

# Mechanical Properties, Bone Mineral Content, and Bone Composition (Collagen, Osteocalcin, IGF-I) of the Rat Femur: Influence of Ovariectomy and Nandrolone Decanoate (Anabolic Steroid) Treatment

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**Summary.** Nandrolone decanoate (ND) is an anabolic steroid with a positive effect on bone mass in osteoporotic patients. The mechanism of action, (i.e., reduction of bone resorption and/or stimulation of bone formation), the ultimate effect on mechanical properties, and the most effective dosage are not yet clear. To address these issues, dose-related effects of the long-term effect of ND on serum and bone biochemistry, bone mineral content, and bone mechanical properties in ovariectomized (OVX) rats (12 weeks old at the start of the experiment) were studied for 6 months. The results were compared with those obtained in age-matched, intact, and OVX rats. OVX caused in the femur a significant increase in net periosteal bone formation and net endosteal bone resorption of bone collagen content and torsional strength, and of serum alkaline phosphatase, osteocalcin, and insulin-like growth factor-I (IGF-I) levels, whereas cortical bone density and calcium/creatinine and phosphorus/creatinine in 24-hour urine were significantly reduced.

Treatment of OVX rats with 1 mg ND/14 days resulted in a significant increase in periosteal bone formation, femur length, cortical and trabecular bone mineral content and density, torsion stiffness and strength, and bone IGF-I content, and a decrease in serum osteocalcin, urinary calcium/creatinine levels, and bone collagen content compared with OVX controls. The higher ND dosage of 2.5 mg/14 days did not improve the results. ND treatment did not reverse all changes induced by OVX to the level of the intact controls.

These results indicate that ND acts as an antiresorptive drug and as a bone formation stimulating drug. Furthermore, the increased bone mass and bone mineral density is associated with improved bone strength and stiffness and the presence of an increased amount of IGF-I. IGF-I is a growth factor considered to play a role in the maintenance of normal skeletal balance by a paracrine or autocrine mechanism.

**Key words:** Bone – Nandrolone decanoate – Ovariectomy – Bone mechanics – IGF-I – Rat.

The ultimate goal of therapy for osteoporosis is to reduce fracture rate. Although a main determinant of bone strength is bone mass, an increase in bone mass is not always asso-

ciated with a reduced fracture rate, as was recently shown in fluoride-treated patients [1]. Most drugs used for osteoporosis have antiresorbing activity and thus prevent further loss of bone without increasing bone mass. Of the drugs that increase bone mass, sodium fluoride and anabolic steroids are most often used for the treatment of established osteoporosis. Nandrolone decanoate (ND), an anabolic steroid with an acceptable side-effect profile, has shown its effectiveness in established osteoporosis with vertebral crush fractures and in corticosteroid osteoporosis [2–5]. Geusens and Dequeker [2] demonstrated in a double-blind study with 34 patients with symptomatic osteoporosis that ND statistically significantly increased the bone mineral content (BMC) at the radius and reduced the endosteal bone loss at the metacarpals and the fracture rate in the second year observation period. Hassager et al. [6] showed that ND therapy could achieve an increase in BMC in postmenopausal women, but this was maintained only for as long as therapy was continued. ND is also effective in the prevention of corticosteroid-induced osteoporosis. This was evidenced by Adami et al. [5] who measured increased bone mass and decreased urinary excretion of hydroxyproline in patients on corticosteroid therapy receiving ND compared with control patients on corticosteroid therapy alone. Although the direct effect of androgens on bone has not been proved *in vivo*, Colvard et al. [7] demonstrated that human bone cells of the osteoblastic lineage contain receptors not only for estrogens but also for androgens, giving support for a direct action. Androgens are shown to stimulate human and murine osteoblastic cell proliferation *in vitro*, and to induce expression of the osteoblast-line differentiation marker alkaline phosphatase, presumably by an androgen receptor-mediated mechanism [8]. Furthermore, Fitzsimmons et al. [9] reported that androgens, as well as the androgenic steroid ND, stimulate proliferation and differentiation of human osteoblast-like cells *in vitro*.

To broaden our understanding of the quality of bone, we report studies on mechanical properties, mineral content, and matrix composition of bone and serum, and urine biochemical characteristics from rats that have been subjected for 6 months to ovariectomy (OVX) and to two different dosages of ND (1.0 or 2.5 mg of ND subcutaneously/2 weeks). The mechanical characteristics of bone have been assessed by measurements of torsion stiffness, torsion strength, toughness, and maximal angular deformation, and were investigated using an impact torsion test to failure. Hydroxyproline, insulin-like growth factor-I (IGF-I) and osteocalcin content, BMC, periosteal and endosteal width, and

serum and urinary parameters relevant to the calcium metabolism have also been measured, and the relationship between these and the physical measurements have been examined.

## Materials and Methods

### Animals

For the experiment, 120 sexually mature (12 weeks old) female Wistar rats (mean weight  $239 \pm 10$  g) obtained from Harlan CPB, Zeist, The Netherlands were used. One day after arrival the animals were divided into four groups ( $n = 30$ ) by a block design using body weight as discriminator. One group was sham operated and the other groups were OVX. Treatment was started immediately after the operation. The sham-operated and one group of OVX rats were injected subcutaneously once every 14 days with 0.25 ml arachis oil. The remaining animals were injected subcutaneously with 1.0 or 2.5 mg ND (Deca-Durabolin, Organon Int. B.V. Oss, The Netherlands) once every 14 days. During the experimental period of 27 weeks the animals were kept individually in Macrolon cages type 3 (Komeco, Alkmaar, The Netherlands), were pair fed, had free access to tap water, and were weighed once a week.

