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**EARLY PREVENTIVE EFFECTS OF RISEDRONATE IN A RAT
MODEL WITH MASSIVE BONE LOSS DUE TO ORCHIDECTOMY AND
LOCALIZED DISUSE.**

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ABSTRACT

Alterations on lean, fat mass, bone mass and architecture were maximized on the paralysed side of a model combining orchidectomy and hindlimb paralysis induced by botulinum neurotoxin. Risedronate have protective effects on trabecular bone but failed to preserve cortical bone on the paralysed side.

Introduction: Orchidectomy (ORX) in rat and unilateral hindlimb paralysis induced by botulinum neurotoxin (BTX) are respectively suitable models for hypogonadism and disuse induced osteoporosis. ORX and BTX models were combined to see if their effects were cumulative and if bone loss could be prevented by risedronate or testosterone.

Materials and Methods: 4 groups of 12 rats were examined for 1 month: SHAM operated; ORX and paralyzed in right hindlimb; ORX+BTX+risedronate (5 μ g/kg/d), ORX+BTX+testosterone (30 μ g/kg/day). Modifications of bone, lean and fat mass were examined by DXA on whole body, regional left and right hindlimbs and on excised tibia and femur. BV/TV, Tb.Th, Tb.N, Tb.Sp and microarchitectural parameters were assessed by histomorphometry. Trabecular 3D and cortical 2D measurements were assessed by X-ray microtomography.

Results: Whole body BMC and lean mass were decreased in ORX-BTX and respectively preserved by risedronate but not by testosterone, by testosterone but not by risedronate. On paralysed side, decrease of lean mass was maximized and could not be prevented by testosterone. In right tibial metaphysis, bone loss was maximized: -33.5% BMC vs. -11.2% on left side. BMC is completely preserved by risedronate. BV/TV and Tb.N decreased and Tb.Sp increased on both sides whereas Tb.Th was significantly lower only on the paralyzed side. Alterations of 2D and 3D architecture were significantly highest on the paralysed side. Risedronate preserved trabecular volume and architecture but not trabecular thickness.

Testosterone was less effective except for trabecular thickness. Cortical area, thickness and cross-sectional moment of inertia were altered with maximum changes on the paralysed side. Risedronate preserved cortical bone on left side but not on paralysed side. Testosterone was ineffective in preserving cortical bone.

Conclusions: The combined model appears suitable to evaluate pharmacological compounds since bone loss occurs rapidly. Risedronate have early and protective effects on trabecular bone mass and bone architecture.

KEY WORDS:

Osteoporosis

Orchidectomy

Disuse

Risedronate

Bone histomorphometry

MicroCT

INTRODUCTION

Sex hormones are recognized as important factors in the maintenance of bone mass and architecture. An increased bone-remodeling rate is observed in women after menopause or surgical castration. In men, the prevalence of osteoporosis has often been underestimated. Epidemiological studies have shown that about 30% of hip and 20% of vertebral fractures occur in men.^(1,2) American studies have estimated that the lifetime risk of hip fracture in 50 year-old white men is about 6% and the risk of vertebral fracture about 5%.^(3,4) In the aging man, a decrease in blood testosterone levels or an increase in its complexing protein (Sex Hormon Binding Globulin – SHBG) have been found to occur in a number of patients.^(5,6) Reduced testosterone level is associated with modifications of the body composition and fat, lean and body masses have been found to be altered.⁽⁷⁻⁹⁾ These changes are similar to those described in patients with primary or acquired hypogonadism and appear highly correlated with androgen levels.⁽¹⁰⁻¹²⁾ Bone mineral density was reported to be correlated with fat body mass.^(13,14) Other studies have found that both fat and lean body mass have important effects on bone mass, depending on the bone parameter considered, the skeletal site measured and the hormonal status.⁽¹⁵⁻¹⁷⁾ The mechanisms governing the fat and lean masses and their relationships to the decrease in bone mass with age and gender are still unclear.⁽¹⁸⁾ Muscular activity is well known to exert positive effects on bone mass^(19,20) and hormones released by adipocytes (leptin and adiponectin) are also known to control bone mass.⁽²¹⁾

Generalized bone loss, developed in the course of disuse, is a well recognized complication after para or tetraplegia in human.⁽²²⁾ A localized loss of bone is also documented in patients with regional disuse after fracture⁽²³⁾, unilateral amputation⁽²⁴⁾, stroke or poliomyelitis⁽²⁵⁾ and reduced physical activity^(26,27). However, in men, other risk factors are commonly observed and intricaded in the pathogenesis of osteoporosis

