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Tamoxifen Impairs Both Longitudinal and Cortical Bone Growth in Young Male Rats

Elham Karimian,[1] Andrei S Chagin,[1] Jennifer Gjerde,[2,3] Terhi Heino,[1] Ernst A Lien,[2,3] Claes Ohlsson,[4] and Lars Sävendahl[1]

ABSTRACT: Tamoxifen (Tam) has been used experimentally to treat boys with gynecomastia and girls with McCune-Albright syndrome. This drug was recently shown to inhibit the growth of cultured fetal rat metatarsal bones and thus might also affect bone growth in vivo. Four-week-old Sprague-Dawley rats were gavaged daily with vehicle alone (peanut oil), Tam (40 mg/kg/d; 1 or 4 wk), or estradiol (40 μg/kg/d; 4 wk). Five of the 10 rats in each group were killed after 4 wk and the other five after 14 wk of recovery. Bone growth was followed by repeat DXA scans, whereas other bone parameters and spine length were evaluated by pQCT and X-ray at the time of death. Four-week Tam treatment significantly decreased body weight, nose-anus distance, spinal and tibial bone lengths, trabecular BMD, cortical periosteal circumference, and bone strength and also reduced serum IGF-I levels (424 ± 54 versus 606 ± 53 ng/ml in control; $p < 0.05$). Analysis of the tibial growth plate of treated rats showed elevated chondrocyte proliferation (BrdU) and apoptosis (TUNEL), as well as decreases in the number of hypertrophic chondrocytes and in the size of terminal hypertrophic chondrocytes. Despite a complete catch-up of body weight after 14 wk of recovery, the tibia was still shorter ($p < 0.001$) and its cortical region was smaller. We conclude that, when administered at a clinically relevant dose, Tam causes persistent retardation of longitudinal and cortical radial bone growth in young male rats. Our findings suggest that this inhibition results from local effects on the growth plate cartilage and systemic suppression of IGF-I production. Based on these rat data, we believe that Tam, if given to growing individuals, might compromise cortical bone growth, bone strength, and adult height.
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Key words: selective estrogen receptor modulator, growth and development, chondrocyte, bone QCT, apoptosis

INTRODUCTION

Both of the estrogen receptors (ER)α and ERβ are expressed by bone[1–3] and growth plate cartilage of humans and other species.[4,5] Estrogens play an essential role in the regulation of longitudinal bone growth and maintenance of bone mineralization,[6] which is illustrated by osteoporosis, continuation of bone growth into adulthood, and lack of epiphyseal growth plate fusion observed in a man with a mutation in the *ERα* gene that renders this receptor unresponsive to estrogens.[7] A similar phenotype is found in men and women who carry a mutated *CYP19* gene associated with a deficiency in aromatase activity.[8]

Selective estrogen receptor modulators (SERMs) give rise to agonistic and antagonistic estrogenic effects that are tissue specific as a consequence of their differential binding affinities to ERα and/or ERβ and subsequent tissue-specific recruitment of co-activators or co-repressors.[9] Thus, SERMs may potentially influence both longitudinal bone growth and BMD. Indeed, we showed previously that raloxifene, a SERM that has been approved for clinical treatment and prevention of osteoporosis in postmenopausal women,[10] promotes growth plate fusion in the rabbit.[11]

Tamoxifen (Tam), another SERM, is used clinically for the treatment and prevention of ER-positive and -negative breast cancers.[12,13] Unfortunately, even after >35 yr of such use, the possible effects of Tam on bone physiology and growth in young individuals remain unclear. When administered for treatment of pubertal gynecomastia in boys, this drug has been reported both not to affect[14] and to decrease[15] longitudinal bone growth. Girls with McCune-Albright syndrome who receive Tam exhibit a decreased rate of growth.[16] Furthermore, treatment of gonadoectomized male and female rats with Tam causes a narrowing of the growth plate.[17,18]