After 27 weeks of treatment, blood from the retro-orbital plexus and 24-hours urine specimens were collected and stored at  $-20^{\circ}\text{C}$  until biochemical determinations (see below). The subsequent day the rats were sacrificed. Both femora, tibia, and vertebrae were dissected. The left femurs were immediately frozen and stored at  $-20^{\circ}\text{C}$  until determination of the biochemical composition of the bone, that is, mineral, collagenous and noncollagenous protein content (see below). The right femurs were cleaned from adherent muscles, placed in sterile saline, and stored for 8–16 hours at  $4^{\circ}\text{C}$ . Left tibia and vertebrae of 12 and 8 randomly selected animals per group, respectively, were fixed with B rkhart fixative and used further for histomorphometrical analysis (see below).

### Serum and Urinary Biochemistry

Serum calcium (Ca), phosphorus (P), alkaline phosphatase, osteocalcin, and IGF-I were determined. Plasma was acid-ethanol extracted before IGF-I determination. Osteocalcin and IGF-I were measured by radioimmunoassay (RIA) as described below. Analyses of urinary Ca, phosphate, and creatinine (Cr) concentrations were performed by standard techniques (Multistat II centrifugal Analyser, Instrumentation Laboratory Italy, and Encore centrifugal Analyser, Baker Instruments USA).

### Biochemistry of Bone

**Preparation of Bone Tissue.** The left femurs were cleaned, and epiphyses and bone marrow were removed. The diaphyses were defatted for 2 days in trichlorethylene (100%) which was renewed twice a day. Pulverization was carried out with a beater mill cooled with liquid nitrogen. Particle size of bone powder can affect the results of the analyses, as reported earlier [10], hence, bone powder of unknown particle size may give rise to confusing results. To avoid this problem, we powdered the bone up to particles between 40 and 160  $\mu\text{m}$ . Bone powder was stored at  $-20^{\circ}\text{C}$  until required for analysis.

**Analytical Procedures.** Small amounts (approximately 10 mg) of bone powder were ashed at  $700^{\circ}\text{C}$  for 6 hours. The resident ash weight was determined and expressed as percentage of dry bone weight. Subsequently, the samples were dissolved in 2.5 ml 1 N HCl and diluted (factor 10) before Ca and P analysis. Both automatic colorimetric measurements were done by a BM/Hitachi System 717 apparatus (Boehringer Mannheim GmbH, Germany).

The dry bone samples were analyzed for their hydroxyproline content according to the method of Kivirikko et al. [11].

Osteocalcin and IGF-I were measured after extraction of the bone powder. For osteocalcin, 10 mg of each dry bone powder sample was extracted in 1.0 ml ammonium-EDTA 0.5 M containing protease inhibitors (benzamidine 5 mM, 6-aminocaproic acid 10 mM, p-hydroxymercuribenzoic acid 100  $\mu\text{M}$ , pH 6.2) at  $4^{\circ}\text{C}$ . For IGF-I, about 15 mg of each dry bone powder sample was extracted in 1.5 ml ammonium-EDTA 0.5 M containing the same protease inhibitors cocktail. The extraction was carried out overnight in microcentrifuge tubes by end-over-end rotation. After 18 hours, the solution was centrifuged (12,000 rpm, 30'), and the noncollagenous supernatant was separated from the collagenous residue and used for RIA measurements of osteocalcin and IGF-I. After a second extraction of the first collagenous residue with ammonium-EDTA or formic acid, no measurable amounts of the noncollagenous proteins (osteocalcin, IGF-I) could be found in this extract. After a second extraction of the first residue with guanidine-EDTA, less than 5% of the IGF-I concentration in the first extract could be found in this second extract.

Osteocalcin was measured directly with an in-house RIA in the 1:5000 diluted EDTA extract as described earlier [12], based on the method of Gundberg et al. [13]. The RIA had an intraassay variation of 5.9%, an interassay variation of 5.2%, and the detection limit was 6.45 pmol osteocalcin/tube. The analytical recovery was  $100 \pm 2\%$  (mean  $\pm$  SEM).

Before IGF-I determination, the EDTA extracts were desalted in Sephadex PD-10 columns (Pharmacia) and lyophilized in a Speed Vac Concentrator (Savant). The samples were solved in RIA buffer, and IGF-I was determined as described by Verhaeghe et al. [14] using antibodies against recombinant human IGF-I raised in a guinea pig. Inter- and intraassay coefficients of variation were 7.7 and 7.4% respectively, with a detection limit of 1.3 ng IGF-I/tube. The recovery was  $100 \pm 2\%$  (mean  $\pm$  SEM); no interference with IGF-binding proteins was observed after gel filtration over superose 12 (Pharmacia).

### Bone Histomorphometry

The left tibia and vertebrae were fixed for 24 hours with B rkhart fixative, cut sagittally into 2 equal halves with a diamond saw (Buehler Isomet, low speed saw, Chicago, IL, USA), dehydrated with methanol and embedded in methylmetacrylate. Trabecular bone volume (TBV) of the secondary spongiosa of the metaphysis was measured in sections (thickness 5  $\mu\text{m}$  and stained according to von Kossa's method) with an interactive image analysis system (Context Vision, Link ping, Sweden). TBV was measured in an area of minimally 4  $\text{mm}^2$ .

### Bone Mass Measurements

BMC of the right femurs was measured using single photon absorptiometry using a lunar bone mineral analyser (model SP2 single photon rectilinear scan; Lunar Radiation Corporation, Madison, WI). The source of the mono-energetic radiation was a  $^{125}\text{I}$ -crystal. The calibration of BMC units (gBMC/cm longitudinal bone length) was performed using the standards supplied by the manufacturer. Manufacturer-supplied software as used in the clinical situation was used to calculate BMC. Prior to scanning, femurs were mounted on a plexiglass stage, the bone being supported only at the distal and the proximal end, with the posterior side downwards. The stage was placed on the scanner deck, so that the diaphysis of the bone was oriented perpendicular to the path of the photon beam. Measurements were made at the middle of the femur (F50), mainly containing cortical bone. Eight scan lines were performed at a speed of 0.25 mm/second and interval steps of 1.25 mm. Mean BMC and bone width (W) were measured and bone mineral density ( $\text{BMD} = \text{BMC}/\text{W}$ ) was calculated for the total scanned area. Reproducibility for BMD was 2.2% [15].