(smoking, alcohol abuse, previous gastric surgery, glucocorticosteroid therapy...). The additive and cumulative effects of these risk factors have been advocated in male osteoporosis. ⁽²⁸⁾

Animal models have significantly contributed to understand the pathogenesis of bone loss induced by various clinical conditions. The orchidectomized rat (ORX) has been proposed to simulate osteoporosis due to hypogonadism ⁽²⁹⁻³²⁾. Animal models of disuse osteoporosis have also received considerable attention (see review by Jee ⁽³³⁾). At the present time, non surgical methods such as hindlimb immobilisation by casting or bandaging ⁽³⁴⁾, tail suspension ⁽³⁵⁾ or paralysis of a single muscle of the right hindlimb by the Botulinum neurotoxin ⁽³⁶⁾ are favored because of the lack of surgical trauma or regional acceleratory phenomenon. ⁽³⁷⁾

The aim of the present study was to measure the regional variations of bone, lean and fat mass in a combined model associating ORX with a localized disuse induced by BTX. Subcutaneous injections of testosterone or risedronate (a third generation aminobisphosphorate) were used as counter-measures and their effects were investigated in a short time study (4 weeks after ORX and BTX). Dual energy X-ray absorptiometry (DXA), histomorphometry and X-ray microtomography (microCT) were used.

MATERIAL AND METHODS

Animals

Forty-eight males Wistar rats (Charles River, Cléon, France), aged 18-19 weeks and weighting 507 ± 33 g were acclimated for 2 weeks to the local vivarium conditions (24°C and a 12-h/12-h light/dark cycle). Rats were given a standard laboratory food containing 0.8%

calcium (UAR; Villemoisson sur Orge, France) and water *ad libitum*. Rats were randomised into 4 groups (12 animals per group).

On day 0, 36 rats were anaesthetized with halothane, orchidectomized bilaterally by scrotal incisions and ligation of testicular arteries. Incisions were closed with three clips. At the time of surgery, they were injected intramuscularly with *Clostridium Botulinum* type A neurotoxin (Laboratoire Transphyto, Clermont-Ferrand, France). Each animal received 2U of BTX dissolved in saline (0.4 ml) in the *quadriceps femoris* of the right hindlimb. They constituted the ORX-BTX animals. 12 rats were similarly anaesthetized, sham operated and received an injection of saline in the right hindlimb (0.4 ml). They served as control (SHAM group).

On day 1, countermeasures were initiated by subcutaneous administration during 4 weeks of either sodium risedronate (Procter and Gamble Pharmaceuticals), testosterone (water-soluble testosterone, Sigma T5035) or saline. The ORX-BTX rats were divided into 3 groups of 12 animals. The ORX-BTX-RISE group received risedronate (dissolved in saline) 5 µg/kg/day; 5 days/7. The ORX-BTX-TESTO group received testosterone (30 µg/day; 7 days/7). The ORX-BTX group received 0.5 ml of saline, 5 days/7.

Rats were weighted weekly. Four weeks after the beginning of the study, they were sacrificed with chloroform. At the time of sacrifice, whole body DXA measurements were made soon after euthanasia. Tibia and femur were dissected, defleshed and fixed in 70% ethanol with 1% acetic acid for 24hr at 4°C. Excized bones were measured again by DXA before other techniques.

Dual energy X-ray absorptiometry

Measurements were done on a Hologic QDR 4500A (Hologic Inc., Waltham, MA) with a small animal software (release V8-26h). Before each series of measurements, a tissue calibration scan was performed with the Hologic phantom. Several parameters were obtained:

whole body bone mineral content (BMC), total lean and fat masses. Lean and fat masses were also determined on interactively drawn regions of interest: each right hind limb (R) and each left hind limb (L) (fig 1). BMC of the excised tibia and femur were measured using a proximal height resolution option with 0.0267 cm line spacing. Bones were placed in a plastic dish containing water at a constant depth for all measurements. The analysis of DXA scans was performed on the whole tibia, and in three regions of interest on each tibia centered on the proximal metaphysis (R1), diaphyseal shaft (R2), and distal metaphysis (R3). The lower limit of R1 was fixed at 20mm from the articular surfaces and the upper limits of R3 was fixed at the insertion of the fibula.

