS/BS. Bone

formation rate (BFR/BS, $\mu m^3/\mu m^2.day-1$) was calculated as the product of MS/BS and MAR. Five 8-µm thick sections were stained with 4,6-Diamidino-2-Phenylindole Dihydrochl to measure adipocyte number (Ad.N, number of cells/mm^2) and relative volume of fat in the marrow cavity (Ad.V/MV, adipocyte volume/marrow volume).

The aforementioned parameters of bone resorption and formation were measured with a semiautomatic system made of a digitizing table (Summasketch-Summagraphics, Paris, France) connected to a personal computer and to a Reichert Polyvar microscope equipped with a drawing system (Camera Lucida; Reichert-Jung Polyvar).

Bone geometric characteristics

Due to the asymmetric shape of the femoral and tibial shaft, 2D bone slice at mid-diaphysis obtained by microcomputed tomography can be characterized by an ellipsoid shape. An ellipse yields two diameters, a large one corresponding to the medio-lateral (ML) direction and a small one corresponding to the antero-posterior (AP) direction. These two diameters were assessed at the mid-diaphysis (=50% of the femur or tibia length) of the left femur and left tibia. Cortical width of the long bone is an average of the cortical width measured in ML and AP direction.

Inner and outer cortical width was measured on 5 slices located at 50% of the total height. The results represent an average of those 5 slices. Geometric measurements are illustrated in figure 1.

Statistical analysis

Results are presented as means ± SEM. Bone mineral density of the tibia and femur were analyzed using a one-way ANOVA with repeated measurements at baseline, 3, 6 and 9

weeks. A one-way ANOVA test was used to compare the groups for geometric data, BMD, architectural parameters, biochemical analyzes and histomorphometric parameters. If needed, Post hoc differences were determined with the Newman-Keuls test and correlations were performed using the Pearson's test. Significance was defined as $p<0.05$.

RESULTS

General observations:

For all groups, the body mass of rats increased from baseline to the end of the treatment. Despite receiving a similar amount of food as the SHAM group, OVX and OVX PRO 20mg groups were 5% heavier at the end of the treatment. Animals of OVX PRO 0.1mg and 5mg groups increased their body mass similar that of SHAM group (Table 1).

At necropsy, the uterine mass in all OVX animals was reduced by more than 75% compared with the SHAM, indicating a successful ovariectomy (Table 1).

We did not observed significant difference between groups for the gastrocnemius and extensor digitorum longus but we notice a lower soleus mass in OVX PRO 20mg compared to OVX group (Table 1).

Tibial, femoral and vertebral BMD

Longitudinal BMD measurement at the tibia and femur revealed a significantly higher BMD gain in OVX PRO 0.1mg group compared to OVX group. At the tibia, animals treated with 5mg of propanolol revealed a higher BMD increase compared to OVX group (not noticed in the femur). Femoral BMD decreased more significantly in the OVX PRO 20mg group than in OVX animals (not noticed in the tibia) (Fig. 2). At the end of the study, BMD

measurements at tibial, femoral and vertebral sites revealed a significantly lower BMD in OVX and OVX PRO 20mg groups compared to SHAM, OVX PRO 5mg and OVX PRO 0.1mg (Table 2). For each bone site no significant difference was observed between SHAM, OVX PRO 5mg and OVX PRO 0.1mg groups.

Tibial and femoral metaphysis of OVX PRO 0.1mg group had similar BMD than the SHAM group, both were significantly higher than OVX and OVX PRO 20mg groups. Animals of the OVX PRO 5mg group, presented a significantly lower tibial metaphysis BMD compared to the SHAM group, whereas there was non significant difference on the femur metaphysis BMD. Tibial and femoral metaphysis BMD in the OVX PRO 20mg group did not differ from the OVX group.

Concerning the diaphysis BMD, there was non significant difference between groups in the tibia. We observed lower femoral diaphysis BMD in the OVX PRO 20mg group compared to OVX PRO 5mg and OVX PRO 0.1mg groups.

Trabecular bone microarchitecture

Proximal tibia. At the end of the treatment, when compared to the SHAM animals, 3D trabecular structure of the OVX rats revealed a 14.65% loss of trabecular thickness and a 50.90% loss of trabecular number. OVX animals yielded an overall reduction in trabecular bone volume fraction of 54.65% (Fig. 3). Trabecular bone proportion in OVX PRO 0.1mg had increased by +50.35% compared to the OVX group, and BV/TV finally was similar to that of the SHAM group. We observed comparable differences concerning trabecular number (Fig. 3). Trabecular thickness increased by +6.81% in OVX PRO 0,1mg compared to OVX group. The SMI increase from baseline was significantly higher in the OVX group (+77.45%, $p<0.001$) compared to the SHAM group (+10.72%). SMI in the OVX PRO 0.1mg group was

54% lower than that of the OVX group and was similar compared to the SHAM group. The DA increased significantly in the OVX group (+48,98%, $p<0.01$) compared to OVX PRO 20mg, 5mg and 0.1 mg groups where the values were similar compared to the SHAM group. For all other tibial microarchitecture parameters, the OVX PRO 20mg group did not differ significantly from the OVX group (Fig 3). Microarchitecture parameters of OVX PRO 5mg group were comparable to OVX PRO 0.1mg group except for the Tb.Th which was not significantly different compared to the OVX group (Fig 3).

Distal femur. At the end of the treatment, femoral microarchitecture parameters differences between groups were comparable to those observed in the tibia. (Table 3).

L4 Vertebrae. The OVX group displayed lower BV/TV, Tb.N, Tb.Th (-31.20%, –24.83% and -8.26%; $p < 0.001$ respectively) but also higher Tb.Sp (+22.92, $p < 0.001$) and SMI (+29.51%, $p < 0.001$) than the SHAM group, indicating a loss of architectural integrity. No significant difference was found between the SHAM and OVX PRO 20mg group, except for BV/TV which was significantly lower in the OVX PRO 20mg group (-17.53%, $p < 0.01$). But the OVX PRO 20mg group had significantly higher BV/TV than the OVX group (+16.67%, $p < 0.01$). Animals of OVX PRO 0.1mg had higher BV/TV (+32.26%, $p < 0.01$) compared to the OVX group. Structural model index in the OVX PRO 0.1mg group was significantly lower (-30.18%, $p < 0.01$) than in the OVX group. Microarchitecture parameters of OVX PRO 5mg group were comparable to OVX PRO 0,1mg for BV/TV and Tb.N but not for SMI and Tb.Th which were not significantly different compared to the OVX group (Table 3).