### Radiogrammetry

The outer (D) and inner (d) diameter of the cortical bone of the left

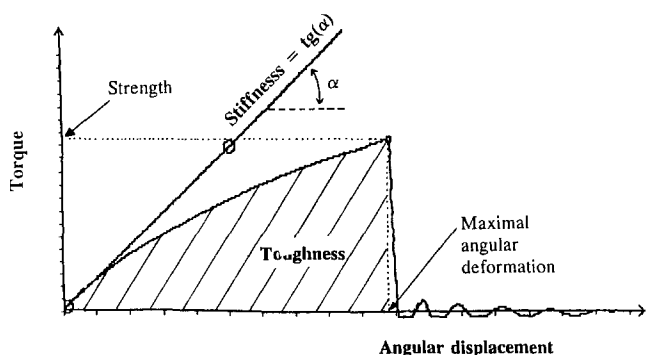


Fig. 1. A typical torque-angular displacement graph of the tested rat femurs. Deformation energy to fracture and stiffness can be derived from this curve. The graph displays most of the mechanical properties of the torsionally loaded bone.

and right femurs were measured at 50% of the length of the femur, using an X-ray caliper with a readout of 0.1 mm [16]. Reproducibility was 1.4% for D and 2.5% for d. The femur length was measured as the distance between the trochanter top and the end of the lateral condyles, with a reproducibility of 0.5%. Cortical width (D-d), cortical area of cross-section ( $D^2-d^2$ ), and percentage cortical area of cross-section ( $D^2-d^2/D^2$ ) were calculated from D and d.

### Mechanical Measurements

The epiphyseal ends of the right femurs were embedded in rectangular epoxy blocks, fitting the grips of the torsional testing machine. Impact torsional loading tests of whole bones were performed with loading time less than 0.1 second. The torsion bench used was a copy of the one described by Burstein and Frankel [17] and Martens et al. [18], with adapted dimensions for small size rat bones. This test is appropriate for whole bone structures as the site of fracture is not predetermined by the testing procedure.

Data from strength and angular displacement transducers were registered with a Biomatron Waveform Recorder model 1015 and processed using a personal computer. Applied torque and angular deformation as a function of time were registered, from which torque versus angular displacement curves were calculated. From the last curve the following whole bone (structural) mechanical variables were derived (Fig. 1): (1) maximum strength (torque at fracture); (2) ultimate angular deformation; (3) torsional stiffness as the ratio of applied torque to resultant angular deformation in the linear region of the curve; stiffness = torque/angular deformation; (4) deformation energy to fracture as the area under the curve (toughness). These parameters do not measure the material properties of the bone tissue, but reflect the mechanical properties of the whole bones which are determined by geometry as well as bone mass and quality. Because the fractures all occur at midshaft positions, torsional strength is due to the cortical bone as no trabecular bone is available in this region. Therefore, the mechanical experiments reflect the influence of ovariectomy and medication on the cortical bone part of the rat femur.

### Statistical Analysis

A software computer program (SAS, SAS Institute Inc., Cary, NC, USA) was used. Intergroup differences were analyzed by one-way analysis of variance (ANOVA) and when significant ( $P < 0.01$ ), Tukey's studentized Range test was used to compare pairs of means. These parametric statistical tests could be used because the data were normally distributed (checked by Shapiro-Wilk statistics). Bone histomorphometry results ( $n = 8/\text{group}$ ) were analyzed by nonparametric statistics using ANOVA and Wilcoxon tests.

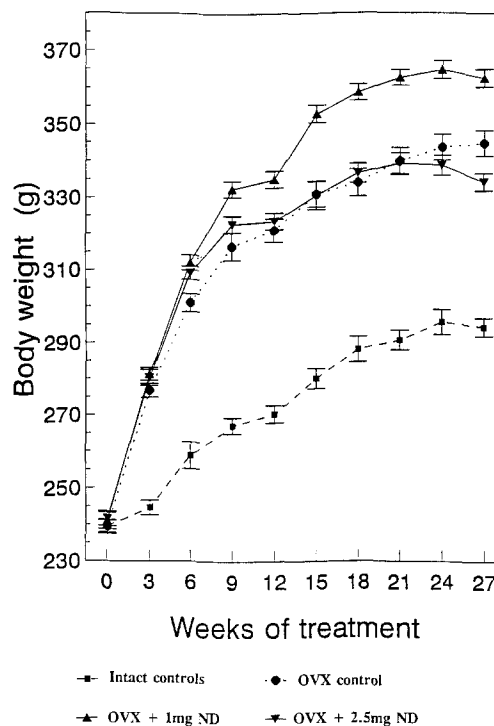


Fig. 2. Evolution of the mean body weight (mean  $\pm$  SD) of each treatment group during the experimental period of 27 weeks.

## Results

### Evaluation of Body Weight during Experiment

The gain in body weights of the four groups during the experimental period is presented in Figure 2. Body weight was significantly higher in all the OVX groups compared with the intact controls from 3 weeks postovariectomy til the end of the experiment. Treatment with 1 mg ND raised body weight gain significantly compared with the OVX control group, from 6 weeks postovariectomy onwards. The higher dose level (2.5 mg ND/14 days) did not result in significantly increased body weight gain compared with the OVX control group.