X-ray microtomography

MicroCT was performed on the distal femur with a Skyscan 1072 X-ray computed microtomograph (Skyscan, Aartselaar, Belgium). Samples were fixed on brass stubs and analysed with a magnification of 26.95 (a pixel corresponding to 11.41 μm) with the fan beam mode. For each samples 219 section images were obtained every 11.41 μm covering 2.5 mm, i.e., the cumulated height of the primary and secondary spongiosa. The 2D section images were stored in the .bmp format. After interactive segmentation, the 3D models were constructed from the stack of 2D images with a surface-rendering program (Ant, release 2.0.5, Skyscan, Aartselaar, Belgium). The 3D measurements were obtained with the CtAn software (release 2.5 - Skyscan - Aartselaar, Belgium). The volume of interest (VOI) was designed by drawing interactively polygons on the 2D gray images before reconstruction. Trabecular volume (BV/TV_{3D}) and the structural model index (SMI) were calculated. SMI characterizes a 3D bone structure composed of a certain amount of plates and rods. The SMI has a value between 0 and 3. In an ideal plate model, the SMI value is 0, and in an ideal

cylindrical rod structure, the SMI is 3, independent of the physical dimensions of the structure.

Measurements on cortical bone were performed on a 2D section image at 4mm under the growth cartilage. Image was analysed with Image J software (release 1.34s-NIH-USA). After thresholding, three measurements were obtained: cortical area (Ct.Ar), average cortical thickness (Ct.Th) and outer diameter (B.Dm). Cross-sectional moment of inertia (CSMI) was calculated using the assumption that bones were roundly shaped ⁽³⁸⁾.

$$\text{CSMI} = (\pi/64) \times (\text{B.Dm}^4 - (\text{B.Dm} - 2\text{Ct.Th})^4)$$

Bone histomorphometry

The fixed tibias were dehydrated in acetone and embedded undecalcified in methylmethacrylate. Sections (7 µm thick) were cut dry on a heavy-duty microtome equipped with tungsten carbide knives (Leica Polycut S, with 50° knives) and stained with a modified Goldner trichrome. Histomorphometric analysis was done on a Leica Quantimet Q570 image processor (Leica, France) equipped with a CCD camera (Sony 930). Bone sections were placed on an X-ray light box after equalization of light intensity. Digitized images of a complete section were stored in the grey image processor at a magnification of x6. After interactive thresholding, a binary image was displayed on the control monitor. The growth plate was identified interactively and the region of interest (comprising only the secondary spongiosa) was automatically recognized (1.5→3.5 mm from the growth plate). Artefacts created during the surgical and histotechnological steps were eliminated by automatic and interactive procedures. Trabeculae were disconnected from the endosteal surfaces by an interactive procedure as previously described ⁽³⁹⁾. Static and 2D microarchitectural histomorphometric measurements were obtained as previously reported ⁽⁴⁰⁾ Trabecular Volume (BV/TV_{2D}), Trabecular Thickness (Tb.Th), Trabecular Number (Tb.N), Trabecular

Separation (Tb.Sp), Free End Count (FEC), Node Count (NC), Star volume of marrow space ($V_{m.space}^*$), Star volume of trabeculae (V_{Tb}^*), Euler-Poincaré's Number (E), Kolmogorov's fractal dimension (D).

Statistical analysis

Statistical study was performed using Systat statistical software, release 10.0 (SPSS inc. Chicago, IL). All data were expressed as mean \pm SEM. At each studied time, differences between the SHAM and different ORX-BTX subgroups were analyzed by ANOVA with the Fisher's Least Significant Difference post-hoc test. Data from left and right hindlimbs were compared using paired sample *t* test. Differences were considered as significant when $p < 0.05$.

RESULTS

DXA-whole body measurements (Fig. 2)

Body BMC

BMC of the whole body was significantly decreased in the ORX-BTX group (-7.2% $p < 0.005$) and in the ORX-BTX-TESTO group (-5.2% $p < 0.005$) versus SHAM. No difference could be observed after treatment with risedronate in the ORX-BTX-RISE group versus SHAM animals.

Lean body mass

Lean body mass was considerably decreased with a significant difference in the ORX-BTX group (-14% $p < 0.005$) and in the ORX-BTX-RISE group (-10.9% $p < 0.005$) versus SHAM animals. No significant difference could be observed after treatment with testosterone in the ORX-BTX-TESTO animals compared with the SHAM group .

Fat body mass

No significant change in fat body mass could be evidenced between ORX-BTX, ORX-BTX-RISE and ORX-BTX-TESTO versus SHAM.

DXA - hindlimb measurements (Fig. 3)

In the 3 groups ORX-BTX, ORX-BTX-RISE and ORX-BTX-TESTO, there was a significant loss of lean mass between the right (paralyzed hind limb) and left (intact hind limb). Lean mass was significantly decreased with a considerable difference of -20% on L and -43% on R in the 3 groups versus the comparable limbs in the SHAM group.