Cortical parameters:

Tibia. MicroCT analyses revealed that the OVX group significantly decreased the cortical width compared to SHAM (-6.21%, $p < 0.01$) (Table 4). Animals of OVX PRO 0,1mg and SHAM groups increased their cortical width respectively by +3.33% and +4.01% compared to baseline values (non significant). OVX and OVX PRO 20mg groups had significantly decreased their cortical width by -3% and -9% compared to baseline values ($p<0.01$).

At the end of the treatment, when compared to the SHAM group, the cortical structure revealed a higher pore diameter (+9.67%, non significant) and a higher pore number (+47.62%, $p < 0.01$) in the OVX group, which yielded an overall higher cortical porosity of +51.66% ($p<0.05$). Cortical porosity tended to be lower in OVX PRO 0.1mg (-63.63% of Ct.Po, compared to OVX) than in OVX PRO 5mg (-11,00% of Ct.Po, compared to OVX). Pore number was significantly lower in OVX PRO 0.1mg group compared to OVX, OVX PRO 5mg and OVX PRO 20mg groups. Animals of OVX PRO 20mg had similar cortical parameters than the OVX group.

Femur. Cortical width in the OVX group (392.0µm) did not differ significantly from the SHAM group (373.7µm). Animals of OVX PRO 20mg had significantly lower cortical width (344.9µm) compared to the OVX group ($p < 0.05$). Cortical width of OVX PRO 5mg (365.5µm) and 0.1mg (374.3µm) groups did not significantly differ from OVX and SHAM groups. The other cortical parameters did not significantly differ between groups but the same trend was observed in the tibia.

L4 vertebrae. Inner cortical width in the OVX group (215.8µm) was significantly lower than in the SHAM group (240.0µm). Animals of OVX PRO 20mg group (194.3µm) did not significantly differ from the OVX group. Inner cortical thickness of OVX PRO 5mg (248.1µm) and 0.1mg (263.3µm) groups were significantly higher than OVX animals. The

same statistical difference was observed between groups for the outer cortical width and for the cortical area.

Histomorphometry measurement

Trabecular bone volume and structure in the proximal tibial metaphyses. Static histomorphometry results were close to the morphometric parameters obtained by µCT (data not shown). Correlations of BV/TV, Tb.N, Tb.Sp and Tb.Th between histomorphometry and µCT were all significant (r ranged between 0.67 and 0.85, $p < 0.001$).

Bone formation and resorption. At the end of the experiment, the mineralized surface of the OVX group had significantly increased by 61% compared to baseline. Animals of OVX PRO 5mg and 0.1mg groups had lower MS/BS than OVX and OVX PRO 20mg groups, but MS/BS were significantly higher than in the SHAM group.

After 10 weeks, the osteoid surface was significantly higher in OVX, OVX PRO 0.1mg, OVX PRO 20mg groups compared to SHAM and OVX PRO 5mg groups ($p<0.001$).

There was no difference between MAR of the OVX and SHAM groups. Animals of OVX PRO 0.1mg, 5mg and 20mg groups had significantly higher MAR compared to SHAM and OVX groups. MAR was significantly higher ($p<0.001$) in OVX PRO 0.1mg (+35.92%, compared to OVX) than in OVX PRO 5mg (+22.00%, compared to OVX).

From baseline, osteoclast surface and osteoclast number increased by 70% and 73.2%, respectively, in the OVX group and remained high in the OVX PRO 20mg group. Animals of the OVX PRO 5mg group had significantly lower osteoclast surface compared to the OVX PRO 20mg group but significantly higher Oc.S/BS than the SHAM group. Animals of OVX PRO 0.1mg had similar Oc.S/BS and Oc.N than the SHAM group (Fig. 5).

At the end of the treatments, the N.At and At.S/Ma.Ar values of the OVX and OVX PRO 20mg groups were significantly higher than those of the SHAM, OVX PRO 5mg and 0.1mg groups. A significant increase of the At.S/Ma.Ar in all the groups was observed with a higher increase in OVX and OVX PRO 20mg groups (89.33% and 89.20%, respectively) than in SHAM, OVX PRO 5mg and 100µg groups (71.26%, 68.10% and 74.47%, respectively) (Fig. 5).

N.At and At.S/Ma.Ar correlated with the bone resorption parameters (Osteoclast number, $r = 0.77$, $p < 0.001$ and osteoclast surface, $r = 0.61$, $p < 0.001$), but not with the bone formation parameters.

DISCUSSION

The main findings of this study was that propranolol effects on the skeleton are dose and site dependant in rats models. Propranolol 0.1mg bred better preventive effects on trabecular and cortical bone in ovariectomized rats. The dose of 5mg of propranolol prevents the effects of ovariectomy on architecture, but not on the femoral BMD and the tibial osteoclast surface. Globally, the high – dose treatments (OVX PRO 20mg group) did not induce any effect, except, for tibial BMD and osteoclast surface which are respectively lower and higher than in OVX. The low BMD observed in the tibia of the group treated by propranolol 20mg compared to the OVX group are associated with a lower gastrocnemius muscle mass. The lower muscle mass is probably due to a decreased activity of the animals which presented a lower heart rate and lower motricity than the other groups (analysis were made with an electrocardiogram and an open field test, unpublished data). The fact that patients with muscular dystrophy are osteopenic, provides further evidence for a functional association between decreased muscle mass and low bone mass (Aparicio et al., 2002). Our

results with animals treated by propranolol 20mg caution the findings obtained on β1β2-adrenergic receptor KO mice in appendicular bone (tibia, femur) where no preventive effect of propranolol on BMD and in metaphysis architecture were shown (Pierroz et al., 2005) (Elefteriou et al., 2005a). However, we noticed an increase of BMD L4 and a higher trabecular bone volume in the axial bone (vertebrae L4) of OVX PRO 20mg group compared to the OVX group. These results on the vertebrae are in accordance with the results of Takeda et al. and Levasseur et al. (Takeda et al., 2002) (Levasseur et al., 2003). This suggests that bone cell of the spine responds to propranolol 20mg in a fundamentally different manner than cells of the appendicular bone. Another possible explanation is the potentially osteogenic effects of propranolol on long bone are overcome by the consequences of the muscle mass and physical activity decrease. In fact, the preventive effect of propranolol on axial bone cannot be effective if there is a decrease of muscle mass and activity. Regardless tentative explanations, it is clear that propranolol at a dose of 20mg has different effects on axial and appendicular skeleton.