### Biochemical Characteristics

The plasma and urine parameters relevant to Ca metabolism, measured after 27 weeks of treatment, are summarized in Table 1. The OVX rats differed from intact rats by significantly increased levels in serum alkaline phosphatase, osteocalcin, and IGF-I, and decreased levels of 24 hour urinary calcium/creatinine (Ca/Cr) and P/Cr ratio.

ND reversed the OVX-induced increase of serum osteocalcin and the decrease in the 24 hour urinary P/Cr ratio. However, not all changes induced by ovariectomy were reversed by ND therapy. The increase in alkaline phosphatase after OVX was not influenced by ND therapy although serum IGF-I was slightly increased after 1 mg ND treatment. The significant reduction by OVX in serum Ca concentration was not corrected and urinary Ca and Ca/Cr ratio were even more reduced under ND therapy.

The bone biochemical parameters are shown in Table 2. No significant change in the degree of mineralization (ash,

**Table 1.** Plasma and urine parameters (mean  $\pm$  SD) measured in OVX rats treated with ND compared with intact and OVX controls

Fluid	Parameter	Intact control	OVX control	OVX + 1 mg ND	OVX + 2.5 mg ND
Plasma	Calcium (mmol/liter)	2.42 $\pm$ 0.07	2.38 $\pm$ 0.10	2.35 $\pm$ 0.13 <sup>a</sup>	2.29 $\pm$ 0.12 <sup>c,e</sup>
	Phosphorus (mmol/liter)	1.3 $\pm$ 0.2	1.4 $\pm$ 0.1	1.4 $\pm$ 0.2	1.4 $\pm$ 0.1
	Alkaline Phosphatase (U/liter)	40.6 $\pm$ 11.2	73.1 $\pm$ 15.4 <sup>c</sup>	68.9 $\pm$ 14.6 <sup>c</sup>	70.0 $\pm$ 9.7 <sup>c</sup>
	Osteocalcin ( $\mu$ g/liter)	36.8 $\pm$ 8.7	53.5 $\pm$ 11.1 <sup>c</sup>	36.4 $\pm$ 7.1 <sup>f</sup>	22.5 $\pm$ 4.2 <sup>c,f</sup>
	IGF-I ( $\mu$ g/liter)	0.57 $\pm$ 0.10	0.67 $\pm$ 0.06 <sup>c</sup>	0.72 $\pm$ 0.10 <sup>c,d</sup>	0.64 $\pm$ 0.06 <sup>c</sup>
Urine	Calcium (mmol/liter)	3.75 $\pm$ 2.33	2.16 $\pm$ 1.85 <sup>c</sup>	0.59 $\pm$ 0.43 <sup>c,f</sup>	0.55 $\pm$ 0.37 <sup>c,f</sup>
	Phosphate (mmol/liter)	47.0 $\pm$ 24.3	34.1 $\pm$ 18.7 <sup>a</sup>	30.5 $\pm$ 13.4 <sup>b</sup>	37.4 $\pm$ 17.6
	Creatinine (mmol/liter)	11.7 $\pm$ 5.4	12.9 $\pm$ 5.0	10.2 $\pm$ 4.2	9.1 $\pm$ 4.0
	Calcium/Creatinine	0.35 $\pm$ 0.25	0.18 $\pm$ 0.15 <sup>c</sup>	0.06 $\pm$ 0.06 <sup>c,f</sup>	0.06 $\pm$ 0.03 <sup>b,e</sup>
	Phosphate/Creatinine	4.17 $\pm$ 1.1	2.6 $\pm$ 0.9 <sup>c</sup>	3.2 $\pm$ 0.9 <sup>c</sup>	4.2 $\pm$ 0.9 <sup>f</sup>

<sup>a</sup>  $P < 0.05$ ; <sup>b</sup>  $P < 0.01$ ; <sup>c</sup>  $P < 0.001$  versus intact control<sup>d</sup>  $P < 0.05$ ; <sup>e</sup>  $P < 0.01$ ; <sup>f</sup>  $P < 0.001$  versus OVX control**Table 2.** Biochemical characteristics (mean  $\pm$  SD) of the left femur bone in OVX rats treated with ND compared with intact and OVX controls

Biochemical parameter	Intact control	OVX control	OVX + 1 mg ND	OVX + 2.5 mg ND
Ash (% of dry bone)	69.2 $\pm$ 0.95	68.8 $\pm$ 0.8	69.0 $\pm$ 0.9	69.2 $\pm$ 1.2
Calcium (% of dry bone)	25.7 $\pm$ 0.4	25.7 $\pm$ 0.5	25.7 $\pm$ 0.4	25.7 $\pm$ 0.4
Phosphorus (% of dry bone)	11.9 $\pm$ 0.2	11.9 $\pm$ 0.2	11.9 $\pm$ 0.2	11.9 $\pm$ 0.2
Hydroxyproline ( $\mu$ g/mg dry bone)	19.09 $\pm$ 0.90	19.55 $\pm$ 0.94 <sup>a</sup>	19.08 $\pm$ 0.75 <sup>c</sup>	19.02 $\pm$ 0.74 <sup>c</sup>
Osteocalcin ( $\mu$ g/mg dry bone)	1.06 $\pm$ 0.17	1.13 $\pm$ 0.22	1.09 $\pm$ 0.18	0.97 $\pm$ 0.13 <sup>d</sup>
IGF-I (ng/mg dry bone)	3.77 $\pm$ 0.79	4.01 $\pm$ 0.62	4.72 $\pm$ 0.69 <sup>b,d</sup>	4.57 $\pm$ 0.66 <sup>b,d</sup>

<sup>a</sup>  $P < 0.05$ ; <sup>b</sup>  $P < 0.001$  versus intact control<sup>c</sup>  $P < 0.05$ ; <sup>d</sup>  $P < 0.001$  versus OVX control

Ca, and P concentration) between the different treatment groups was observed. After OVX, the hydroxyproline content was significantly increased whereas osteocalcin increased nonsignificantly. These changes were prevented by ND therapy. Bone IGF-I content increased significantly in the ND treatment groups compared with intact and OVX controls. OVX itself had no effect on the level of bone IGF-I.

