Doping dose of salbutamol and exercise training: impact on the skeleton of ovariectomized rats

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Bonnet N, Laroche N, Beaupied H, Vico L, Dolleans E, Benhamou CL, Courteix D. Doping dose of salbutamol and exercise training: impact on the skeleton of ovariectomized rats. J Appl Physiol 103: 524–533, 2007. First published May 3, 2007; doi:10.1152/japplphysiol.01319.2006.—Previous studies in healthy rats have demonstrated a deleterious bone impact of β2-agonist treatment. The purpose of this study was to examine the trabecular and cortical effects of β2-agonists at doping dose on treadmill exercising rats with estrogen deficiency. Adult female rats were ovariectomized (OVX; n = 44) or sham operated (n = 12). Then, OVX rats received a subcutaneous injection of salbutamol (SAB) or vehicle with (EXE) or without treadmill exercise for 10 wk. Bone mineral density (BMD) was analyzed by densitometry. Microcomputed tomography and histomorphometric analysis were performed to study trabecular bone structure and bone cell activities. After 10 wk, SAB rats presented a much more marked decrease of BMD and trabecular parameters. Exercise did not change the high level of bone resorption in OVX EXE SAB compared with OVX SAB group (both on COOH-terminal collagen cross-links and osteoclast number). These results confirm the deleterious effect of β2-agonists on bone quantity (femoral BMD gain: OVX EXE, +6.8%; vs. OVX EXE SAB, −1.8%; P < 0.01) and quality (−8.0% of femoral trabecular thickness in OVX EXE SAB vs. OVX EXE), indicating that SAB suppresses the effect of EXE in OVX rats. Furthermore, we notice that the slight beneficial effect of exercise was mainly localized in the tibia. These findings indicate the presence of a bone alteration threshold below which there is no more alteration in structural bone quantity and quality. The negative effects of SAB on bone observed in this study in trained rats may indicate potential complications in doping female athletes with exercise-induced amenorrhea.

β2-agonist; estrogen deficiency

THE WIDESPREAD ABUSE of doping agents that increase lean body mass of athletes might be responsible for menstrual irregularities and low bone density (19). Salbutamol (SAB), a β2-adrenergic agonist, is the most commonly prescribed medication for bronchospasm and exercise-induced asthma, affecting ~10–20% of the athletes (42). Systemic administration of SAB is prohibited by the World Anti-Doping Agency because of its possible advantages on the user’s performance. Animal experiments using β2-agonists at doses 10- to 20-fold higher than therapeutic doses showed a muscle anabolic effect (29). Those experiments also described several toxicological effects such as an increased heart weight associated with inflammation, muscle fibers hypertrophy, focal myocardial necrosis, and fibrosis.

At the present time, researchers are more and more interested in investigating the bone effect of β2-agonists. First, studies have demonstrated the presence of β2-agonist receptor on osteoblast and osteoclast cells (2, 32, 40). Second, these substances are widely used in therapeutics for the treatment of asthma. In 2006, De Vries Pharm et al. observed in the Dutch Pharmo database that patients using higher doses of β2-agonists (but still at therapeutic dose) had increased the risk of hip/femur fracture despite adjustments for disease severity (1.46; 95% confidence interval, 1.02–2.08) (15). Some athletes also used β2-agonists for bronchodilator, muscle anabolism and lypolitic effects (13, 16). Finally, the bone impact of the β2-agonist family is thought to have contradictory results depending on age, estrogen, and muscle status.

An increase in muscle strength possibly associated with a higher muscle mass usually induces an anabolic effect on bone tissue. This effect is mediated by the so-called mechanostat system regulating bone mass (17). Therefore, according to Frost’s theory of muscle-bone interaction, an increase in muscle mass caused by β2-agonists action would be expected to result in a subsequent gain in bone tissue (17).

Clenbuterol, a powerful β2-agonist, has been shown to reduce net bone loss in denervated (44) or tail-suspended rats evaluated by long-bone mineral density (BMD) (1). Caruso et al. performed, in a human study, 40 days of unilateral limb suspension with the left legs otherwise refraining from normal ambulatory activity (10). While performing left leg strength training 3 days/wk, subjects received a concurrent placebo or SAB (16 mg/day) treatment. They demonstrated that SAB, associated with resistance exercise, reduces bone loss induced by immobilization (10). Preliminary data published by Patak et al. demonstrated that SAB may increase the trabecular bone volume (BV/TV) in ovariectomized rats (37).

A previous study carried out in the author’s laboratory demonstrated a deleterious effect of SAB on the vertebral trabecular architecture in young rats evaluated by microtomography (8). Our data showed a decrease in BV/TV (~19.7% vs. placebo), and biomechanical tests revealed a lower ultimate force (~15.5% vs. placebo) (8). Consecutively, the deleterious effect of SAB was confirmed in the adult rat, and we could show that a running session was able to counterbalance the deleterious effect of SAB (6). Kitaura et al. reported that
clenbuterol treatment inhibited longitudinal bone growth and decreased bone mineral content (BMC) in growing rats (27). Takeda et al. showed that isoproterenol, a nonspecific β-agonist, decreased bone mass in mice (40). They showed a decrease of BV/TV (−34.3% vs. placebo) due to a lower bone formation rate (−23.6%) and osteoblast number (−41.7%) (40). Preliminary data on the effect of β2-agonists seemed to indicate that when β2-agonists are injected in an osteopenic animal model they exerted beneficial effects on bone (37). Conversely, β2-agonists induced harmful effects on unaltered bones (8). Accordingly, it would be interesting to check whether the controversies shown in the literature might be due to a differential effect of SAB since, in the aforementioned studies, an estrogen deficiency vs. any deficiency was reported.