It was shown that 5mg or 0.1mg of propranolol prevents the deterioration of the cancellous bone due to ovariectomies. However, the general positive effects observed in OVX rats treated with propranolol 5mg or 0.1mg differ for several parameters particularly in the cellular activity. Animals treated by propranolol 0.1mg marked a similar mineralized surface than OVX and OVX PRO 5mg groups but a higher osteoblast activity compared to SHAM, OVX and OVX PRO 5mg groups. (MAR + 40%, compared to OVX and + 18%, compared to OVX PRO 5mg). Animals treated with propranolol 5mg have similar osteoclast surface than OVX. Whereas, propranolol 0.1mg treatment prevents the increase in osteoclast surface. Other drugs classically used in therapeutics, act only on bone resorption or on bone formation. Propranolol 0.1mg effect on osteoblast and osteoclast is close to the decoupling effect

described for the strontium ranelate (Marie, 2005) a new molecule, which has been announced to play a main role in the future management of osteoporosis (Reginster et al., 2005). However, the effect of propranolol on the osteoblast did not indicate if there is an increased of osteoblast number or a decreased of apoptosis of this cell.

The net results of these cellular changes were a tibial BMD decrease in the OVX group whereas SHAM BMD did not change during the experiment. Animals treated with propranolol 5mg and 0.1mg had the same tibial BMD evolution as SHAM whereas OVX PRO 20mg group had a higher tibial BMD decrease than the OVX group. On the proximal metaphysis BMD, only the animals treated by 0.1mg of propranolol had higher values than the OVX group. Non significant difference was observed on the diaphysis BMD between groups. Total vertebrae BMD difference between groups were similar to total femur.

Previous reports (Levasseur et al., 2003) did not include bone microarchitecture analysis. In the present study, microarchitectural parameters revealed a severe microarchitectural alteration in OVX and OVX PRO 20mg groups in proximal tibia, as demonstrated by lower Tb.N and Tb.Th (-51% and -15%, compared to SHAM, respectively, in both groups). Perforation of trabeculae and subsequent cavity enlargement by osteoclasts have been shown to be responsible for this loss of cancellous structural integrity (Abe et al., 1999). We observed the same alterations on the vertebrae, but only in the OVX group and with less severity. Propranolol at 5mg and 0.1mg doses, totally prevent the effects of OVX on all the microarchitectural parameters in proximal tibia and distal femur. In vertebrae, it was possible to separate the microarchitectural effect of 0.1mg and 5mg of propranolol. Animals treated by propranolol 5mg had Tb.Th and SMI in the same range as the OVX group, whereas propranolol 0.1mg treatment had significant higher Tb.Th and higher proportion of plate shapes than the OVX group.

Propranolol 5mg or 0.1mg treatment had also a preventive effect on adipocyte proliferation observed in OVX rats. Propranolol is suggested to inhibit the differentiation of the adipocyte lineage and therefore to contribute by this mechanism to the inhibition of the bone deterioration induced by estrogen deficiency (Vicennati et al., 2002). The high adiposity observed in OVX PRO 20mg contrasts the last suggestion of Vicennati et al (Vicennati et al., 2002). But bone marrow adipogenesis is known to increase with hindlimb unloading (Ahdjoudj et al., 2002) (Justesen et al., 2001). Thus, the high adiposity observed in the tibia of OVX PRO 20mg group was not surprising, given the decrease of physical activity and therefore gastrocnemius muscle mass.

Scanning electron microscopy and μCT analysis revealed that cortical porosity was primarily located close to the endocortical surface at mid-diaphysis, whereas they were more present in periosteal surface at the proximal diaphysis. Animals treated with propranolol 0.1mg had lower cortical porosity, pore number and higher space between pores compared to the OVX group. This observation respond to Reid et al. questioners who suggested a hypothetic effect of propranolol on cortical properties (Reid et al., 2005).

The usual contradictory results reported in the literature concerning the relation-ship between β blockers and human bone might be explained by different doses of treatment as was reported by De Vries et al. in there preliminary data regarding the use of β blockers and the potential risk of hip and vertebral fracture (De Vries et al., 2005). The study of Pasco et al. and Reid et al. are limited by a lack of information with respect to the duration and doses of β blockers treatment (Pasco et al., 2004) (Reid et al., 2005). Furtheremore, based upon the study of Kondo and Togari, it was suggested (Reid et al., 2005) that the dose of propranolol necessary to block the effects of sympathetic activation on bone must be doses reaching 10 fold higher than for a therapeutical treatment for hypertension (20 mg.kg-1) (Kondo and Togari, 2003). Accordingly to Reid et al. the present results suggest a dose effect of

propranolol showing a better effect with the lowest dose (Reid et al., 2005). In parallel to the bone investigation we have made an evaluation of the cardiac hemodynamic functions. As expected the various parameters of cardiac functions were affected in rats treated with 20mg.kg-1.day-1 propranolol, few of them were affected by 5mg.kg-1.day-1 propranolol but none were changed with the dose of 0.1mg.kg-1.day-1 (unpublished data). These cardiac investigation are in accordance with the main study achieve on heart and β-blockers (Yaoita et al., 2002). These results, consistent with the dominant nature of the adrenergiqueβ*2* receptor-deficient mice bone and cardiac phenotype suggested that low doses of β-blockers affecting only β2 receptor may be an efficacious osteoporosis treatment. Our results are comparable to those reported by Minkowitz et al., suggesting a preventive effect of β blockers with a dose of 0.1mg.kg-1 body mass (Minkowitz et al., 1991). In addition, our results describe the specific impact of β blockers propranolol on bone architecture and cellular activity, showing its effect on the cortical features of ovariectomized rats. These data must be confirmed by a clinical study taking into account the dose and duration of such treatment.

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Legends for Figures

FIG. 1 : Geometric measurement of long bone and vertebrae. Inner and outer cortical widths were measured by microcomputed tomography on 5 slices located at 50% of the total height. On long bone, 2D bone slice at mid-diaphysis obtained by microcomputed tomography can be characterized by an ellipsoid shape. An ellipse yields two diameters, a large one corresponding to the medio-lateral (ML) direction and a small one corresponding to the antero-posterior (AP) direction. These two diameters were assessed at the mid-diaphysis (=50% of the femur or tibia length) of the left femur and left tibia.