#### Bone Histomorphometry and Bone Mass Measurements

TBV was significantly decreased in both tibia and vertebrae after OVX (Table 3). ND therapy could reverse this OVX-induced decrease to the level of the intact controls.

Results of BMC measured by single photon absorptiometry at the middle of the femur (F50)—mainly representing cortical bone—are also shown in Table 3. OVX decreased bone width and BMD. ND therapy in OVX rats increased BMC, BMD, and bone width significantly. There was no dose relationship; the maximum effect was obtained with 1 mg and a lesser effect with the 2.5 mg dosage.

#### Radiogrammetry

The effect on the bone surface—periosteal and endosteal—was evaluated by radiogrammetry at the middle of the femur and the results are shown in Figure 3. OVX induced a significant increase in periosteal (D) and endosteal (d) diameter, resulting in a small increase in cortical area ( $D^2 - d^2$ ) and a decrease in percentage cortical area ( $(D^2 - d^2)/D^2$ ) (both not significant). Cortical width (D-d) did not alter significantly after OVX. ND therapy significantly increased the periosteal diameter further with no concomitant increase in endosteal diameter, resulting in a significant increase in cortical width and area. A nonsignificant increase in percentage cortical

area was found. ND treatment further stimulated femoral longitudinal growth (L) very significantly.

#### Mechanical Properties

The results of mechanical properties assessed from torque-deflection traces (stiffness, strength, deformation energy to fracture, and angular deformation) are shown in Figure 4. OVX did not change the torsional stiffness but significantly increased the torsional strength, deformation energy to fracture, and angular deformation. ND significantly increased torsional stiffness, strength, and deformation energy to fracture compared with intact controls. The positive effect of OVX on maximal angular deformation was also maintained, except for the high dosage of ND.

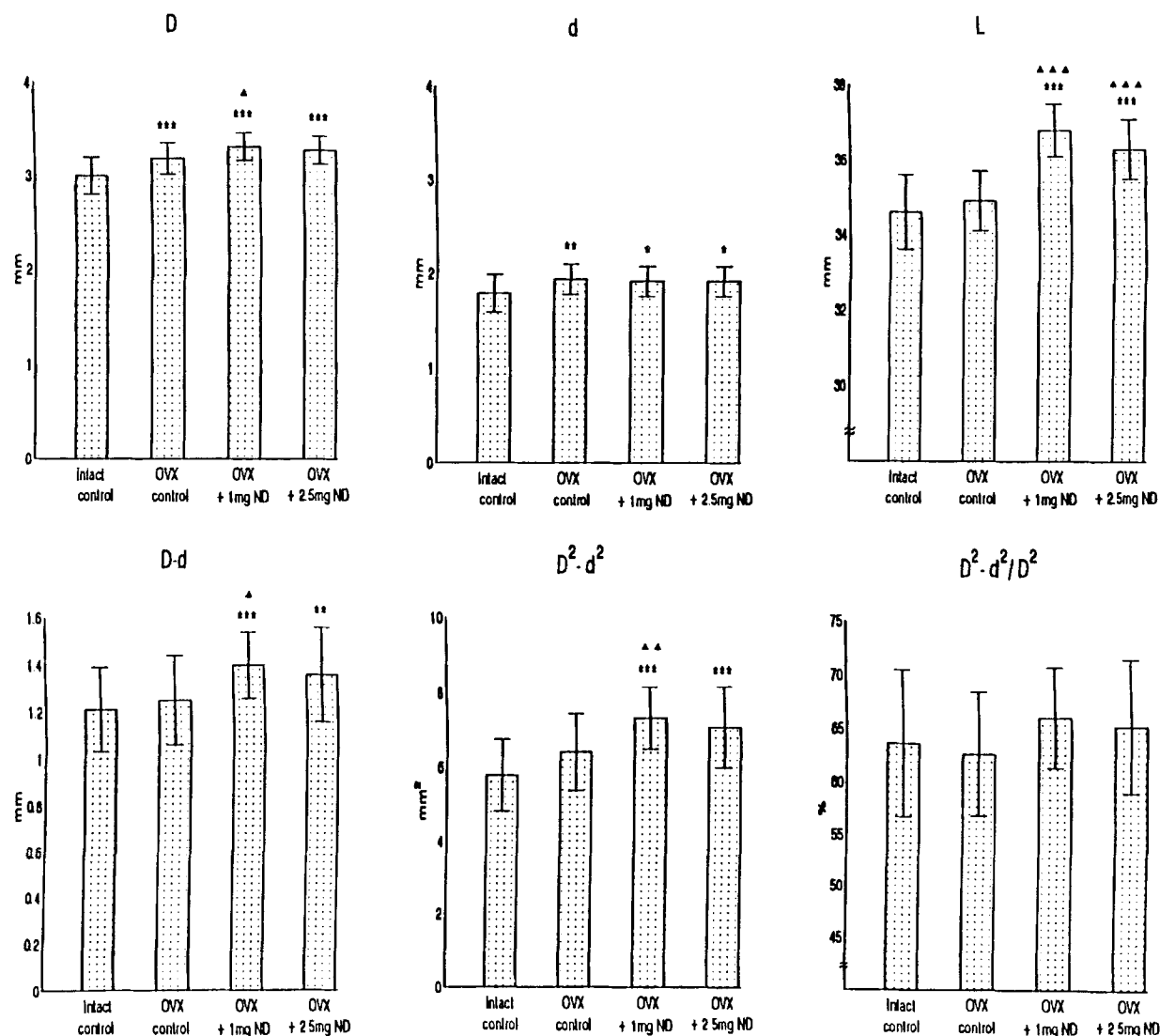
#### Discussion

The present study of mechanical properties reveals that with OVX, despite the fact that there was no increase in BMC and even a decrease in cortical bone mineral density, increases of torsion strength, toughness of the whole bone structure (and not of the material alone), and maximal angular deformation compared with the sham-operated group were observed. This may be explained on one hand by the important increase in periosteal diameter changing the bone mass, shape, and outer volume (geometry), and on the other hand by material properties alterations. The strength of bone tissue therefore is determined also by the chemical composition and the physical characteristics of the solid mineralized phase, and thus not only by the bone mass.