To our knowledge, no study has investigated the effects of β2-agonists at doping dose on treadmill exercising rats with estrogen deficiency, which is the real condition of use of this substance in amenorrheic women athletes. Moreover, few studies have evaluated the effects of SAB on the skeleton, especially regarding bone metabolism and bone trabecular micro-architecture (8, 37).

The objective of this study is to assess to the effect of SAB on mature bone with or without exercise in osteopenic rats (ovariectomized model: OVX). Compared with our study of SAB on sham rats (non-OVX model), our hypothesis is that SAB will not add a deleterious effect on bone status in osteopenic rats, since Benoit et al. (5) demonstrated a lower effect of SAB on fat cells in absence of estrogen, and that, during exercise, mechanical loading combined with the SAB muscle mass anabolic effect would reduce the net bone loss due to OVX.

**MATERIALS AND METHODS**

**Animals and Treatment**

Fifty-six female Wistar rats aged 33 wk (animal production center, Olivet, France) were acclimatized for 2 wk before the experiment and maintained under constant temperature (21 ± 2°C) and under 12:12-h light-dark cycles during the experiment. The rats were housed in groups of three in standard cages and provided with a commercial standard diet. At 35 wk of age, animals were either ovariectomized (OVX; n = 44) or sham-operated (SHAM; n = 12). Bilateral ovariectomies were performed dorsally under pentobarbital anesthesia (0.60 ml/kg). Sham operations were performed by exteriorizing the ovaries. The whole body BMD of rats was determined by dual-energy X-ray absorptiometry (DEXA; Hologic QDR-1000W) to match all groups. Twenty OVX rats were constrained to perform exercise and were immediately treated with saline or SAB (OVX EXE, n = 10; and OVX EXE SAB, n = 10). The remaining untrained rats were randomized to receive treatment with saline or SAB (SHAM, n = 12; OVX, n = 12; OVX SAB, n = 12). Two different SAB doses are used in animals: therapeutic regimen (16 mg/kg, Sigma-Aldrich Chimie) 5 days/wk for 10 wk. The dose of SAB used in this study was therefore equivalent to a doping dose. The control groups (SHAM, OVX, OVX EXE) were treated with sterile saline at an identical dose regimen, injected subcutaneously 5 days/wk for 10 wk. Food consumption was recorded weekly for the SHAM group, and this amount was then fed to the OVX rats over the following week. Treadmill exercise and SAB and saline treatment started at 36 wk of age.

For histomorphometric parameters, bone labeling of the rats was performed by an intraperitoneal injection of tetracycline (30 mg/kg body wt) 14 days and 4 days before death. At the end of the study, all groups were killed by a pentobarbital overdose. Immediately after death, the hindlimb muscles (soleus, gastrocnemius, and extensor digitorum longus), uterus, and heart were weighed. In all rats, the femurs and tibias were excised and cleared of fat and connective tissues. The right tibias were immediately fixed in 10% formaldehyde for 48 h at +4°C. The other bones were placed in plastic tubes and frozen at −20°C for subsequent microarchitectural and biomechanical tests. The procedure for the care and killing of animals was in accordance with the European Community standards on the care and use of laboratory animals (IAUC, Ministere de l’agriculture, France, Authorization INSERM 45-001).

**Exercise Protocol**

The rats (OVX EXE and OVX EXE SAB) were trained 5 days/wk for 10 wk. During the first week, the rats were familiarized with the treadmill locomotion by gradually increasing the speed and the duration of each running session from 8 m/min for 15 min to 13 m/min for 60 min. The rats were then constrained to run at 13 m/min for 60 min/day during wk 9. This protocol corresponded to moderate exercise for the age of these rats (25). Untrained rats (OVX, OVX SAB) were handled twice daily at 1-h intervals to mimic the stress induced by handling before and after running.

**Body Mass, Fat Mass, and Lean Mass**

Body mass was recorded at weekly intervals throughout the study. Lean and fat masses were measured at −1, 3, 6, and 9 wk by DXA using specific rat body composition software (line spacing, 1.5 mm; resolution, 0.7 mm). Since muscle mass represents 94–96% of lean mass, it is generally accepted to extrapolate muscle mass from lean mass. The coefficients of variation (CV = standard deviation/mean) were determined for these parameters from seven repeated measurements with repositioning on an animal cadaver. The CVs were 4.76% and 1.64% for fat and muscle masses, respectively.

**BMC and BMD Measurements**

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

For all bone densitometric measurements, CVs were determined by seven repeated measurements on one femur and one tibia over several days, with repositioning for each scan. The CVs for BMC and BMD measurements ranged from 0.33% to 4.64%, depending on the bone site.