FIG. 2 : Time course of total BMD change in tibia and femurs. —□— OVX, —◇— SHAM, —▲— OVX PRO 20mg.kg-1, —■— OVX PRO 5mg.kg-1, —X— OVX PRO 0.1mg.kg-1. The notes a, b, c, d, e express significantly statistical comparisons between groups. a: comparison to SHAM group ($p<0.05$). b: comparison to OVX group ($p<0.05$). c: comparison to OVX 0.1mg.kg-1 ($p<0.05$). d: comparison to OVX 5mg.kg-1 ($p<0.05$). e: comparison to OVX 20mg.kg-1 ($p<0.05$). Means ± SEM

FIG. 3 : Trabecular bone structure parameters of the proximal tibia assessed by microcomputed tomography. The notes a, b, c, d, e, f express significantly statistical comparisons between groups. a: comparison to SHAM group ($p<0.05$). b: comparison to OVX group ($p<0.05$). c: comparison to OVX 0.1mg.kg-1 ($p<0.05$). d: comparison to OVX 5mg.kg-1 ($p<0.05$). e: comparison to OVX 20mg.kg-1 ($p<0.05$). f: comparison to baseline ($p<0.05$). Trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp, µm), trabecular thickness (Tb.Th, µm), structure model index (SMI, 0-plate to 3-rod), degree of anisotropy (DA). Histograms represent means ± SEM.

FIG. 4 : Scanning electron micrographs taken from tibial proximal-diaphysis. Pore geometry image represents the two levels of pore diameter: large pore (>40µm), small pore (<10µm).

FIG. 5 : Histomorphometry parameters of the proximal tibia after 10 weeks of propranolol treatment. The notes a, b, c, d, e express significantly statistical comparisons between groups. a: compared to SHAM group ($p<0.05$). b: compared to OVX group ($p<0.05$). c: compared to OVX 0.1mg.kg-1 ($p<0.05$). d: compared to OVX 5mg.kg-1 ($p<0.05$). e: compared to OVX 20mg.kg-1 ($p<0.05$). Baseline group have the same difference than SHAM group. Osteoclastic surfaces (Oc.S/BS), mineral apposition rate (MAR), mineralizing surface per bone surface (MS/BS), adipocyte number (N.At, number of cells/mm²). Histograms represent means ± SEM.

TABLE 1. Effect of propranolol on body, muscle and uterine weight. Mean ± SD. The notes a, b, c, d, e design significant statistical comparisons. a: comparison to SHAM group ($p<0.05$). b: comparison to OVX group ($p<0.05$). c: comparison to OVX 0.1mg.kg-1 group ($p<0.05$). d: comparison to OVX 5mg.kg-1 ($p<0.05$). e: comparison to OVX 20mg.kg-1 ($p<0.05$). Body weight 5 weeks correspond to measurement done 5 weeks after ovariectomy (3 weeks after the beginning of the treatment).

Weight (g)	SHAM	OVX	OVX 0.1mg.kg-1	OVX 5mg.kg-1	OVX 20mg.kg-1
Body before OVX	325.00 ± 25.92	340.42 ± 40.21	318.78 ± 24.59	317.20 ± 27.29	329.53 ± 31.34
Body 5 weeks	358.76 ± 21.34 [c,d]	384.92 ± 9.68 [c,d]	327.41 ± 29.51 [a,b,e]	323.64 ± 26.96 [a,b,e]	378.60 ± 27.73 [c,d]
Body 10 weeks	359.15 ± 16.73 [b,e]	407.32 ± 40.93 [a,c,d]	339.89 ± 34.94 [b,e]	340.18 ± 20.55 [b,e]	398.24 ± 36.48 [a,c,d]
Soleus	0.11 ± 0.01	0.12 ± 0.01 [e]	0.09 ± 0.01	0.10 ± 0.01	0.08 ± 0.01 [b]
Heart	0.77 ± 0.09	0.91 ± 0.09	0.87 ± 0.09	0.89 ± 0.07	0.85 ± 0.09
Uterine	0.73 ± 0.15 [b,c,d,e]	0.20 ± 0.10 [a]	0.14 ± 0.02 [a]	0.15 ± 0.04 [a]	0.18 ± 0.05 [a]

TABLE 2. Bone densitometry at the femur, tibia and L4 vertebrae in the five groups of rats: sham, ovariectomized with placebo, or receiving propranolol 20mg, 5mg and 0.1mg.kg-1. Bone density (BMD) are expressed by mean ± SEM. The notes a, b, c, d ,e express significantly statistical comparisons between groups. a: comparison to SHAM group ($p<0.05$). b: comparison to OVX group ($p<0.05$). c: comparison to OVX 0.1mg.kg-1 ($p<0.05$), d: comparison to OVX 5mg.kg-1 ($p<0.05$); e: comparison to OVX 20mg.kg-1 ($p<0.05$).

	BMD (mg/cm²)	SHAM	OVX	OVX 0.1mg.kg-1	OVX 5mg.kg-1	OVX 20mg.kg-1
FEMUR BMD	Total	244 ± 2.5 [b,e]	224.8 ± 2.7 [a,c,d]	240.5 ± 2.8 [b,e]	240.1 ± 4.6 [b,e]	222.3 ± 2.7 [a,c,d]
	Distal metaphysis	220.1 ± 4.5 [b,e]	188.3 ± 3.7 [a,c,d]	217.0 ± 3.6 [b,e]	217 ± 4.1 [b,e]	183.9 ± 3.1 [a,c,d]
	Diaphysis	222.0 ± 2.4	213.4 ± 3.2	223.8 ± 3.8 [e]	225.7 ± 5.5 [e]	211.0 ± 3.0 [c,d]
TIBIA BMD	Total	229.9 ± 2.1 [b,e]	208.0± 2.0 [a,c,d]	226.2 ± 2.5 [b,e]	223.0 ± 3.2 [b,e]	207.8 ± 1.9 [a,c,d]
	Proximal metaphysis	194.3 ± 1.9 [b,e]	176.5 ± 2.0 [a,c,d]	188.2 ± 3.3 [b,e]	181.2 ± 4.2 [e]	171.8 ± 2.5 [a,c,d]
	Diaphysis	203.6 ± 2.2	196.0 ± 2.9	203.7 ± 3.7	202.3 ± 3.1	198.4 ± 2.3
VERTEBRAE	L4	242.6 ± 3.5 [b,e]	216.0 ± 3.8 [a,c,d]	238.6 ± 4.1 [b,e]	235.4 ± 5.2 [b,e]	211.3 ± 3.1 [a,c,d]

TABLE 3. Trabecular bone structural parameters of the distal femur and L4 vertebrae by microcomputed tomography in sham, ovariectomized with placebo (OVX), or receiving propranolol (PRO) 20mg, 5mg and 0.1mg and at baseline. The notes a, b, c, d, e, f express significantly statistical comparisons between groups. a: comparison to SHAM group ($p<0.05$). b: comparison to OVX group ($p<0.05$). c: comparison to OVX 0.1mg.kg-1 ($p<0.05$). d:comparison to OVX 5mg.kg-1 ($p<0.05$). e: comparison to OVX 20mg.kg-1 ($p<0.05$). f: comparison to baseline ($p<0.05$).