Moreover, we recently reported that Tam induces permanent growth arrest of cultured fetal rat metatarsal bones, an effect associated with specific elimination of chondrocytes, primarily in the resting zone of the growth plate, through apoptosis.[19] These findings raise concerns about potential long-lasting negative side effects of Tam on bone physiology and linear growth. The aim of this study was to

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[1]Pediatric Endocrinology Unit, Department of Woman and Child Health, Karolinska Institutet, Stockholm, Sweden; [2]Hormone Laboratory, Haukeland University Hospital, Bergen, Norway; [3]Section for Endocrinology, Institute of Medicine, University of Bergen, Bergen, Norway; [4]Division of Endocrinology, Department of Internal Medicine, Sahlgrenska University Hospital, Gothenburg, Sweden.

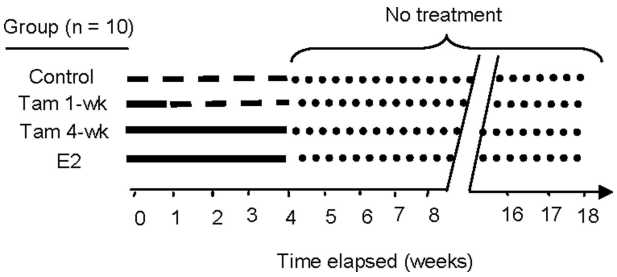

FIG. 1. Schematic illustration of the design of the study. The vehicle (i.e., peanut oil alone; control group), Tam (40 mg/kg/d) for 1 (Tam 1-wk) or 4 wk (Tam 4-wk), or 17β-estradiol (E2; 40 μg/kg/d for 4 wk) dissolved in peanut oil was administrated by gavage. Five animals from each group except in the case of the E2 group, where $n = 4$, were killed after 4 wk of treatment, and the remaining rats were killed after 14 additional wk without any treatment. In both cases, BrdU (50 mg/kg) was injected into each animal 2 and 16 h before death. Solid line, period of drug administration; dashed line, vehicle administration; dotted line, follow-up without any treatment.

evaluate the long-term effects of Tam on bone growth and mineralization, using young intact male rats as a model organism.

MATERIALS AND METHODS

Animals

Four-week-old male Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) were randomly divided into four different experimental groups, each containing 10 animals, which were treated as follows: group 1 (control) received peanut oil for a period of 4 wk (control), group 2 (Tam 1-wk) was treated with Tam (40 mg/kg/d) for 1 wk, group 3 (Tam 4-wk) received the same dose of the drug for 4 wk, and group 4 (E2) was administered 17 β-estradiol (40 μg/kg/d) for 4 wk. For this treatment, Tam and 17-β estradiol (both obtained from Sigma-Aldrich, Stockholm, Sweden) were dissolved in peanut oil with gentle warming and administered by gavage.

One of the rats in the E2 group died after 1 wk of treatment as a result of aspiration of peanut oil during gavage. After 4 wk of treatment, five animals in each group (except for the E2 group; see above) were killed, whereas the remaining five rats in each group were maintained with no treatment at all for an additional 14 wk before their death (Fig. 1). After death by inhalation of CO_2, blood and tissue samples were collected for subsequent analysis.

Throughout these studies, rats received a soy bean–free diet (R70; Lactamin AB, Stockholm, Sweden) and tapwater ad libitum and were subjected to a 12-h light/dark cycle. These experiments were preapproved by the local ethical committee at Karolinska Institutet, Stockholm, Sweden.

Collection of blood and tissue samples

Immediately after death, blood was collected directly from the heart of each animal, allowed to clot for 1 h at room temperature, and thereafter centrifuged to obtain the serum, which was stored at −80°C for later analysis. The femur and tibia bones were dissected out and fixed in 4% phosphate-buffered formalin for 24 h, followed by decalcification in 10% EDTA and storage in 70% ethanol until being embedded in paraffin. Five-micrometer-thick sections were prepared from these paraffin blocks. In addition, the cartilaginous regions of the sternum and the costochondral junction were also dissected out, snap-frozen in liquid nitrogen, and stored at −80°C for later processing. Finally, the testes and brain were removed and weighed.