**Morphological and Topological Characteristics of Trabecular Bone**

Microarchitecture of the femoral and tibial trabecular bone was investigated using a microcomputed tomograph. The characteristics and methods have already been described elsewhere (7). The X-ray source was set at 75 kV and 100 μA, with a pixel size at 11 μm. 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 low noise and the relatively good resolution of the data sets, we used simple global thresholding methods (34). On the femur, 250 slices were selected from the distal growth plate to the shaft proximally. On the tibia, 250 slices were selected from the proximal growth plate to the shaft distally. The trabecular bone was extracted by drawing ellipsoid contours with “computer tomography analyzer” software (Skyscan, Aartselaar, Belgium). BV/TV (%) and trabecular number were calculated by the mean intercept length method. Trabecular thickness (Tb.Th; μm) and...
trabecular separation (μm) were calculated according to the method of Hildebrand and Ruegssegger (21). The trabecular bone pattern factor (TBPF) was calculated to determine the prevalence of platelike or rodlike trabecular structures: the higher the TBPF, the more trabecular bone is organized in the form of rodlike structures. The degree of anisotropy (DA) was calculated by superimposing parallel test lines in various directions on the three-dimensional image. DA defines the magnitude of the preferred orientation of the trabeculae. The higher the DA, the more trabeculae are preferentially oriented.

Morphological Characteristics of Cortical Bone

Cortical bone of the femoral and tibial middiaphyses was analyzed by microcomputed tomography. The characteristics and methods have already been described elsewhere (30). We used the same acquisition characteristics as for trabecular bone. After reconstruction, the cortical bone was extracted by drawing polygon contours with computed tomography analyzer software. Before inversion of the image, we applied simple global thresholding methods, and the algorithms developed for trabecular bone analysis were used to characterize the porosity network. Porosity was labeled Ct.Po (%). The pore number was measured by the mean intercept length method. The pore diameter (μm) and pore spacing (μm) were derived from the Hildebrand method and Po/S/PoV (pore surface on volume) from the triangulation method.

For analysis of the femoral cortex, 100 slices were selected starting 12 mm from the distal growth plate on the shaft proximally, corresponding to the distal diaphysis region. For analysis of tibial cortex, 100 slices were selected starting 12 mm from the proximal growth plate on the shaft distally, corresponding to the proximal diaphysis region.

Diameters and cortical width of the mid-diaphysis (equal to 50% of the femur or tibia length) were measured by microcomputed tomography software. The bone area (μm²), cortical bone area (μm²), and bone marrow area (μm²) were measured at the mid-diaphysis using computer tomography-analyzer software.

Bone Histomorphometry

After 48 h of fixation, the right tibia was dehydrated in absolute acetone and embedded in methylmethacrylate at low temperature according to the method developed by Chappard et al. (12). 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 Goldner’s trichrome and used for measurement of the following parameters in secondary spongiosa according to the ASBMR histomorphometry nomenclature (36) using an automatic image analyzer (BIOCOM): BV/TV, Tb.Th, trabecular number, trabecular separation, osteoid surface (%), and osteoid thickness (μm). Five 8-μm-thick sections were stained with tartrate-resistant acid phosphatase activity to measure active osteoclastic surfaces and osteoclast number. Histodynamic parameters were determined on five unstained, 12-μm-thick sections under ultraviolet light: mineral apposition rate (MAR; μm/day), 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; μm³/μm²·day⁻¹) was calculated as the product of MS/BS and MAR.

All bone remodeling parameters were measured using a semi-automated analysis consisting of a digitizing table (Summasketch-Summagraphs) connected to a personal computer and a Reichert Polyvar microscope equipped with a drawing system (Camera Lucida; Reichert-Jung Polyvar).

Bone Mechanical Tests

Mechanical properties of the left femur were assessed by three-point bending tests. Four hours before mechanical testing, the bones were thawed at room temperature. Each bone was secured on the two lower supports of the anvil of a UniverSAB Testing Machine (Instron 4501, Instron, Canton, MA). The diameter of these supports is 4 mm, and the distance between the two supports is 20 mm. The cross-head speed for all tests was 1 mm/min, and the upper roller contacted the femur at the anterior mid-diaphysis with the load direction perpendicu lar to the medio-lateral diameter. Load-displacement curves were recorded using specialized software (Instron 4501 software). Biomechanical properties were calculated from these curves: ultimate force (the maximum force supported by the bone before fracture; in N); energy to ultimate force (work required to fracture the bone; in J); stiffness (extrinsic rigidity of the femur; in N/mm). Owing to the irregular shape of the femoral diaphysis, the femoral diameter used in the calculation was the mean of medio-lateral and antero-posterior femoral mid-diaphysis diameters. Ultimate stress (MPa) and Young’s modulus (MPa, modulus of elasticity) were determined by the equations previously described by Turner and Burr (41). Because the indirect calculation of strain results in incorrect values, one cannot analyze the complete stress-strain curve on the three-point bending test (a strain gauge positioned on the bone is needed to obtain correct values) (41). Therefore, results concerning toughness data are not presented here. In fact, the imprecision of the measure could have induced errors in the interpretation, and this variability does not permit one to point out the differences between groups. To ensure comparable testing sites at mid-diaphysis, the femur was always mounted so that the cross-head could be applied just in the middle of the bone, as previously described by Turner and Burr (41).