In the 3 groups ORX-BTX, ORX-BTX-RISE and ORX-BTX-TESTO, there was a significant increase of fat mass in the right (paralyzed hind limb) when compared to the left side. No significant difference for fat mass (L or R) was noted in the 3 groups versus SHAM.

DXA of excised bones; comparison between R/L bones (table 1)

The mean difference (Δ in %) between the R and L bones was used to simplify comparisons. ($\Delta = (R - L) * 100 / L$). When the whole bone was taken into consideration, a marked reduction of BMC was evidenced on the immobilized R side; this was verified for the tibia and femur, bone loss was in the same order of magnitude in the different groups. No R/L difference was noted in the SHAM group. When Δ were calculated with the corresponding bones of SHAM animals, the influences of ORX and BTX were clearly evidenced. Also, the additive deleterious action of the two factors was highly shown in the ORX-BTX group and the bone loss was similar for the tibia and femur. In rats treated with risedronate, the bisphosphonate prevented bone loss excepted in the R tibia where Δ BMC reached significance. Bone loss prevention by testosterone was ineffective since bone gain was minimal on the left side ($\Delta = +5.6\%$ vs. ORX-BTX group, $p = 0.15$) and on the right side ($\Delta = +3.8\%$ vs. ORX-BTX

group, $p = 0.36$). When the subregions of the tibia were considered, bone loss was hereagain found to be maximized when the conjoint actions of ORX and BTX acted on the right side with a considerable bone loss of -33.5% in the R1 region of interest in the ORX-BTX group. The difference was also highly significant for the R2 region (mostly cortical) ($\Delta = -20.8\%$) and the R/L difference was noted, confirming the additional effect of disuse and hormone deprivation. On the other hand, no difference was observed with SHAM rats and no R/L difference could be evidenced for the R3 region (exclusively cortical). Animals which had received risedronate had a complete preservation of bone mass in the R1 region on the right bone and presented a +9.0% increase on the left side (with an highly significant difference between the two sides, $p < 0.001$). Interestingly, the bisphosphonate had little effect on the R2 region since the values were slightly improved, but did not reached significance, when compared to the ORX-BTX group. The R/L difference was not significant. No effect was observable on R3.

Testosterone slightly improved R1 values in the ORX-BTX-TESTO group but bone loss remained dramatically reduced on the right side. In the R2 and R3 regions, testosterone appeared not significantly effective on bone loss.

Histomorphometric analysis (table 2)

In the ORX-BTX group, BV/TV and Tb.N were significantly decreased ($p < 0.001$) and conversely, Tb.Sp increased ($p < 0.005$). No significant R/L difference was evidenced in this group. Tb.Th was not modified on the L-side but decreased on R-side submitted to both disuse and hormonal deprivation ($p < 0.05$). Microarchitectural changes in this group indicated the occurrence of trabecular perforation and removal of complete trabeculae evidenced by an increased of $V_{m.space}^*$ ($p < 0.001$), a decreased of NC ($p < 0.001$), FEC ($p < 0.001$) and V_{trab}^* (only on the R-side, $p < 0.05$). The fractal dimension D was significantly reduced on the two

sides ($p < 0.001$) and confirmed the loss of complexity and connectivity of the trabecular network.

In the ORX-BTX-RISE group, risedronate appeared effective in preserving bone mass since BV/TV, Tb.SP were not modified when compared to SHAM; Tb.N was over-compensated on the two hindlimb ($p < 0.005$) but trabecular thickness remained at lower values than in SHAM animals. No significant R/L difference was evidenced. All other parameters values were improved and significantly differed from those of the ORX-BTX group ($p < 0.005$). All the 2D microarchitectural parameters were indicated that trabecular architecture was preserved by thin and more numerous trabeculae with a lower V_{trab}^* ($p < 0.05$ on the L side).

In the ORX-BTX-TESTO group, BV/TV and Tb.N remained significantly decreased ($p < 0.001$) and Tb.Sp was not modified when compared to SHAM values. Tb.Th and Tb.N from the L side were significantly increased and Tb.Sp from both L and R side decreased when compared to ORX-BTX. It is noticeable that trabecular thickness was not different from control animals. The architectural parameters were still altered and exhibited a loss of trabeculae with increased separation and loss of connectivity vs. SHAM, the difference did not reach significance when compared with the ORX-BTX group except V_{trab}^* from the R side ($p < 0.05$). In both L and R side, it appeared that testosterone treatment was significantly less effective than risedronate except for trabecular thickness.