Analysis of serum IGF-1 and liver function

The serum level of IGF-1 was measured by a double-antibody IGF-binding protein-blocked commercial RIA kit (Mediagnost, Tubingen, Germany). Routine biochemistry was applied to measure serum albumin (ALB) and alanine transaminase (ALT) using colorimetric and enzymatic methods, respectively (Beckman LX Instrument; Beckman Coulter, Fullerton, CA, USA).

Measurements of BMD, femur growth, and spine length

BMD and femur length were analyzed using a high-resolution DXA scanner (pDXA Sabre; Norland, Fort Atkinson, WI, USA). The animals were anesthetized with isoflurane (Forene; Abbott), and the right femur of each animal was scanned at the beginning of the experiment and 2, 4, 6, 8, and 18 wk later. After being killed, a digital spinal X-ray image was captured, and the distance from the second cervical vertebrae (axis) to the last lumbar vertebrae was measured using the software of the picture archiving communication system (PACS).

Measurements of Tam and its metabolites in serum and cartilage

The concentrations of Tam and its metabolites in serum and cartilage were measured using high-performance liquid chromatography–tandem mass spectrometry as described previously,[20,21] with the modifications that the cartilage samples were first cut into small pieces and disrupted in ice-cold, 50 mM Tris-Cl, pH 7.4, with an ultrasonic homogenizer before assay.

Quantitative histology of the growth plate

For this purposes, sections prepared from the tibial growth plate were stained with Alcian blue/Van Gieson, and histological parameters were quantified as described previously.[22] In brief, the height of the growth plate, numbers of proliferative and hypertrophic chondrocytes per column, and size of terminal hypertrophic chondrocytes were determined by analysis of digital images from the center two thirds of the growth plate with Microimage software (Olympus Optical, Hamburg, Germany). In this connection, chondrocytes >7 μm were considered to be hypertrophic. All values presented are means of 24 such measurements on each individual growth plate.

Evaluation of cell proliferation

To this end, each rat received an intraperitoneal injection of BrdU (50 mg/kg) 2 and 16 h before death, and subse-

quent detection of $BrdU^+$ cells in the growth plate was achieved using a cell proliferation kit (Amersham Biosciences, Buckinghamshire, UK), as described previously.[23] The extent of chondrocyte proliferation in the growth plate was expressed as the number of $BrdU^+$ cells per unit area.

Detection of apoptosis

Apoptotic cells in sections prepared from the growth plate were identified using the TUNEL technique (TdT-FragEL DNA fragmentation kit; Calbiochem, Damstadt, Germany), as described previously.[23] The number of apoptotic cells (DAB^+ stained cells) was counted per section and normalized per growth plate area. The data were confirmed by streptavidin conjugated to Alexa Fluor 546 (Invitrogen). Slides were embedded in DAPI-containing mounting medium (Vector Laboratories). Alexa-546–positive cells (red florescence) detected apoptotic chondrocytes.[19]

Immunohistochemical detection of type X collagen and PTH-related peptide

Immuno-detection of type X collagen and PTH-related peptide (PTHrP) were performed in paraffin-embedded sections of the growth plate as previously described.[19,24] The primary anti-type X collagen antibody was purchased from Quartett (Berlin, Germany) and the rabbit polyclonal anti-PTHrP from Oncogene Science (Cambridge, MA, USA). The secondary anti-mouse and -rabbit biotinylated antibodies were from DakoCytomation (Glostrup, Denmark).

pQCT

Tomographic measurements were performed using the Stratec XCT Research M (software version 5.4B; Norland Medical Systems) adapted especially for examination of small bone specimens.[25] Parameters associated with the trabecular and cortical bones were evaluated as described previously.[22]

Mechanical testing of bone strength

The mechanical properties of the femur shafts were tested with a previously described three-point bending device with a span length of 13 mm.[26] Each bone was compressed at a constant rate of 0.155 mm/s until breakdown. Mechanical parameters, including ultimate strength (maximal load in N) and energy absorbed by the bone tissue representing structural toughness (area under the load deformation curve, $Nm \times 10^{-3}$) were calculated.