The distal metaphysis of the right femur was tested in compression using the same material testing system used for the bending test. To extract specimens at the same relative position of each femur, the distal metaphysis was cut to a length of 2.5 mm using a standardized procedure: the location of each specimen was standardized from image analysis of the micro-CT to extract the same region of interest used to evaluate bone microarchitecture. The location of the point at which the primary spongiosa below the epiphyseal growth plate transitioned to fully cancellous bone was determined from the image. From the distance measured between this point and the distal end of the femur, we calculated the ratio of this distance to the total length of the femur for each bone. These data were then averaged for all bones. The overall average ratio was multiplied by the length of each femur to define the value of s for each bone. The first cut was made at that point, and the more proximal cut was then made to produce specimens nominally 2.5 mm long. Both cuts were made perpendicular to the long axis of the bone using a low-speed diamond blade wafering saw under continuous irrigation (Buehler Isomet 4000).

The specimens were loaded between flat parallel plates by compression. The load was applied in the cranio-caudal direction using a steel disk (5 cm) at a nominal deformation rate of 0.5 mm/min (35) (22). Load-displacement curves were recorded during testing. We directly measured extrinsic parameters from the force-displacement curve: ultimate force (in N), displacement at ultimate force, energy to ultimate force (in J), and stiffness (in N/mm). Extrinsic properties reflected the combined effects of bone size and shape and tissue material properties. Intrinsic properties referred to the tissue-level material behavior and were derived by adjusting the extrinsic properties to the size and shape of the specimen, using appropriate engineering analysis and assumptions. In the present study of the metaphysis, intrinsic properties represented combined contributions from cancellous and cortical bone. Therefore, the area used to calculate intrinsic parameters was the total bone area determined from micro-CT images. The following intrinsic properties were calculated assuming purely uniaxial loading: ultimate stress, ultimate strain, intrinsic energy to ultimate strain (toughness), and Young’s modulus.
Biochemical Analyses

Osteocalcin (a marker of bone formation) and COOH-terminal collagen cross-links (CTX; a marker of bone resorption) were assayed in duplicate by enzyme-linked immunosorbent assay (Nordic Bioscience Diagnostics, Herlev Hovedgade, Denmark). The within-assay and between-assay CV were <10% in our laboratory.

Statistical Analysis

Results are expressed as means ± SE. Body composition, BMD, geometric data, architectural parameters, biochemical analyses, and femoral mechanical properties were analyzed by two-way ANOVA. When necessary, post hoc differences were determined with the Newman-Keuls test. Correlations were performed using Pearson’s test.

RESULTS

Body Mass and Muscle Mass

In all groups, the body mass of the rats increased normally during the experimental period as commonly observed in the literature (Fig. 1). To normalize data to baseline body mass, fat mass and muscle mass are expressed as percentages changes from baseline (Fig. 1).

At the end of the protocol, we observed higher body mass gains in OVX (+16.4%), OVX SAB (+25.4%), and OVX EXE (+17.6%) groups compared with SHAM (+10.6%, \( P < 0.05 \)) and OVX EXE SAB groups (11.8%).

We also noted a lower total muscle mass gain in OVX group compared with OVX SAB, OVX EXE, and OVX EXE SAB groups (DXA).

A lower fat mass gain was observed in SHAM (+1.6%), OVX EXE (+13.3%), and OVX EXE SAB (−15.1%) groups vs. OVX (+39.9%) group. The fat mass gain in OVX SAB (+20.2%, \( P = 0.06 \)) tended to be lower than in the OVX group. The fat mass gain in the OVX EXE SAB group was significantly lower than in the OVX SAB and OVX EXE groups but did not differ from the SHAM group (Fig. 1).

At necropsy, the uterine mass in all OVX animals was more than 75% lower than that of the SHAM group, indicating successful ovariectomy. SAB groups (OVX SAB and OVX SAB EXE) had higher muscle mass for gastrocnemius and soleus compared with their respective control groups (OVX and OVX EXE). No significant difference for extensor digitorum longus was observed between groups. We also observed a higher gastrocnemius mass in the OVX EXE group than in the OVX group (Table 1).

BMD

Longitudinal analyses of tibia BMD indicated a significant difference between the BMD decrease in OVX (−3.3%) group compared with the BMD increase of SHAM (+4.5%, \( P < 0.01 \)) and OVX EXE (+8.9%, \( P < 0.01 \)) groups (Fig. 2). The BMD gain was higher in the OVX SAB (+4.0%) group than in the OVX group, but after adjustment of BMD gain for the muscle mass of the gastrocnemius the difference disappeared (BMD at 9 wk after adjustment, OVX: 0.2217 g/cm²; OVX SAB: 0.2259 g/cm²; \( P > 0.05 \)). We observed a lower BMD gain in the OVX EXE SAB group (+2.7%) than in the OVX EXE group (+8.9%, \( P < 0.01 \)).