MicroCT analysis

3D images reconstructions performed by microCT on the right side of the tibia showed the bone trabecular loss in ORX-BTX compared to SHAM (Fig. 4A and B). Trabecular network was preserved in ORX-BTX-RISE group (Fig. 4C) but not in ORX-BTX-TESTO group (Fig. 4D). These results were confirmed by 3D measurements summarized in table 2. BV/TV_{3D} decreased and SMI increased significantly in the ORX-BTX group versus SHAM and the

values were maximized on R side. The bisphosphonate preserved bone mass and microarchitecture since no significant difference was observed with SHAM animals and the difference was highly significant with the ORX-BTX-RISE group. No significant differences between R/L were found in this group. In the ORX-BTX-TESTO group, microCT confirmed that testosterone was ineffective in preserving bone mass and architecture; the R/L difference was significant. SMI increased, emphasizing the increased conversion of trabeculae from plates to rods.

When cortical parameters were considered (Table 2, Fig. 5), it was noticeable that no significant change in outer diameter could be evidenced between the 4 groups. Ct.Ar, Ct.Th and CSMI were decreased on the R side in the ORX_BT X group. On the L side, only Ct.Ar and CSMI were significantly decreased. The R/L difference was significant for the 3 parameters: Ct.Ar, Ct.Th and CSMI. Risedronate preserved cortical bone on the L side but only slightly improved Ct.Ar, Ct.Th and CSMI values from the R side as these values remained lower compared to SHAM. Testosterone was ineffective in preserving cortical bone. Ct.Ar, Ct.Th and CSMI were also found significantly lower compared to those in SHAM.

DISCUSSION

Several factors are known to control bone mass in humans and animals. Genetic influences, nutritional factors (such as protein or calcium intake), physical activity and endocrine status have been shown to play key roles in human ⁽⁴¹⁾. Changes in body composition (lean and fat mass) and BMC have been repeatedly reported being associated with a decrease in blood estrogen level in women ^(13,42) In men, a decreased androgen level is associated with decreased lean body mass; an increase in fat mass is associated with

aging⁽⁸⁾. Acquired hypogonadism has also been found to induce similar modifications⁽¹¹⁾. In hypogonadic men, the increase in bone density and lean body mass during testosterone therapy have been reported in patients whose age ranged from 22 to 69 years⁽¹²⁾. Similarly, testosterone was found to reduce bone remodeling and increase muscle mass in castrated animals in hypogonadic patients.⁽⁴³⁾

The aged male rat appears a useful model for studying the age-related changes in body composition, bone or muscle mass^(44,45). The ORX rat model has been found appropriate for investigating the bone, lean and fat mass changes induced by androgen deficiency⁽⁴⁶⁾. DXA is a sensitive and reproducible technique to measure bone loss in small laboratory animals. In the aged rat, it was previously reported that ORX influenced BMC, lean mass but had no effect on fat mass^(45,46). Testosterone appeared to compensate the lean mass, but its action was not sufficient to be visible on the right or left hind limb when compared to SHAM animals. A significant difference exists between the two hind limb: risedronate or testosterone have no effect on lean mass in each limb separate.

Risedronate is a third generation aminobisphosphonate with potent antiresorptive activity. It was found to inhibit bone loss due to hormone deprivation, and has proven highly effective in patients with osteoporosis.^(47,48)

Injection of BTX only in the right hindlimb reduced body BMC. Testosterone had no effect on whole body bone loss. Risedronate compensated entirely the whole body bone loss. The results were more intensive and more precocious on the whole body when we compared with the experimentation with only ORX⁽⁴⁶⁾ and on the right limb, immobilized by BTX. The effects of ORX and BTX was cumulative on bone loss and lean mass loss.

In the excised bone (Fig 6) the effect of ORX and BTX was cumulative on bone loss in ORX-BTX group on the immobilized (right) side. Risedronate did compensate the loss of BMC in the

non-immobilized side, but the dose is not sufficient to compensate the great loss in the immobilized side.

Bone loss is also associated with an alteration of bone architecture, which can be appreciated by histomorphometric methods^(36,39,40). The World Health Organisation, Guidelines for preclinical evaluation and clinical trials in osteoporosis (WHO) considered that the histomorphometric technique remains the “gold standard” to quantify bone loss in animals⁽⁴⁹⁾. ORX-induced bone loss is due to the removal of complete trabeculae by an increased osteoclastic resorption as evidenced by decreased Tb.N and increased $V_{m.space}^*$ while the thickness of trabeculae is not altered (Tb.Th and V_{tb}^* remain constant)⁽⁵⁰⁾.