Statistical analysis

All data are expressed as means ± SE. Differences between control and treated groups were evaluated with the one-way ANOVA followed by the Holm-Sidak post test, and $p < 0.05$ was considered statistically significant.

RESULTS

Levels of tamoxifen and its metabolites in serum and cartilage

When assessed 24 h after the last daily administration of Tam, the concentration of Tam was 25 ± 2 ng/ml in serum and 64 ± 11 ng/mg in rib cartilage (Tam 4-wk group, $n = 5$). The corresponding concentrations of the metabolites 4-OH-tamoxifen, 4-OH-*N*-demethyltamoxifen, *N*-demethyltamoxifen, and *N*-dedimethyltamoxifen were 27 ± 3, 148 ± 24, 79 ± 12, and 3.4 ± 0.4 ng/ml in serum and 23 ± 5, 143 ± 27, 91 ± 16, and 3.0 ± 0.8 ng/mg in rib cartilage, respectively. Fourteen weeks after the final administration of Tam, no trace of this drug or its metabolites was detected in either serum or cartilage.

Serum levels of IGF-I and liver function tests

After 4 wk of treatment, serum levels of IGF-I were clearly decreased (–30%, $p < 0.05$) in the Tam 4-wk group and elevated (+35%, $p < 0.01$) in the E2 group (Table 1). In both cases, these values had returned to control levels by the end of the 14-wk recovery period. Four-week treatment with Tam did not change the serum albumin and ALT levels compared with controls (Table 1). After 18 wk, no significant difference was observed between the experimental groups, confirming that Tam at this dose did not impair liver function in our rats (Table 1).

Body weight and food intake

Subsequent to either continuous 4-wk treatment with Tam or 1 wk of such administration followed by 3 wk of treatment with the vehicle (peanut oil) alone, both the body weight and relative body mass index (BMI; weight/body length^2) of treated rats were significantly lower than for untreated animals ($p < 0.05$ and $p < 0.001$, respectively; Figs. 2A and 2B). However, when treated animals were allowed to recover for 14 wk, an obvious catch-up with respect to both of these parameters occurred (Figs. 2A and 2B). During the initial 4-wk period, total food intake was lower in the Tam 4-wk group than for untreated animals (17 ± 0.3 versus 26 ± 0.2 g/rat/d, respectively; $p < 0.001$), but there was no significant difference in food intake relative to body weight (101 ± 8 versus 88 ± 3 mg/g body weight/d, respectively). Seventeen weeks after initiation of the experiment (i.e., 13 wk after the final administration of Tam), the rats in the Tam 4-wk group consumed the same amount of food as the control animals (34.8 ± 0.3 versus 35.4 ± 1.5 g/rat/d, respectively). Treatment with E2 did not affect body weight, the relative BMI, or food intake (Figs. 2A and 2B and data not shown).

Effects of Tam on axial skeletal growth

To detect any influence of Tam on axial skeletal growth, the distance between the nose and anus of each animal was measured every other week. Growth restriction was already apparent after 2 wk in the case of both the Tam 1-wk ($p < 0.001$ versus control) and Tam 4-wk ($p < 0.001$ versus control) groups (Fig. 2C). After 14 wk of recovery, the nose-anus distance for the Tam 1-wk animals was the same as for

TABLE 1. TIBIAL GROWTH PLATE MORPHOMETRY AND SERUM LEVELS OF IGF-I, ALBUMIN, AND ALANINE TRANSAMINASE IN RATS TREATED WITH TAM OR E2