Analysis of the femur showed a lower BMD gain in OVX (+1.9%), OVX SAB (+3.2%), OVX EXE group (+6.8%), and OVX EXE SAB (−1.8%) groups than in the SHAM group (+12.4%; \( P < 0.05 \)). We did not observe any difference in BMD gain between OVX, OVX SAB, and OVX EXE SAB groups. OVX EXE group (+6.8%) had a higher BMD gain compared with OVX (+1.9%; \( P < 0.05 \) and OVX EXE SAB (\( P < 0.01 \)).
Trabecular Bone Microarchitecture

Proximal tibia. At the end of the treatment, OVX rats had a 7.1% loss of Tb.Th and a 48.7% loss of trabecular number compared with SHAM animals. OVX animals showed a 53.9% lower BV/TV fraction, whereas BV/TV fraction was higher in the OVX EXE and OVX EXE SAB groups (+38.6% and +21.7%, respectively) compared with the OVX group. The OVX EXE SAB group presented a lower BV/TV (-21.5%) compared with the OVX EXE group; however, we did not notice a significant difference in all the trabecular parameters measured between OVX and OVX SAB groups.

Tb.Th was +16.8% higher in the OVX EXE group compared with the OVX group. Nevertheless, Tb.Th was −5.9% lower in the OVX EXE SAB compared with the OVX EXE group. SAB inhibited the effect of exercise on Tb.Th in the OVX EXE SAB group (Fig. 3).

Distal femur. At the end of the treatment, the differences in femoral microarchitecture parameters indicated the same trend that was observed in the tibia (Table 2). We did not observe significant difference between SAB treatment and placebo group in sedentary OVX, whereas there was an alteration of the trabecular parameter in OVX EXE SAB group compared with OVX EXE. Tb.Th was significantly lower and TBPF higher in OVX EXE SAB group compared with OVX EXE group (~8.0% and +36.1%, respectively; \( P < 0.05 \)). Despite the same trend, exercise induced a lower effect on the femur than on the tibia. For example, femoral Tb.Th was +10.1% higher in the OVX EXE SAB group compared with the OVX group, whereas a +16.8% difference of Tb.Th was observed in the tibia.

Most of the analysis of variance revealed significant overall SAB and EXE effects (\( P < 0.05 \)), and a significant interaction between SAB and EXE was detected, suggesting that SAB effect on trabecular bone parameters is different between sedentary and exercise groups.

Cortical Investigation

No significant difference for tibial mid-diaphysis cortical width was observed between the OVX (536.5 ± 19.8 μm), OVX SAB (530.82 ± 18.4 μm), OVX EXE (567.4 ± 20.3 μm), and OVX EXE SAB (543.4 ± 19.7 μm) groups (Fig. 3). Conversely, the cortical porosity in the OVX EXE (0.63% ± 0.18) and OVX EXE SAB (0.69% ± 0.21) groups was significantly lower than in OVX (1.8% ± 0.23; \( P < 0.05 \)) and OVX SAB (2.2% ± 0.21; \( P < 0.05 \)) groups.

No significant difference for femoral mid-diaphysis cortical width was observed between the OVX and OVX SAB groups or between the OVX EXE and OVX EXE SAB groups. However, we observed a significantly higher cortical width in OVX EXE and OVX EXE SAB vs. OVX SAB. The OVX EXE and OVX EXE SAB groups had lower cortical porosity (−45.8% and −37.5%, respectively; \( P < 0.01 \)) than the OVX group (Table 2).

Cellular Activity

Higher resorption and formation activities were observed in the OVX group than in the SHAM group (5% in SHAM group vs. 12.5% in OVX group of osteoclastic surfaces and +66.6%...
of BFR/BS in OVX group). SAB treatment did not alter the histomorphometric data in the OVX SAB group. We observed an effect of exercise on both bone formation parameters and osteoclast surface. The OVX EXE group had greater mineralized apposition rate compared with the OVX group. The osteoclast surface in the OVX EXE group was 39% lower than in the OVX group (Fig. 4).

No significant difference was observed between OVX EXE/OVX EXE SAB. Nevertheless, the significant difference existing between OVX/OVX EXE for bone formation rate and mineralized apposition rate did not exist anymore between OVX/OVX EXE SAB (Fig. 4), indicating that SAB appeared to slow down the bone formation effect of EXE.

**Biomechanical Parameters**

*Bending test.* Bending tests revealed no significant difference between OVX and OVX SAB groups except for the energy to ultimate failure, which was higher in OVX SAB group compared with OVX group (Table 3). We observed significantly higher energy to ultimate force and Young's modulus in OVX EXE (27.1% and 34.9%, respectively; \( P < 0.05 \)) vs. OVX. The difference in Young's modulus disappeared when we compared the OVX group to the OVX EXE SAB group but remained significant for the energy to ultimate force (Table 3). Analysis of variance revealed that energy to ultimate force was higher in rats treated by SAB and equally in sedentary and exercise rats.

**Compression test.** Results obtained from the compression test showed that OVX lowered ultimate force, stress, displacement, extrinsic energy to ultimate, intrinsic energy to ultimate, stiffness, and Young's modulus compared with the SHAM group. Extrinsic and intrinsic biomechanical properties of the metaphysis revealed no significant differences between OVX and OVX SAB groups (Table 4). The OVX EXE group has a higher ultimate force and stiffness (+23% and +33.1%, respectively; \( P < 0.05 \)) compared with OVX and did not differ from the SHAM group, whereas ultimate force and stiffness were significantly lower in the OVX EXE SAB group (−23.6% and −43.1%, respectively; \( P < 0.05 \)) compared with SHAM. Furthermore, stiffness of the OVX EXE SAB group was lower compared with OVX EXE (−40.7%; \( P < 0.05 \)). Analysis of the variance revealed significant overall exercise and treatment effects (\( P < 0.05 \)), and a significant interaction between exercise and treatment was detected, suggesting that SAB biomechanical effect is different in sedentary and exercise rats.