Because destructive mechanical studies cannot be applied on human bone *in vivo*, we have to rely on observations in experimental animals. To what extent our work on rats

**Table 3.** Results of bone histomorphometry and bone mass measurements (mean  $\pm$  SD) in OVX rats treated with ND compared with intact and OVX controls

Place	Parameter	Intact control	OVX control	OVX + 1 mg ND	OVX + 2.5 mg ND
Tibia	TBV (%)	12.09 $\pm$ 3.00	3.05 $\pm$ 1.26 <sup>b</sup>	8.45 $\pm$ 2.88 <sup>d</sup>	7.82 $\pm$ 3.57 <sup>d</sup>
Vertebrae	TBV (%)	32.2 $\pm$ 9.4	21.0 $\pm$ 3.2 <sup>a</sup>	30.2 $\pm$ 9.3 <sup>c</sup>	32.3 $\pm$ 9.5 <sup>c</sup>
Femur middle (F50)	BMC (mg/cm)	87.7 $\pm$ 8.2	87.1 $\pm$ 6.5	102.9 $\pm$ 6.8 <sup>b,d</sup>	98.6 $\pm$ 9.7 <sup>b,d</sup>
	Width (cm)	0.46 $\pm$ 0.02	0.48 $\pm$ 0.02 <sup>b</sup>	0.49 $\pm$ 0.02 <sup>b</sup>	0.48 $\pm$ 0.02 <sup>b</sup>
	BMD (mg/cm <sup>2</sup> )	191.7 $\pm$ 9.6	179.9 $\pm$ 8.4 <sup>b</sup>	209.1 $\pm$ 10.3 <sup>b,d</sup>	205.0 $\pm$ 14.9 <sup>b,d</sup>

<sup>a</sup>  $P < 0.05$ ; <sup>b</sup>  $P < 0.001$  versus intact control<sup>c</sup>  $P < 0.05$ ; <sup>d</sup>  $P < 0.001$  versus OVX control**Fig. 3.** Radiogrammetric characteristics (mean  $\pm$  SD) of the femoral bones in OVX rats treated with ND compared with intact and OVX controls. (Periosteal diameter, D; endosteal diameter, d; femur length, L; cortical width, D-d; cortical area of cross-section, D²-d²;percentage cortical area of cross-section, D²-d²/D²). Significances are indicated as follows: \* versus intact controls,  $\blacktriangle$  versus OVX controls (\*,  $\blacktriangle$ :  $P < 0.05$ ; \*\*,  $\blacktriangle\blacktriangle$ :  $P < 0.01$ ; \*\*\*,  $\blacktriangle\blacktriangle\blacktriangle$ :  $P < 0.001$ ).

can be extrapolated to humans is an important question. With the reservation that the rat has a juvenile skeleton in which cortical bone resorption and formation are not necessarily coupled, it is, nevertheless, interesting to compare skeletal effects of estrogen deficiency in rats and humans [19, 20]. The OVX rat may be useful for the initial evaluation

of therapeutic protocols designed to minimize bone loss in the estrogen-deficient state in humans. It is a valuable but imperfect animal model for investigating the role of (sex) steroids in the pathogenesis of estrogen deficiency-induced bone loss [21]. Although cancellous bone remodeling is similar in rats and humans [22], cortical bone remodeling differs