Parameter	*Time elapsed (wks)*	*Experimental group of rats*			
		Control	*E2*	*Tam 1-wk*	*Tam 4-wk*
Proliferative chondrocytes (cells/column)	4	8.1 ± 0.6	8.7 ± 0.9	7.4 ± 0.6	7.3 ± 0.5
	18	5.3 ± 0.2	4.7 ± 0.5	4.8 ± 0.7	4.2 ± 0.3
Hypertrophic chondrocytes (cells/column)	4	5.8 ± 0.6	6.6 ± 0.2	8.4 ± 1.2*	12.0 ± 0.8†
	18	7.3 ± 0.3	8.6 ± 0.2*	9.4 ± 0.6‡	8.6 ± 0.3*
The ratio of proliferative/hypertrophic cells	4	1.45 ± 0.19	1.29 ± 0.10	0.77 ± 0.10‡	0.60 ± 0.06†
	18	0.73 ± 0.03	0.55 ± 0.05*	0.53 ± 0.10	0.49 ± 0.05‡
Average size of terminal hypertrophic chondrocytes (μm)	4	26.3 ± 0.5	26.5 ± 0.4	27.6 ± 0.8	22.5 ± 0.5†
	18	14.8 ± 0.6	14.2 ± 0.4	14.9 ± 0.6	15.5 ± 0.2
Serum levels of IGF-I (ng/ml)	4	606 ± 53	821 ± 35‡	614 ± 62	424 ± 54*
	18	691 ± 12	734 ± 77	664 ± 36	672 ± 8
Albumin (g/liter)	4	18 ± 1.1	15.8 ± 0.8	15.6 ± 1.4	19.8 ± 2.0
	18	17.4 ± 0.5	18.4 ± 0.9	19.6 ± 1.5	20.8 ± 1.5
Alanine transaminase (ALT) (IU/liter)	4	1.08 ± 0.02	0.67 ± 0.19	0.9 ± 0.09	1.04 ± 0.12
	18	1.2 ± 0.07	1.07 ± 0.09	1.27 ± 0.11	1.17 ± 0.08

Quantitative tibial growth plates histology and serum IGF-I, albumin, and alanine transaminase (ALT) measurements were performed immediately after the 4-wk treatment period (week 4) and after 14 wk of recovery (week 18). The treatment regimen is detailed in the legend to Fig. 1. The values are means ± SE ($n = 5$ except for the E2 group at week 4 where $n = 4$).

* $p < 0.05$, † $p < 0.001$, and ‡ $p < 0.01$ compared with the corresponding control value.