**Bone turnover.** At the end of the experiment, the osteocalcin level was 17.6% higher in the OVX EXE group (251.22 ± 18.5

![Graph showing histomorphometric data](image-url)

![Graph showing biomechanical parameters](image-url)

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**Table 2. Femoral trabecular microarchitecture in ovariectomized rats treated or not by salbutamol and with or without exercise**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SHAM</th>
<th>OVX</th>
<th>OVX SAB</th>
<th>OVX EXE</th>
<th>OVX EXE SAB</th>
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<tr>
<td>BV/TV, %</td>
<td>28.38 ± 0.74abcde</td>
<td>15.63 ± 0.72abcdef</td>
<td>16.75 ± 1.54a</td>
<td>19.34 ± 0.84bc</td>
<td>16.00 ± 1.31a</td>
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<td>Tb.Th, μm</td>
<td>105.0 ± 5.4abc</td>
<td>98.3 ± 1.8bcd</td>
<td>97.1 ± 4.3ab</td>
<td>109.04 ± 4.8bcde</td>
<td>100.27 ± 5.2a</td>
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<tr>
<td>TBPI</td>
<td>3.64 ± 1.06abcde</td>
<td>7.64 ± 0.84abcde</td>
<td>7.59 ± 0.55abcde</td>
<td>3.24 ± 1.06abce</td>
<td>5.07 ± 1.29abcd</td>
</tr>
<tr>
<td>Tb.N</td>
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<td>1.59 ± 0.27a</td>
<td>1.72 ± 0.25ab</td>
<td>1.68 ± 0.32a</td>
<td>1.60 ± 0.50a</td>
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<td>Tb.Sp, μm</td>
<td>352.4 ± 34.12abcd</td>
<td>754.0 ± 42.46abc</td>
<td>657.8 ± 40abc</td>
<td>576.1 ± 37.5bc</td>
<td>612.9 ± 38ab</td>
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<td>C width, μm</td>
<td>473.75 ± 35.24abcde</td>
<td>492.79 ± 41.9</td>
<td>466.65 ± 24.7abcde</td>
<td>532.53 ± 46.7abc</td>
<td>550.3 ± 38.6abc</td>
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<td>Po C, %</td>
<td>0.62 ± 0.27b</td>
<td>0.96 ± 0.42abcde</td>
<td>0.83 ± 0.39</td>
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</tbody>
</table>

Values are means ± SE. BV/TV, trabecular bone volume; Tb.Th, trabecular thickness; TBPI, trabecular bone pattern factor (−plate to +-rod); Tb.N, trabecular number; Tb.Sp, trabecular spacing; C width, cortical width; Po C, cortical porosity. Significant statistical differences between groups: acompared with SHAM group (\( P < 0.05 \)); bcompared with OVX group (\( P < 0.05 \)); ccompared with OVX SAB (\( P < 0.05 \)); dcompared with OVX EXE (\( P < 0.05 \)); ecompared with OVX EXE SAB.
ng/ml; P < 0.01) than in the OVX group (206.91 ± 17.3 ng/ml). We did not observed any significant difference for the others groups.

No significant difference for CTx level was observed between SHAM (19.76 ± 2.2 ng/ml) and OVX (25.47 ± 4.1 ng/ml), but one existed between SHAM and OVX SAB (28.36 ± 2.4 ng/ml). CTx level was 26.6% lower in the OVX EXE group (18.7 ± 3.1 ng/ml; P < 0.05) than in the OVX group. CTx level was 31% higher in the OVX SAB group and did not differ from OVX or OVX SAB groups. CTx level was 26.6% lower in the OVX EXE group than in the OVX group (206.91 ± 17.3 ng/ml; P < 0.05); dcompared with OVX EXE SAB; BMD gain, trabecular bone proportion, Tb.Th, etc.) by increases in all body compartments during the first 5 wk after OVX (31). Animals of the OVX SAB EXE groups presented a similar body weight gain to that observed in the SHAM group (Fig. 1). This result indicates that SAB and exercise alone is not enough to counter balance the effect of OVX, whereas combined they suppresses the higher increased of body weight. However, the adjustment of the bone parameter (BMD gain, trabecular bone proportion, Tb.Th, etc.) by body weight gain did not change the previous significant difference observed.

Despite the anabolic effect induced by SAB on the muscle mass of the soleus and gastrocnemius, there was no anabolic effect on total body muscle mass.