	Parameters	Baseline	SHAM	OVX	OVX 0.1mg.kg-1	OVX 5mg.kg-1	OVX 20mg.kg-1
DISTAL FEMUR	BV/TV (%)	26.07 ± 1.21 [b,c,e]	27.66 ± 1.44 [b,c,e]	15.63 ± 0.77 [a,c,d,f]	22.17 ± 1.36 [b,e,f]	24.85 ± 1.11 [b,e]	12.70 ± 0.91 [a,c,d,f]
	Tb.N (1/mm)	2.27 ± 0.06 [a,b,e,c,d]	3.13 ± 0.25 [b,e,f]	1.59 ± 0.07 [a,c,d,f]	2.71 ± 0.10 [b,e,f]	2.94 ± 0.08 [b,e,f]	1.41 ± 0.08 [a,c,d,f]
	Tb.Th (µm)	98.5 ± 2.7	105.0 ± 5.4	98.3 ± 1.8	97.2 ± 2.2	99.8 ± 1.9	90.2 ± 2.1 [a]
	Tb.Sp (mm)	0.37 ± 0.05 [b,e]	0.35 ± 0.04 [b,e]	0.75 ± 0.04 [a,c,d,f]	0.31 ± 0.03 [b,e]	0.29 ± 0.05 [b,e]	0.77 ± 0.04 [a,c,d,f]
	SMI	0.99 ± 0.09 [b,c,d,e]	1.13 ± 0.12 [b,c,d,e]	1.66 ± 0.04 [a,f]	1.64 ± 0.10 [a,f]	1.39 ± 0.06 [a,f]	1.81 ± 0.08 [a,d,f]
	DA	2.10 ± 0.15 [e]	2.14 ± 0.07 [e]	2.69 ± 0.12	1.76 ± 0.18 [e]	1.64 ± 0.14 [e]	3.01 ± 0.15 [a,c,d,f]
L4 VERTEBRAE	BV/TV (%)	31.92 ± 1.46 [b,e]	33.02 ± 1.52 [b,e]	22.69 ± 1.42 [a,c,d e,f]	33.50 ± 1.46 [b,e]	30.72 ± 1.46 [b]	27.23 ± 1.50 [a,b,c, f]
	Tb.N (1/mm)	2.79 ± 0.09 [b]	2.98 ± 0.12 [b]	2.24 ± 0.13 [a,c,d,f]	3.04 ± 0.11 [b]	2.87 ± 0.12 [b]	2.60 ± 0.12
	Tb.Th (µm)	111.40 ± 3.01 [b]	110.08 ± 2.01 [b]	101.01 ± 2.90 [a,c,f]	111.20 ± 2.30 [b]	106.40 ± 1.63	107.10 ± 1.39
	Tb.Sp (mm)	0.28 ± 0.02 [b]	0.27 ± 0.02 [b]	0.35 ± 0.01 [a,c,d,f]	0.27 ± 0.01 [b]	0.27 ± 0.01 [b]	0.33 ± 0.02
	SMI	1.36 ± 0.07	1.12 ± 0.08 [b]	1.59 ± 0.11 [a,c]	1.11 ± 0.11 [b]	1.29 ± 0.11	1.49 ± 0.09
	DA	3.15 ± 0.27	3.25 ± 0.37	5.21 ± 1.68	3.80 ± 0.61	4.37 ± 0.65	2.61 ± 0.20

Trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp, µm), trabecular thickness (Tb.Th, µm), structure model index (SMI, 0-plate to 3-rod), degree of anisotropy (DA) as defined in Parfitt et al. 1987. Mean ± SEM.

TABLE 4. Cortical bone parameters of the tibial diaphysis in sham, ovariectomized without treatment (OVX), or receiving propranolol 20mg.kg-1, 5mg.kg-1 and 0.1mg.kg-1. Cortical bone has been described using a microcomputed tomograph. One hundred slices were selected starting 12 mm far from the proximal growth plate on the shaft distally for cortical tibia analysis, corresponding to the proximal diaphysis region. The notes a, b, c, d, e express significantly statistical comparisons between groups. a: compared to SHAM group ($p<0.05$). b: compared to OVX group ($p<0.05$). c: compared to OVX 0.1mg.kg-1 ($p<0.05$). d: compared to OVX 5mg.kg-1 ($p<0.05$). e: compared to OVX 20mg.kg-1 ($p<0.05$). At baseline microcomputed tomography did not allow to measure cortical porosity.

Parameters	SHAM	OVX	OVX 0.1mg.kg-1	OVX 5mg.kg-1	OVX 20mg.kg-1
Ct.Po (%)	$0.87 \pm 0.10^{b,e}$	1.8 ± 0.23^{a}	1.1 ± 0.18^{e}	1.6 ± 0.21	$2.0 \pm 0.17^{a,c}$
PoN (1/µm)	$0.11 \pm 0.01^{b,d,e}$	$0.21 \pm 0.02^{a,c}$	$0.10 \pm 0.01^{b,d,e}$	$0.20 \pm 0.03^{a,c}$	$0.20 \pm 0.01^{a,c}$
PoDm (µm)	69.00 ± 6.10	76.22 ± 5.41	83 ± 9.00	68.2 ± 5.57	85.3 ± 6.03
PoSp (µm)	361.6 ± 8.00^{e}	346.2 ± 7.01	358.5 ± 7.40	351.0 ± 9.07	323.9 ± 7.12^{a}
PoS/PoV (%)	$93.48 \pm 4.50^{c,e}$	79.74 ± 4.18	77.46 ± 6.22^{a}	89.68 ± 4.06	74.04 ± 4.61^{a}
Cortical width (µm)	$575.21 \pm 5.8^{b,d}$	$536.50 \pm 9.8^{a,c,e}$	$507.77 \pm 9.8^{b,d,e}$	$541.21 \pm 8.0^{a,c}$	$570.73 \pm 12.6^{b,c}$

The porosity was labelled Ct.Po, pore number (PoN), pore diameter (PoDm), pore spacing (PoSp), pore surface on volume (PoS/PoV), Mean ± SEM.