In this study, we found that the effects of ORX and BTX-induced paralysis were cumulative on trabecular bone since BV/TV and Tb.N were considerably decreased on the right side submitted to both deleterious actions. We recently showed that serum TRAcP levels (reflecting osteoclast activity) were higher in ORX-BTX compared to ORX corresponding to a greater bone resorption and explaining the maximal loss in the R limb⁽⁵¹⁾. Risedronate compensates or overcompensates BV/TV and Tb.N but not Tb.Th and V_{tb}^* . Testosterone seems to have an action on the two parameters Tb.Th and V_{tb}^* .

The increase of SMI in the ORX-BTX group, confirmed the increased osteoclastic activity leading to a marked conversion of plates into pillars. This effect was fully abolished by risedronate but testosterone failed to preserve bone mass in the metaphyseal region. Finally, we showed that risedronate was effective to preserve cortical bone mass and mechanical properties in bone loss induced by ORX but unable to prevent cortical bone changes in bone loss induced by the combination of ORX and disuse. Similar results were observed in a long term disuse model in dogs in which risedronate failed to prevent cortical thinning and altered mechanical properties⁽⁵²⁾.

In summary, a massive bone loss can be generated in the male rat by using a non-surgical immobilization of one hindlimb combined with an hormone deprivation induced by castration. An interesting advantage of the model is that disuse is localized on one side, the animal is its own control. It has been shown that the effect of hormonal deprivation and immobilization are additive after 4 weeks.⁽⁵⁰⁾ This combined model is easy to handle and appears very suitable to evaluate pharmacological compounds since the bone loss occurs rapidly. We have shown the early and protective effects of risedronate on the trabecular bone mass and bone architecture.

Table 1: BMC comparison between left and right bones

	SHAM	ORX-BTX	ORX-BTX-RISE	ORX-BTX-TESTO
<i>intragroup comparison of the difference between left and right (in %)</i>				
Δ tibia	0.5 ± 0.7	-8.7 ± 1.4 ^a	-5.5 ± 0.9 ^a	-10.2 ± 1.4 ^a
Δ femur	0.5 ± 0.9	-8.3 ± 2.4 ^a	-4.4 ± 1.1 ^a	-8.3 ± 1.5 ^a
<i>intergroup comparison of the differences of total BMC with SHAM animals (in %)</i>				
Δ Left tibia		-11.5 ^a	-3.7	-6.6 ^a
Δ Right tibia		-19.8 ^a	-9.4 ^a	-16.0 ^a
Δ Left femur		-11.6 ^a	-2.4	-6.6
Δ Right femur		-19.5 ^a	-6.9	-14.0 ^a
<i>intergroup comparison of the differences of regional tibia BMC with SHAM animals (in %)</i>				
Δ Left R1		-11.2 ^a	+9.0 ^a	-9.1 ^a
Δ Left R2		-15.3 ^a	-14.8 ^a	-9.0 ^a
Δ Left R3		-5.5	+0.7	+0.7
Δ Right R1		-33.5 ^a	-1.3	-27.9 ^a
Δ Right R2		-20.8 ^a	-17.2 ^a	-17.6 ^a
Δ Right R3		-3.0	-4.6	-3.0