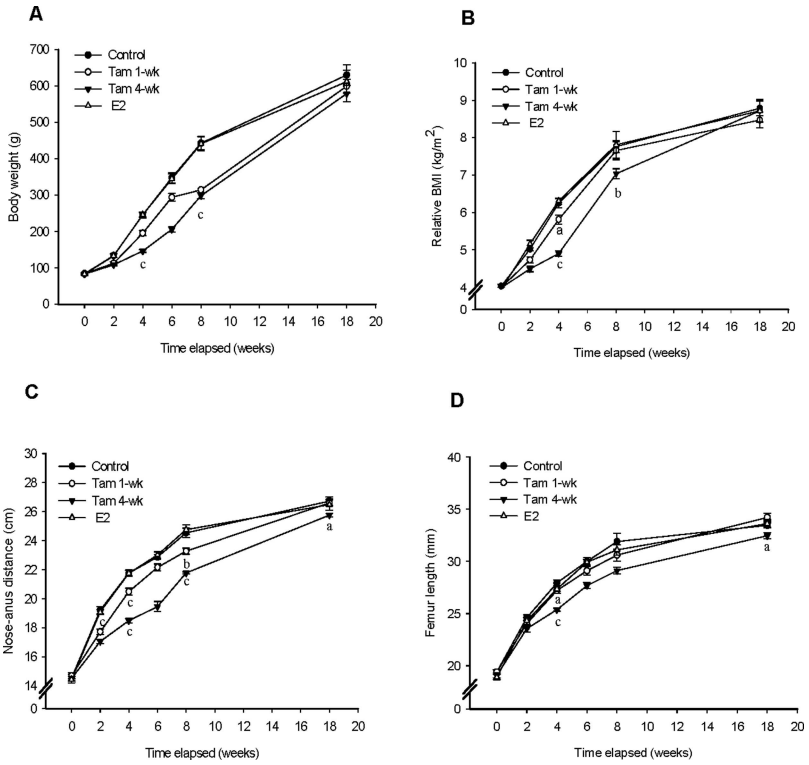

FIG. 2. Body weight (A), relative BMI (B), nose-anus distance (C), and femur length (D) for rats treated with the vehicle peanut oil alone (Control), tamoxifen (40 mg/kg/d) for 1 wk followed by vehicle for 3 wk (Tam 1-wk), the same dose of tamoxifen for 4 wk (Tam 4-wk), or 17β-estradiol (E2; 40 μg/kg/d) for 4 wk and allowed to recover for 14 wk. The values shown are means ± SE ($n = 10$ for the first 4 wk and 5 thereafter, with the exception of the E2 group, for which $n = 9$ between week 0 and 4). [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ compared with the values for untreated rats.

the controls (Fig. 2C), whereas the rats in the Tam 4-wk group were still significantly shorter (25.7 ± 0.1 versus 26.7 ± 0.2 cm, respectively; $p < 0.05$; Fig. 2C). Again, E2 exerted no significant influence on this parameter (Fig. 2C). These findings were confirmed when spinal lengths were measured radiologically after death. After 4 wk, spinal lengths were significantly reduced in the Tam 1-wk and Tam 4-wk groups compared with controls (Fig. 3A). After 14 wk of recovery, Tam 4-wk animals still had shorter spinal lengths than controls (Fig. 3A).

Effects of Tam on appendicular bone growth

To evaluate any possible effect that Tam might have on appendicular bone growth, the right femur of each animal