After OVX, longitudinal BMD analyses of the tibia and femur indicated that SAB did not induce any supplementary deleterious effect. These results are in accordance with Kondo et al., who demonstrated an alteration of bone microarchitecture only in loaded mice treated with isoproterenol and not in unloaded mice (altered bone status) (28). These authors suggested the existence of a threshold of bone alteration below which there is no more alteration of structural bone quantity and quality. Nevertheless, in the present study, we observed a slight beneficial effect of SAB on the femur in the OVX SAB group (BMD gain +2.6% in OVX SAB vs. −3.3% in OVX), but the higher femoral BMD gain in OVX SAB was lost after adjustments for muscle mass (soleus or gastrocnemius). In line with de Souza et al. (14), we suggest that bone cells are more

**Table 3. Influence of exercise on biomechanical properties of the femur in bending test**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SHAM</th>
<th>OVX</th>
<th>OVX SAB</th>
<th>OVX EXE</th>
<th>OVX SAB EXE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate force, N</td>
<td>123.03 ± 4.5</td>
<td>116.68 ± 7.2</td>
<td>112.96 ± 7.3</td>
<td>121.80 ± 7.48</td>
<td>110.04 ± 7.08</td>
</tr>
<tr>
<td>Cross-sectional area, mm²</td>
<td>7.94 ± 0.26</td>
<td>7.38 ± 0.44</td>
<td>7.09 ± 0.37</td>
<td>8.03 ± 0.28</td>
<td>7.25 ± 0.36</td>
</tr>
<tr>
<td>Moment of inertia</td>
<td>6.95 ± 0.38</td>
<td>6.83 ± 0.76</td>
<td>6.73 ± 0.68</td>
<td>7.32 ± 0.53</td>
<td>6.58 ± 0.61</td>
</tr>
<tr>
<td>Stress, N/mm²</td>
<td>144.26 ± 3.8</td>
<td>141.34 ± 21.6</td>
<td>140.89 ± 10.3</td>
<td>157.26 ± 15.27</td>
<td>158.29 ± 17.24</td>
</tr>
<tr>
<td>Energy max, J</td>
<td>64.85 ± 8.5</td>
<td>67.45 ± 8.2</td>
<td>68.81 ± 9.1</td>
<td>69.98 ± 10.05</td>
<td>75.39 ± 7.61</td>
</tr>
<tr>
<td>Energy to ultimate, J</td>
<td>51.77 ± 2.7</td>
<td>47.39 ± 6.6</td>
<td>56.68 ± 6.9</td>
<td>65.02 ± 7.1</td>
<td>69.59 ± 7.9</td>
</tr>
<tr>
<td>Stiffness, N/mm</td>
<td>292.61 ± 24</td>
<td>272.71 ± 15.8</td>
<td>259.4 ± 25.22</td>
<td>280.54 ± 25.1</td>
<td>282.15 ± 39.24</td>
</tr>
<tr>
<td>Young’s modulus, Mpa</td>
<td>7.13 ± 74.4d</td>
<td>6.546 ± 54.3d</td>
<td>6.703 ± 758.8a</td>
<td>10.059 ± 1.678ahc</td>
<td>8.586 ± 1.178</td>
</tr>
</tbody>
</table>

Values are means ± SE. Significant statistical differences between groups: *compared with SHAM group (P < 0.05); +compared with OVX group (P < 0.05); compared with OVX SAB (P < 0.05); compared with OVX EXE (P < 0.05); compared with OVX EXE SAB.
sensitive to mechanical loading when bone is altered compared with our laboratory’s previous study in SHAM (6). In the OVX SAB group, mechanical loading was probably due to the muscle mass anabolic effects of SAB.

Another explanation for this effect could be that the absence of estrogen limited the influence of SAB on bone β2-receptor, as suggested by Benoit et al. (5), resulting in an imbalance between the direct negative effect of SAB on bone receptors and the beneficial effect of SAB on muscle mass inducing a slight positive effect in the OVA SAB group compared with OVX rats. Nevertheless, the absence of estrogens would also have reduced the muscle mass increase, which was not the case. Whatever the explanation, the consequence is that, in OVX rats, we did not notice a deleterious effect of SAB compared with our previous study in SHAM, where we clearly demonstrated an alteration of the BMD and bone microarchitecture (6).

As the potentially lower β2-receptor activity suggested in OVX animals (5), we did not notice any effect of SAB on cortical width or porosity (tibia, femur) compared with our previous study in SHAM (6). Bending and compression tests revealed no significant difference between OVX and OVX SAB groups, except for the bending energy to ultimate force, which is slightly higher in the OVX SAB group compared with the OVX group.

Preventive effects of treadmill exercise on bone have been reported in OVX rats. The present protocol is similar to that described by Iwamoto et al. and demonstrated a significant influence of exercise on bone microarchitecture, cortical width, and porosity in OVX rats, whereas only a trend was observed in our previous study on SHAM rats (24, 25). Our results are in agreement with those reported by Barenholz et al. who demonstrated that exercise had a higher influence on bone BMD and microarchitecture in OVX than in SHAM rats (3, 4). Peng et al. suggested that the higher bone turnover in OVX rats might increase the sensitivity of bone cells to treadmill exercise (38). Rutherford et al. suggested that the bone tissue response to exercise depends on the initial bone status and that exercise is more effective when bone is more fragile (39). Exercise in OVX rats increases Young’s modulus and energy to ultimate in bending tests. The compression tests revealed an influence of exercise on ultimate force and stiffness associated with a beneficial effect on Tb.Pf, BV/TV, and Tb.Th (33). Exercise decreases bone resorption and increases bone formation as shown by osteocalcin and CTX levels and as reflected by an increase of mineralized surfaces and osteoblast activity. The uncoupling of bone resorption and formation in response to exercise must be nuanced since it has been found only in rats, usually young and growing rats (9, 23, 26), and not in humans.