^a p < 0.05 vs. SHAM

Table 2: Histomorphometry and MicroCT data

	SHAM		ORX-BTX		ORX-BTX-RISE		ORX-BTX-TESTO	
	Left	Right	Left	Right	Left	Right	Left	Right
Bone 2D histomorphometry parameters								
BV/TV (%)	12.8 ± 0.8	13.2 ± 1.3	6.0 ± 1.2 ^a	4.2 ± 0.8 ^a	15.6 ± 0.4 ^b	15.5 ± 0.6 ^b	8.44 ± 0.94 ^{a,c}	6.47 ± 1.38 ^{a,c}
Tb.Th (µm)	66.3 ± 1.2	68.6 ± 1.6	62.7 ± 3.4	58.0 ± 2.2 ^a	59.1 ± 1.1 ^a	59.0 ± 2.4 ^a	68.5 ± 1.1 ^{b,c}	66.7 ± 2.2 ^c
Tb.N (/mm)	1.9 ± 0.1	1.9 ± 0.2	0.9 ± 0.2 ^a	0.7 ± 0.1 ^a	2.6 ± 0.04 ^{a,b}	2.6 ± 0.07 ^{a,b}	1.2 ± 0.1 ^{a,b,c}	1.0 ± 0.2 ^{a,c}
Tb.Sp (µm)	473 ± 35	495 ± 46	1542 ± 340 ^a	2824 ± 856 ^a	321 ± 6 ^b	322 ± 9 ^b	823 ± 72 ^b	1680 ± 358 ^b
E	4.36 ± 0.23	4.16 ± 0.24	2.62 ± 0.41 ^a	1.92 ± 0.36 ^a	6.30 ± 0.41 ^{a,b}	6.58 ± 0.37 ^{a,b}	2.79 ± 0.03 ^{a,c}	2.25 ± 0.4 ^{a,c}
NC	1.4 ± 0.2	1.3 ± 0.2	0.6 ± 0.1 ^a	0.3 ± 0.1 ^a	1.8 ± 0.1 ^b	1.8 ± 0.2 ^b	0.8 ± 0.1 ^{a,c}	0.7 ± 0.2 ^{a,c}
FEC	6.4 ± 0.4	6.2 ± 0.6	3.3 ± 0.6 ^a	2.2 ± 0.5 ^a	8.9 ± 0.4 ^{a,b}	9.3 ± 0.5 ^{a,b}	4.1 ± 0.5 ^{a,c}	3.0 ± 0.6 ^{a,c}
V ^{*_{m.space}} (µm ³)	7.7 ± 1.9	9.7 ± 2.0	32.8 ± 6.6 ^a	51.0 ± 10.4 ^a	2.9 ± 0.5 ^b	2.9 ± 0.6 ^b	24.9 ± 5.0 ^{a,c}	45.4 ± 9.8 ^{a,c}
V ^{*_{trab}} (µm ³)	0.031 ± 0.003	0.029 ± 0.003	0.024 ± 0.003	0.017 ± 0.003 ^a	0.023 ± 0.002 ^a	0.022 ± 0.002	0.029 ± 0.002	0.031 ± 0.003 ^{b,c}
D	1.36 ± 0.03	1.34 ± 0.03	1.09 ± 0.05 ^a	0.94 ± 0.08 ^a	1.42 ± 0.02 ^b	1.43 ± 0.02 ^b	1.2 ± 0.05 ^{a,c}	1.11 ± 0.05 ^{a,b,c}
MicroCT measurements								
BV/TV _{3D} (%)	21.0 ± 1.2	21.5 ± 1.2	12.4 ± 1.4 ^a	8.7 ± 1.5 ^a	21.9 ± 1.6 ^b	20.8 ± 1.3 ^b	13.3 ± 1.1 ^{a,c}	9.1 ± 0.9 ^{a,c}
SMI	1.33 ± 0.05	1.31 ± 0.04	1.72 ± 0.06 ^a	1.96 ± 0.08 ^a	1.36 ± 0.06 ^b	1.45 ± 0.06 ^b	1.60 ± 0.05 ^{a,c}	1.83 ± 0.04 ^{a,c}
Ct.Ar (mm ²)	67.4 ± 2.0	68.7 ± 1.5	63.3 ± 1.2 ^a	57.8 ± 1.2 ^a	65.6 ± 0.8	62.5 ± 1.1 ^{a,b}	51.6 ± 1.3 ^{a,b,c}	54.1 ± 1.0 ^{a,b,c}
Ct.Th (µm)	64.7 ± 1.1	66.4 ± 1.1	63.5 ± 1.4	57.2 ± 1.4 ^a	64.8 ± 1.2	61.2 ± 1.3 ^{a,b}	58.9 ± 1.4 ^{a,b,c}	49.9 ± 1.1 ^{a,b,c}
B.Dm (mm)	1.59 ± 0.04	1.58 ± 0.04	1.52 ± 0.03	1.50 ± 0.03	1.54 ± 0.03	1.54 ± 0.03	1.52 ± 0.03	1.50 ± 0.03
CSMI (×10 ⁻²)	9.2 ± 0.8	9.1 ± 0.7	7.7 ± 0.4 ^a	6.8 ± 0.4 ^a	8.2 ± 0.4	7.8 ± 0.5 ^a	6.8 ± 0.4 ^{a,c}	6.0 ± 0.3 ^{a,c}

^a p < 0.05 vs. SHAM, ^b p < 0.05 vs. ORX-BTX, ^c p < 0.05 vs. ORX-BTX-RISE
The gray boxes indicate a significant difference vs. left with p < 0.05

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LEGENDS OF FIGURES:

Figure 1: Whole body image of a wistar rat analysed with the Hologic QDR 4500A. Note the marked difference in intensity between the right (R) and left (L) femur and tibia.

Figure 2: Whole body BMC and lean body mass from DXA measurements.