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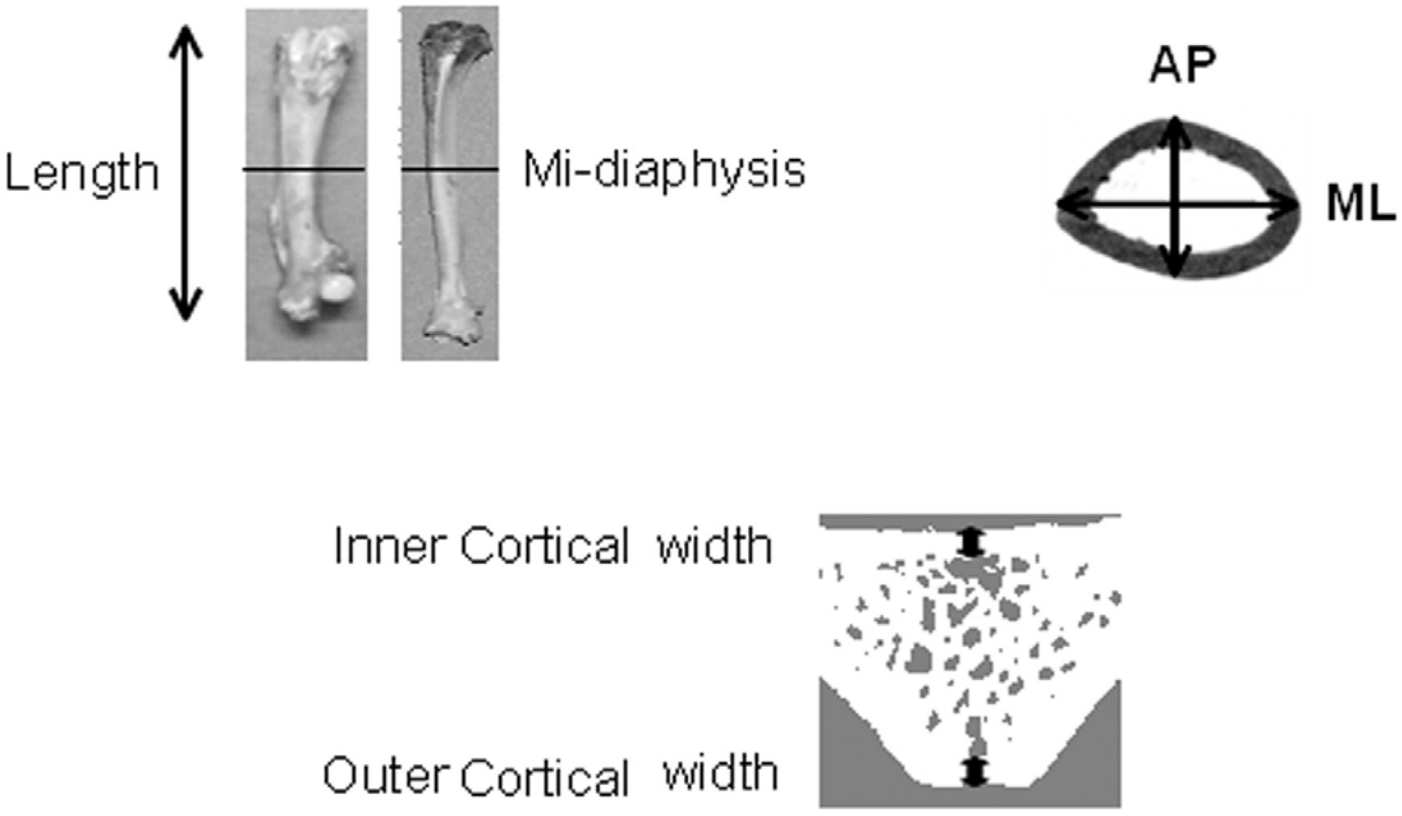

FIG. 1

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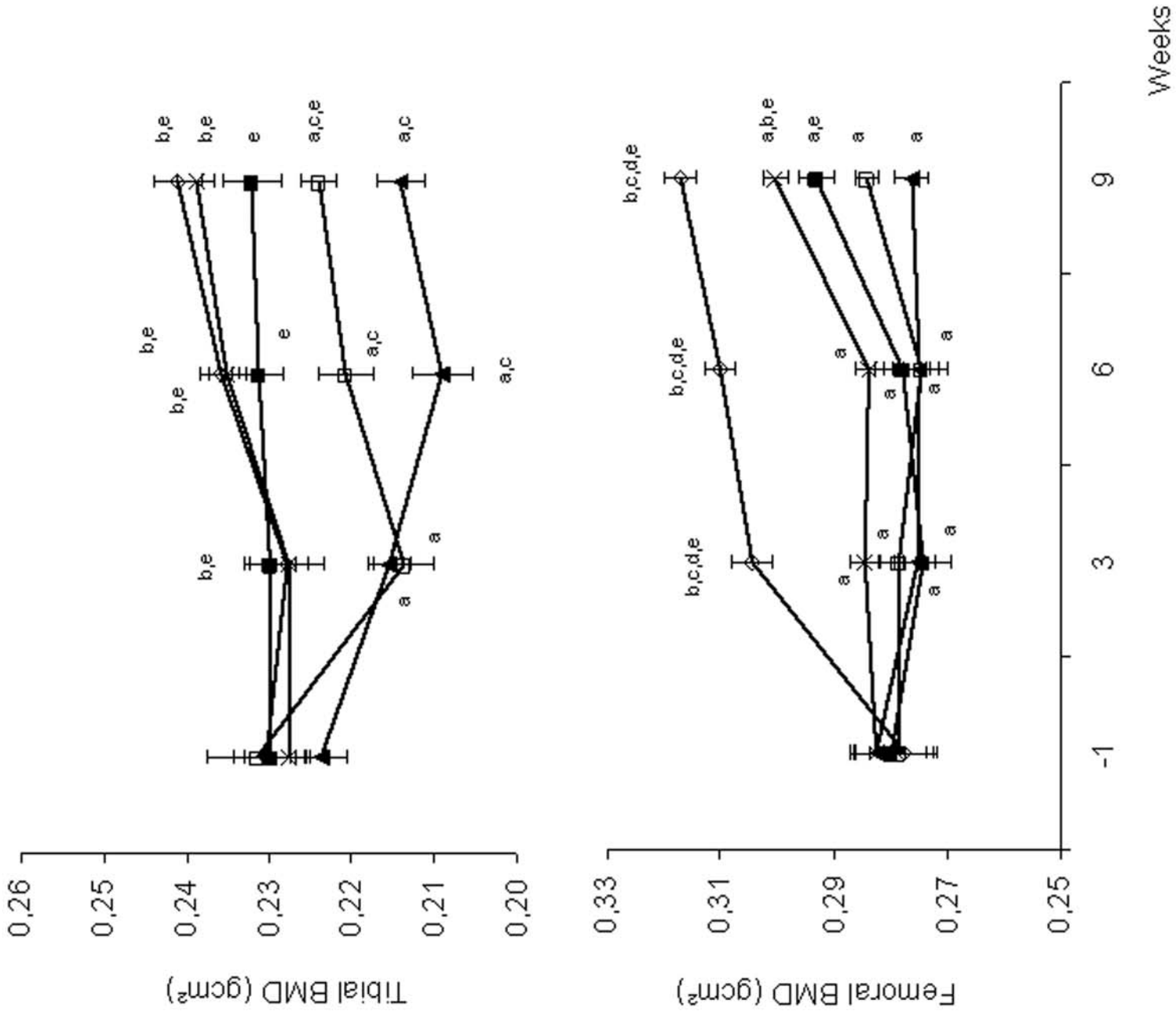