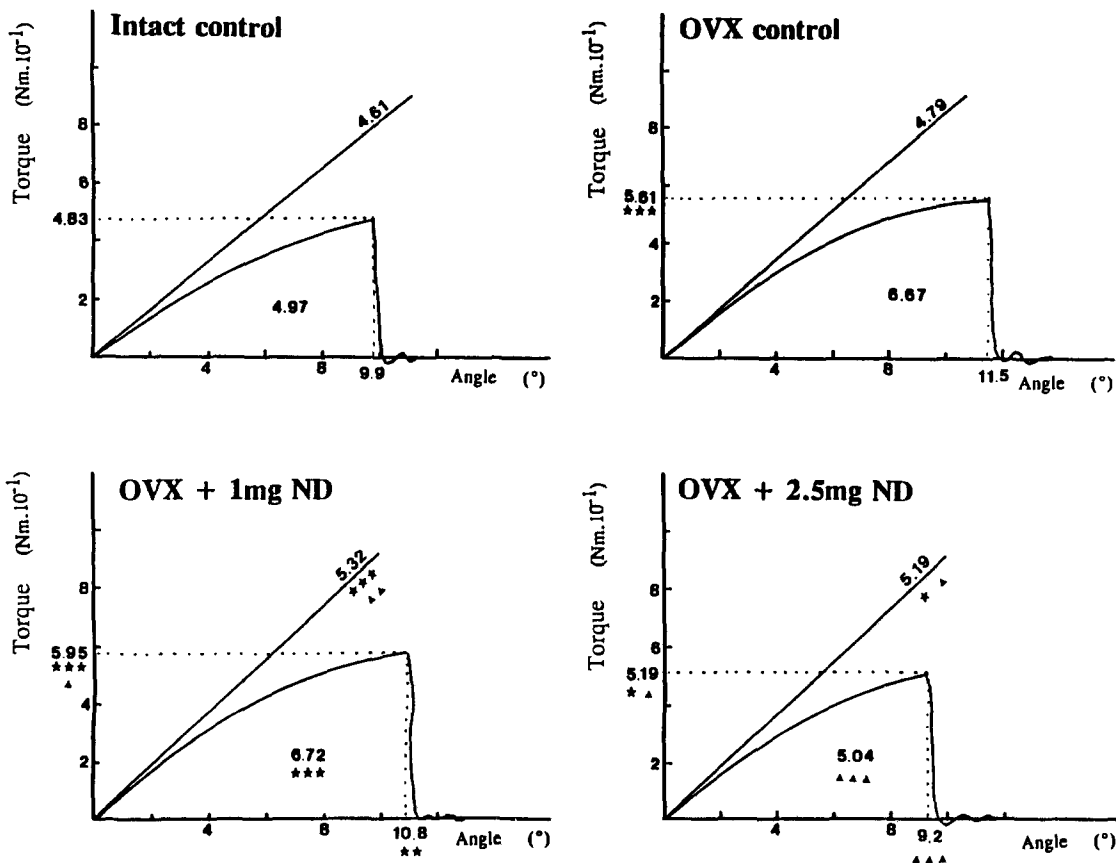


Fig. 4. Mechanical characteristics of the right femurs in OVX rats after long-term treatment with ND compared with intact and OVX controls. The mean results of every group are presented as hand-made torque-angular displacement curves (see Fig. 1), showing the mechanical properties strength ( $10^{-1}$  Nm), maximal angular displacement (degrees), stiffness (Nm/rad), and energy absorption ( $10^{-2}$  J). Significances are indicated as follows: \* versus intact controls,  $\blacktriangle$  versus OVX controls (\*,  $\blacktriangle$ :  $P < 0.05$ ; \*\*,  $\blacktriangle\blacktriangle$ :  $P < 0.01$ ; \*\*\*,  $\blacktriangle\blacktriangle\blacktriangle$ :  $P < 0.001$ ).

placement (degrees), stiffness (Nm/rad), and energy absorption ( $10^{-2}$  J). Significances are indicated as follows: \* versus intact controls,  $\blacktriangle$  versus OVX controls (\*,  $\blacktriangle$ :  $P < 0.05$ ; \*\*,  $\blacktriangle\blacktriangle$ :  $P < 0.01$ ; \*\*\*,  $\blacktriangle\blacktriangle\blacktriangle$ :  $P < 0.001$ ).

in the two species. In the present study, rats were treated during 6 successive months. Under these conditions it can be expected that the effects of OVX and drug treatment on bone balance, architecture, and mass are measured in a steady state situation of the rat bone metabolism. OVX animals gained much more weight than the age-matched, sham-operated controls, confirming earlier rat studies [23]. Increased periosteal and endosteal diameters following OVX are also described by others [24, 25] and may be a consequence of the increased body size and thus lead to reduced BMD, although BMC is unchanged.

The changes observed after OVX in this study are similar to the changes seen after the menopause in women. Increases in alkaline phosphatase activity, osteocalcin levels [26], and periosteal diameter, and a reduction in 24-hour Ca excretion and bone density are also observed in postmenopausal women [27]. The increased osteocalcin levels observed after OVX reflect a high turnover rate, as demonstrated earlier in ovariectomized dogs [28] and rats [29]. Wronski et al. [30] showed in OVX rats of the same age as ours that both the total osteoblast and osteoclast surface of trabecular bone increase during the first month postovariectomy followed by a gradual decline toward control level, indicating a close temporal association between the initial development of osteopenia in OVX rats and a marked increase in bone turnover. It was shown earlier in humans that the temporary postmenopausal increase in bone and serum osteocalcin demonstrates that osteoblastic activity and bone

turnover can be stimulated, but that this increased turnover in the immediate postmenopausal period results in a negative bone balance [31, 32].

Based on biochemical and bone mass parameters measured in the present study, one might conclude that OVX increased bone modeling and remodeling and reduced trabecular bone volume and cortical BMD. An increase in bone turnover would not necessarily result in bone loss unless an imbalance existed between bone resorption and formation with an emphasis on the former process. Such an imbalance occurs in OVX rats as these animals lose bone, as demonstrated by the bone histomorphometric and BMD measurements in our experiment and shown earlier by Wronski et al. [33, 34]. Although we did not observe differences in BMC after OVX, we observed a decrease in BMD due to an increased bone width, indicating the importance of mentioning both parameters.

Interestingly, we found significant elevated IGF-I levels in the serum after OVX compared with intact animals. Although no effect of the menopause can be observed in a study of Yamamoto et al. [32] describing effects of age and sex on plasma IGF-I levels in normal adults, it has been shown that estrogens inhibit IGF-I production in the liver [35, 36]. This is in agreement with our serum IGF-I levels found after OVX where the source of estrogens is removed. IGF-I production is demonstrated in many other tissues beside the liver, leading to a concept of IGF-I action, with growth hormone (GH) considered to act by stimulating

IGF-I production in target tissues, which then act in an autocrine or paracrine manner on local cells [37]. IGF-I can also act in an endocrine manner, with binding proteins determining the circulating levels and possibly their activity. The relative importance of endocrine versus autocrine/paracrine actions may vary from tissue to tissue and, within individual tissues, may vary with developmental or pathological changes [38].

The fact that after OVX IGF-I levels in bone are not increased despite an increase in bone and serum osteocalcin and serum alkaline phosphatase levels (also osteoblast products) indicates that osteoblasts in a different stage of differentiation or in a different microenvironment may produce different molecules. This finding is in line with results obtained by Finkelman et al. [39] who found that OVX lowered the concentration of TGF- $\beta$  in extracts of rat bone but did not change the concentrations of IGF-I or IGF-II.