Figure 3: Lean and fat mass from DXA hindlimb measurements.

$p < 0.0001$, * $p < 0.0001$

Figure 4: X-ray microCT 3D reconstructions of the right inner side of the tibia in: SHAM (A), ORX-BTX (B), ORX-BTX-RISE (C), ORX-BTX-TESTO (D).

Figure 5: X-ray microCT 2D section images from the right inner side of the tibia in: SHAM (A), ORX-BTX (B), ORX-BTX-RISE (C), ORX-BTX-TESTO (D).

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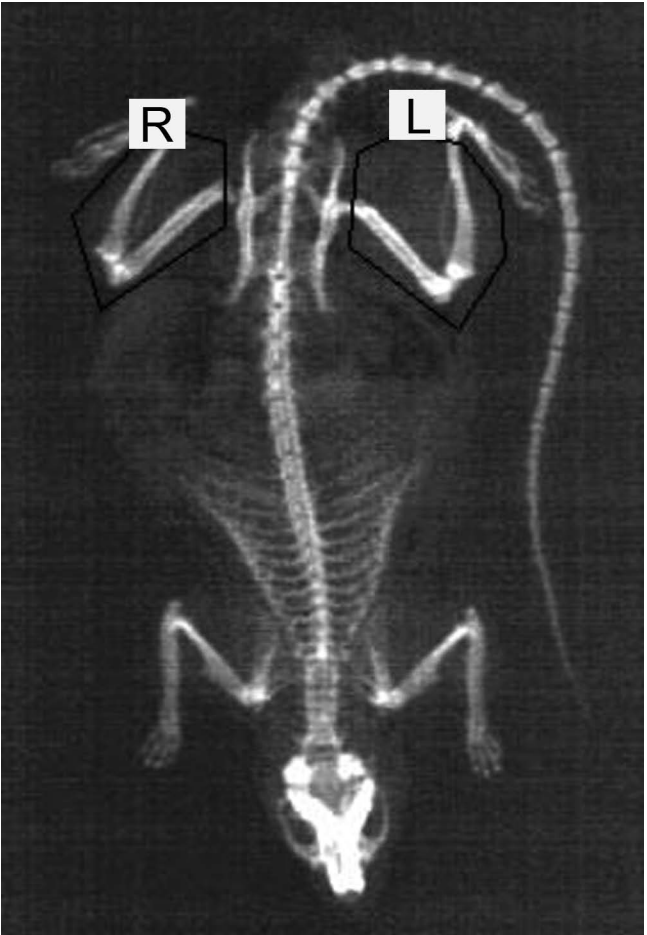
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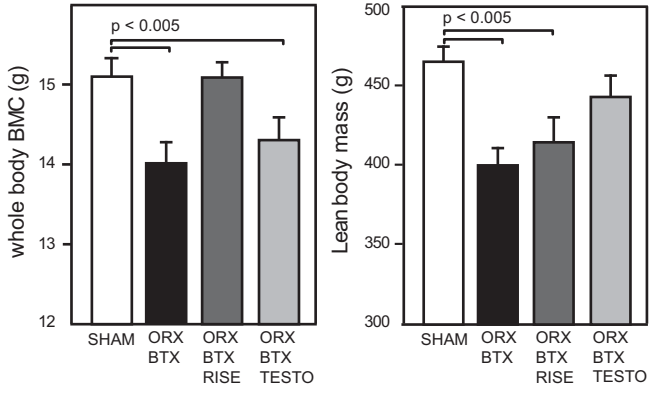


Figure 2.

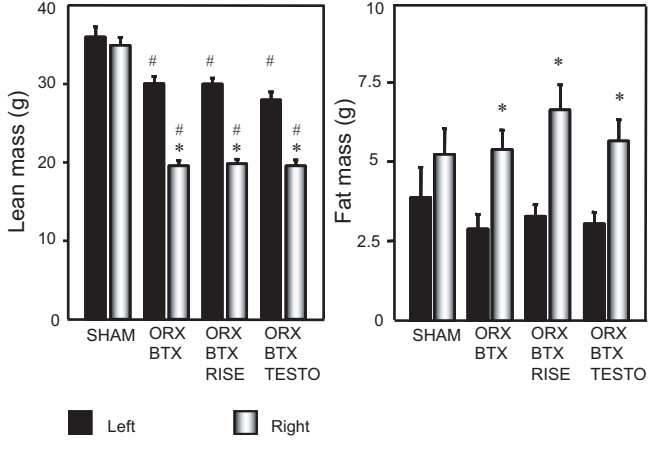


Figure 3.