FIG. 2.

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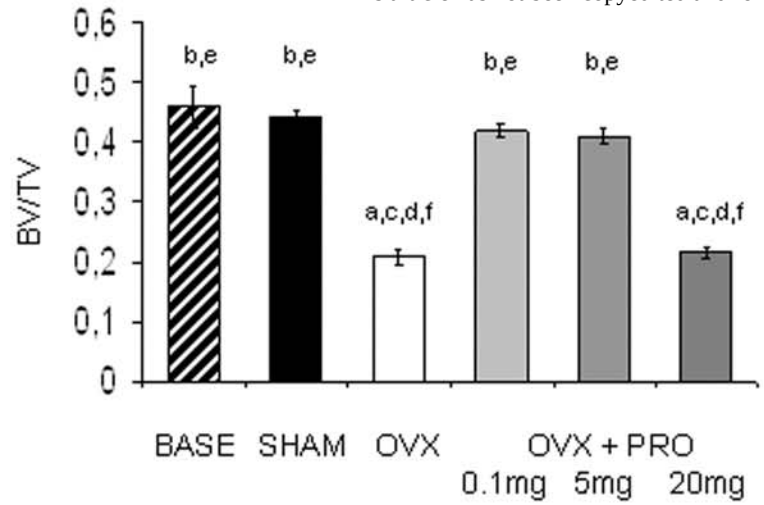