To provide a better understanding of the influence of SAB and physical training on bone tissue, we studied the efficiency of the combination of these two interventions.

We observed that SAB had induced a deleterious effect in OVX EXE, whereas slight effects were observed in sedentary rats. In particular, we observed in the OVX EXE SAB group a lower cross-sectional area in bending and a lower stiffness in compression compared with OVX EXE group.

In OVX EXE rats treated by SAB, no significant difference in Young’s modulus of bending test, ultimate force of compression, and stiffness of compression were observed compared with the OVX group, despite a higher energy to ultimate in bending in OVX EXE SAB vs. OVX; those results suggest an inhibition of the effect of exercise by the SAB treatment. Effectively, we noticed that the decrease of CTx level by EXE was not observed in the OVX EXE SAB group. We observed a similar increase in CTx in sedentary or trained rats with SAB treatment. Our results on bone marker levels are in line with those reported by Cavalié et al. (11), who showed an increase in urinary deoxypyridinoline excretion due to increased bone resorption independently of exercise activity.

However, OVX EXE SAB rats presented a greater tibial Tb.Th and a lower TBPf than OVX SAB rats, suggesting that loading physical activity is more effective on trabecular bone than an increase of muscle mass alone (observed in OVX SAB), as reported by Hamrick et al. in their study on the effect of exercise in myostatin knockout mice (20). It is noteworthy that these results were observed only in the tibia and not in the femur. During running exercise in rats, the tibia is more exposed to mechanical loading (impact) than the femur (23). One explanation is that the tibia is located further away from the rat’s body mass than the femur (18). Bone markers suggest that SAB in OVX EXE increased bone turnover with a higher augmentation of the bone resorption. However, this result must be nuanced since it was not confirmed by histomorphometric data, suggesting that SAB effect is more on the activity of the bone cell than on the number.

The present study demonstrated that SAB did not enhance the alteration of the skeleton in OVX rats, supporting a lower effect of SAB in the absence of estrogen according to Benoit et al. (5). However, the fact that a SAB treatment in OVX EXE rats had induced the same deleterious effect as in SHAM rats suggests (28) the existence of a threshold of bone alteration, below which there is no more alteration of structural bone quantity and quality.

### Table 4. Influence of salbutamol on biomechanical properties of the distal femur metaphysis in compression test

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SHAM</th>
<th>OVX</th>
<th>OVX SAB</th>
<th>OVX EXE</th>
<th>OVX EXE SAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate force, N</td>
<td>693.92 ± 35.58b,c,e</td>
<td>468.01 ± 51.05a,d</td>
<td>509.09 ± 47.00a</td>
<td>607.89 ± 41.83b</td>
<td>529.84 ± 54.5b</td>
</tr>
<tr>
<td>Stress, N/mm²</td>
<td>140.86 ± 10.30b,c,e</td>
<td>92.75 ± 12.62a</td>
<td>102.61 ± 7.75a</td>
<td>103.48 ± 5.55a</td>
<td>102.58 ± 7.50a</td>
</tr>
<tr>
<td>Displacement, mm</td>
<td>0.27 ± 0.04d</td>
<td>0.22 ± 0.02e</td>
<td>0.22 ± 0.02e</td>
<td>0.18 ± 0.02e</td>
<td>0.25 ± 0.04e</td>
</tr>
<tr>
<td>Stiffness, N/mm²</td>
<td>3.57 ± 0.57b,c,e</td>
<td>3.43 ± 0.19d</td>
<td>3.96 ± 0.61c</td>
<td>5.17 ± 0.93b,c,e</td>
<td>3.06 ± 0.66a,d</td>
</tr>
<tr>
<td>Young's modulus, MPa</td>
<td>2.52 ± 0.36b,c,e</td>
<td>1.64 ± 0.37d</td>
<td>1.91 ± 0.41c</td>
<td>2.22 ± 0.42d</td>
<td>1.86 ± 0.34c</td>
</tr>
</tbody>
</table>

Values are means ± SE. Ext, extrinsic energy; Int, intrinsic energy. Significant statistical differences between groups: a compared with SHAM group (P < 0.05); b compared with OVX group (P < 0.05); c compared with OVX SAB (P < 0.05); d compared with OVX EXE (P < 0.05); e compared with OVX EXE SAB.
We are aware of some limitations of this study. First, we have not evaluated other organ system as the growth hormone/IGF-1 axis or the hormone of the energy balance (leptin, cortisol), which could have been changed by the high dose of SAB. Second, it would have been of interest to test therapeutic dose used by athlete for asthma to better explain the result obtain by De Vries Pharm et al. (15).

In conclusion, this study suggests that greater attention should be paid to the side skeletal effects of SAB used at doping doses by athletes. These preliminary results support the hypothesis that β2-agonists, especially their impact on muscle, can induce various bone effects depending much more on the initial bone status than on estrogen deficiency. Further studies are needed to better understand the complex mechanisms of β2-agonists.

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REFERENCES