In this study we demonstrate that in OVX, sexually mature rats, long-term ND treatment increased cortical bone mass (BMC and BMD) of the femur, increased trabecular bone volume of tibia and vertebrae, improved the mechanical properties stiffness and strength of the femur, and enhanced the bone matrix content of the local growth factor IGF-I compared with the OVX controls. These important improvements of bone quality parameters are most likely due to a stimulation of bone formation at the periosteal surface and to changes in material properties.

The high dosage of ND was associated with a decrease of mechanical variables, that is, deformation energy to fracture and maximal angular deformation, despite an increased bone mass, indicating that the chemical composition was altered. An explanation could be that with high dosage ND, bone turnover is so slowed down—and this is in keeping with the significant reduction of serum and bone osteocalcin and decrease in collagen content—that microregions in the bone become more mineralized with aging *in situ* and therefore react differently on mechanical stresses. This observation is further sustained by the OVX group with a high turnover state—characterized by the high serum and bone osteocalcin levels and higher collagen content compared with intact animals—representing young bone tissue with a lesser degree of mineralization of the microregions. On mechanical tests of the whole bone structures, these animals showed results on deformation energy to fracture and angular deformation comparable to a “green wood” type whereas the high-dosed ND animals were more comparable to a “dry wood” type. Green wood can absorb a high amount of energy and can sustain larger angular deformation before fracture. A dry wood type is stiffer but can absorb less energy than green wood, although it may need strength comparable to green wood to break. These observations on mechanical properties of bone are important for choosing the most appropriate dosage of a drug such as ND but also for evaluating the effect of other drugs on the bone quality.

A new and likely important finding of this study is the significant ND-induced increase of the IGF-I content in bone matrix, indicating its effect at the bone cellular level. Osteoblasts are known to produce IGF-I [40]. This locally active growth factor stimulates the proliferation and differentiation of bone cells. Moreover, it has been suggested that on the grounds of a positive correlation between bone growth and levels of IGF-I messenger-RNA in skeletal tissue, locally produced IGF-I plays a more important role in the regulation of skeletal growth than does circulating IGF-I [41]. As we find significantly increased IGF-I levels after ND treatment compared with intact and OVX controls, it seems likely that the mechanism of anabolic actions on bone of ND can be

mediated by IGF-I. The exact mechanism of these actions of ND is not yet known. In our opinion it is unlikely that the ND-induced increased IGF-I content in the bone matrix would be mainly the result of IGF-I produced in the liver or the result of the general stimulating effect of anabolic steroids on protein production as we found different effects on IGF-I concentrations in bone compared with serum after OVX and ND treatment, and we could not observe any correlation between serum and bone IGF-I. The ultimate argument for a local effect on bone IGF-I by ND would be the detection of increased IGF-I mRNA levels in bone cells after ND treatment. The observations by Fitzsimmons et al. [9] that ND *in vitro* increases bone cell proliferation, increases alkaline phosphatase activity, increases IGF-II receptor number, and increases the maximum mitogenic action of IGF-II are in favor of our hypothesis. A recent study [42] reports a reduced BMC and stimulated resorptive activity after 14 days continuous infusion of IGF-I in very old OVX rats. However, Spencer et al. [43] observed reduced osteoclastic activity after IGF-I infusion into the hind limbs of mature (150 g) rats. The contrasting effects of these studies may be a consequence of the multiple roles of IGF-I on endocrine, paracrine, and autocrine levels and/or the different circumstances of these studies (e.g., rat age, hormonal status, IGF-I dosing regimen) and illustrate the complexity of the working mechanism of IGF-I in bone *in vivo*.

The effects of ND treatment on bone mass and bone metabolism observed in this animal study are in line with the effects observed in humans [2–4], in beagle dogs [44], and in aged rats [45], but are not the same as the effects of estrogens. If ND would act similarly on bone as estrogen does, a complete reversal of the changes observed after OVX would be expected. This is not the case. First of all BMC and BMD are significantly increased in ND treatment groups compared with OVX controls whereas the intact group is not significantly different from the OVX group. This finding is similar to results we found earlier in the bones of growing children, [46] where we demonstrated comparable effects of androgens and estrogens on trabecular bone density; however, androgens had a more pronounced effect on both cortical and trabecular BMC and bone sizes. This can be explained by a more pronounced stimulation of periosteal bone growth by androgens than by estrogens. Furthermore, a dissociative effect on biochemical markers for bone formation is observed in the present study. ND does not decrease the high post-OVX serum IGF-I and alkaline phosphatase activity levels whereas serum and bone osteocalcin are significantly reduced under ND therapy. Urinary Ca is further reduced but phosphate excretion is increased, which indicates a more positive Ca balance. The observation that alkaline phosphatase activity is not depressed with ND confirms earlier studies in humans [2, 4] and in animals [44], and is likely the reflection of stimulated bone formation and not of liver activation because no negative effects of ND on liver function have been found [2, 47].

In conclusion, ND has the unique ability to decrease bone turnover—characterized by decreased serum and bone osteocalcin levels—and to stimulate osteoblast activity, expressed by high serum alkaline phosphatase levels and increased IGF-I concentrations in the bone matrix, resulting in an increased cortical bone mass and TBV compared with OVX controls. The ND therapy-associated increase in cortical bone mass and BMD is associated with improved mechanical properties, as expressed by bone stiffness and strength. Furthermore, ND therapy increased IGF-I content in the bone matrix. IGF-I is a growth factor now considered to play a role in the maintenance of normal skeletal balance

by paracrine and/or autocrine mechanisms. New insights into the mechanisms of how ND can influence the bone metabolism in osteoporotic conditions can probably be found in cellular responses on increased IGF-I production by osteoblasts.

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