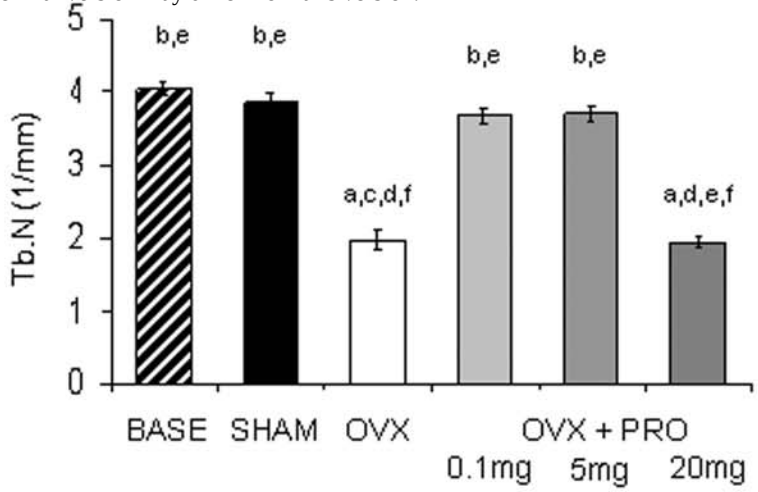

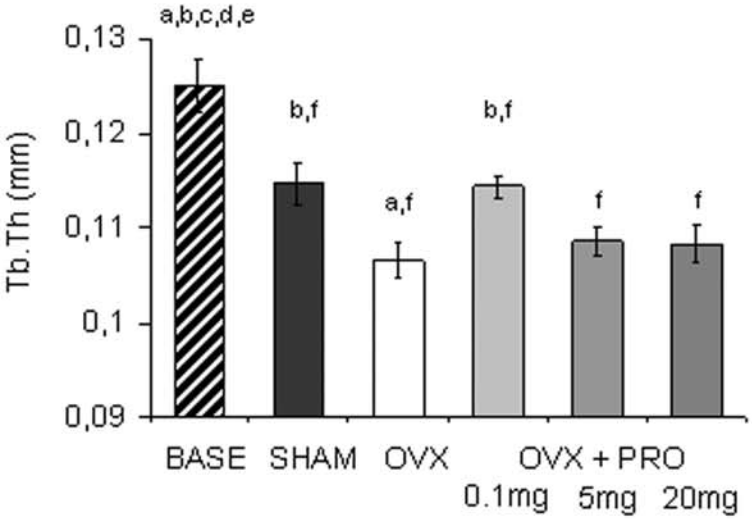

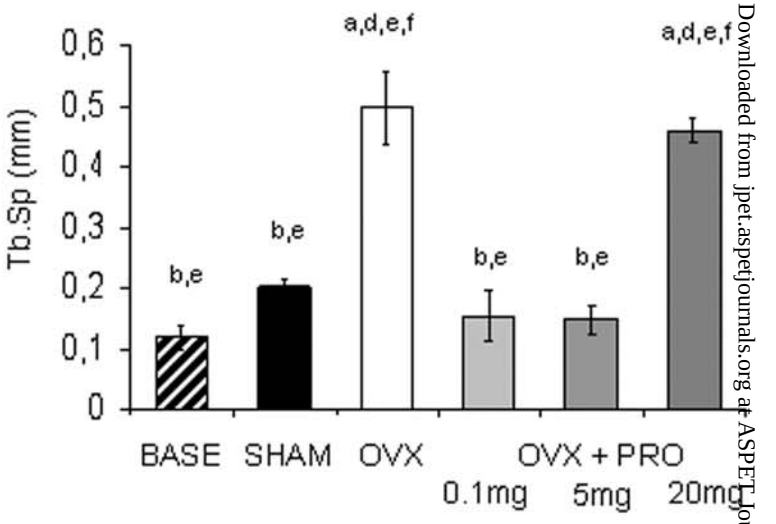

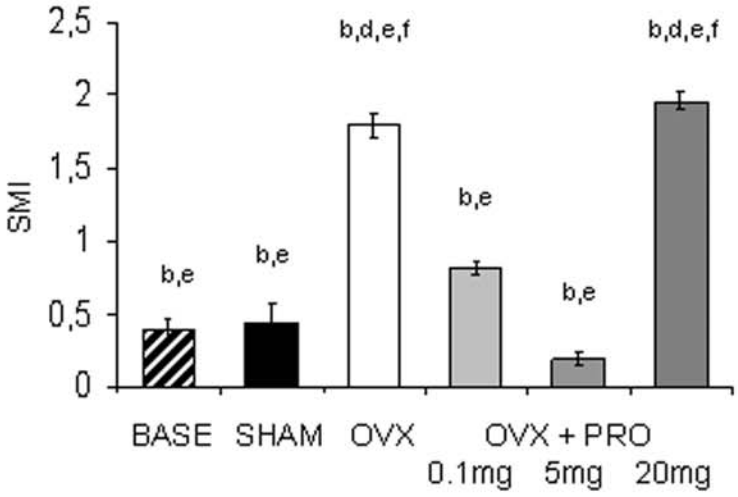

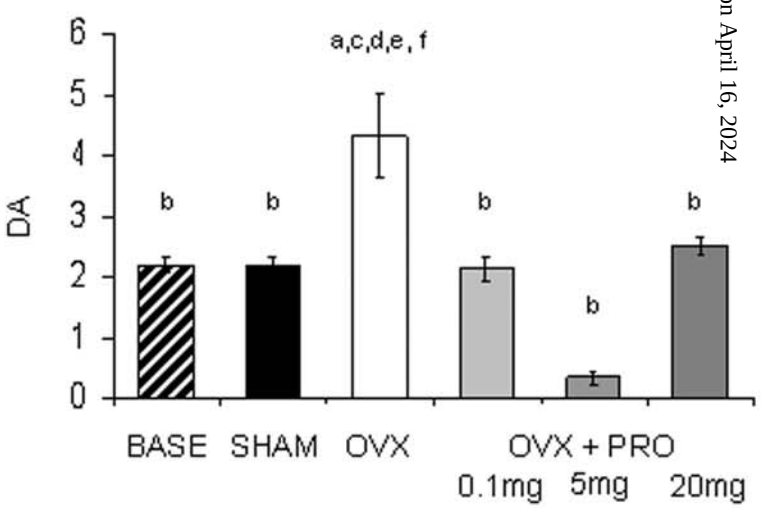

FIG. 3.

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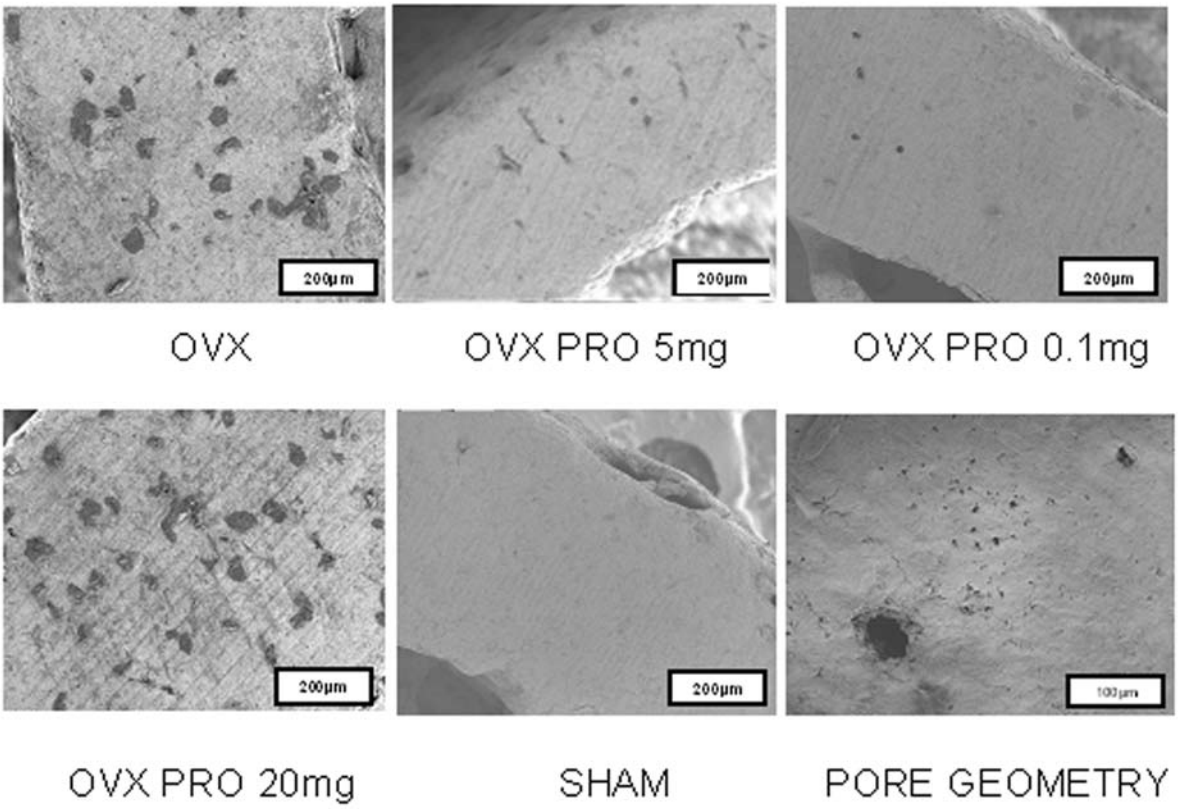

FIG. 4.

FIG. 5.