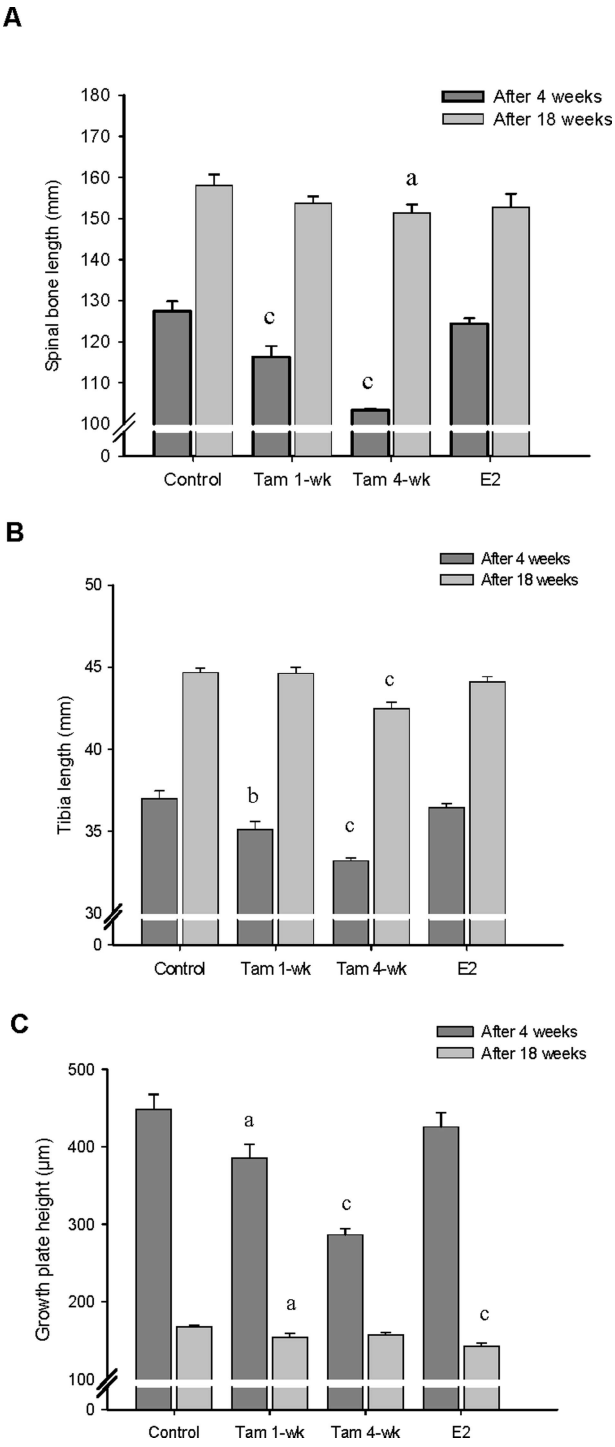

FIG. 3. Spinal length (A), proximal tibial length (B), and growth plate height (C) in control, Tam 1-wk, Tam 4-wk, and E2 animals as measured by digital X-ray, a digital caliper, and histomorphometry, respectively. The values shown are means ± SE ($n = 5$, except for the E2 group at week 4 where $n = 4$). $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ compared with the values for untreated rats.

was subjected to a DXA scan every other week. Treatment with Tam for either 1 or 4 wk significantly reduced femur length in comparison with the control value after 4 wk ($p < 0.05$ and $p < 0.001$, respectively; Fig. 2D). Even after 14 wk of recovery, the femurs of rats in the Tam 4-wk group were still significantly shorter than in controls (32.4 ± 0.2 versus 33.6 ± 0.3 mm, respectively; $p < 0.05$; Fig. 2D). Femur length was unaffected by E2 treatment (Fig. 2D). To confirm the results of the DXA measurements, tibia lengths were also measured with a digital caliper. After 4 wk, animals in both the Tam 1-wk and Tam 4-wk groups had shorter tibias than control rats ($p < 0.01$ and $p < 0.001$, respectively; Fig. 3B), and this was still the case for the Tam 4-wk group after 14 wk of recovery ($p < 0.001$; Fig. 3B). Tibia length was unaffected by E2 treatment (Fig. 3B). Thus, there was excellent agreement between the measurements made using the DXA technique and purely mechanical measurements with a digital caliper.

Effects of Tam on growth plate morphology

In an attempt to understand the mechanism underlying Tam-induced retardation of growth, histomorphometrical analysis of the tibial growth plate was performed at two different time points. After 4 wk, the height of the growth plate in the proximal tibia of Tam 1-wk and Tam 4-wk rats was less than that in control animals ($p < 0.05$ and 0.001, respectively; Fig. 3C and Figs. 5A and 5B), and in addition, the ratio between the numbers of proliferative and hypertrophic chondrocytes was significantly reduced ($p < 0.01$ and $p < 0.001$ versus control, respectively; Table 1). Moreover, at this time point, the terminal hypertrophic chondrocytes were smaller in Tam 4-wk animals ($p < 0.001$ versus control; Table 1). After 14 wk of recovery, the height of the growth plate was still decreased in the Tam 1-wk group ($p < 0.05$ versus control; Fig. 3C). Furthermore, at this same time point, the height was significantly lower in the E2 group ($p < 0.001$ versus control; Fig. 3C), and the ratios between the numbers of proliferative and hypertrophic chondrocytes were reduced in both the E2 ($p < 0.05$) and Tam 4-wk ($p < 0.01$) groups (Table 1).

Chondrocyte proliferation, apoptosis, and expression of type X collagen

In comparison with control tissue, the rate of chondrocyte proliferation was increased in the Tam 1-wk ($p < 0.05$) and Tam 4-wk ($p < 0.01$) groups (Figs. 4A, 5I, and 5J). In addition, 4 wk of treatment with Tam significantly enhanced the frequency of apoptosis among the same cells ($p < 0.01$ versus control; Figs. 4B, 5K, and 5L). To determine whether the differentiation of chondrocytes was also affected, the expression of type X collagen was examined and found to be lowered in the Tam 4-wk group ($p < 0.001$; Figs. 4C, 5E, and 5F). However, when the height of the zone positive for type X collagen staining was expressed as a percentage of the height of the entire growth plate, no significant difference was detected (43 ± 1% versus 45 ± 1% for Tam-4wk and control rats, respectively). To further study the mechanism of Tam-induced growth retardation, the expression patterns of an important paracrine factors and key regulator in the growth plate, PTHrP, were analyzed by immunohistochemistry. In control animals, PTHrP positive cells were only detected in the resting zone (Fig.

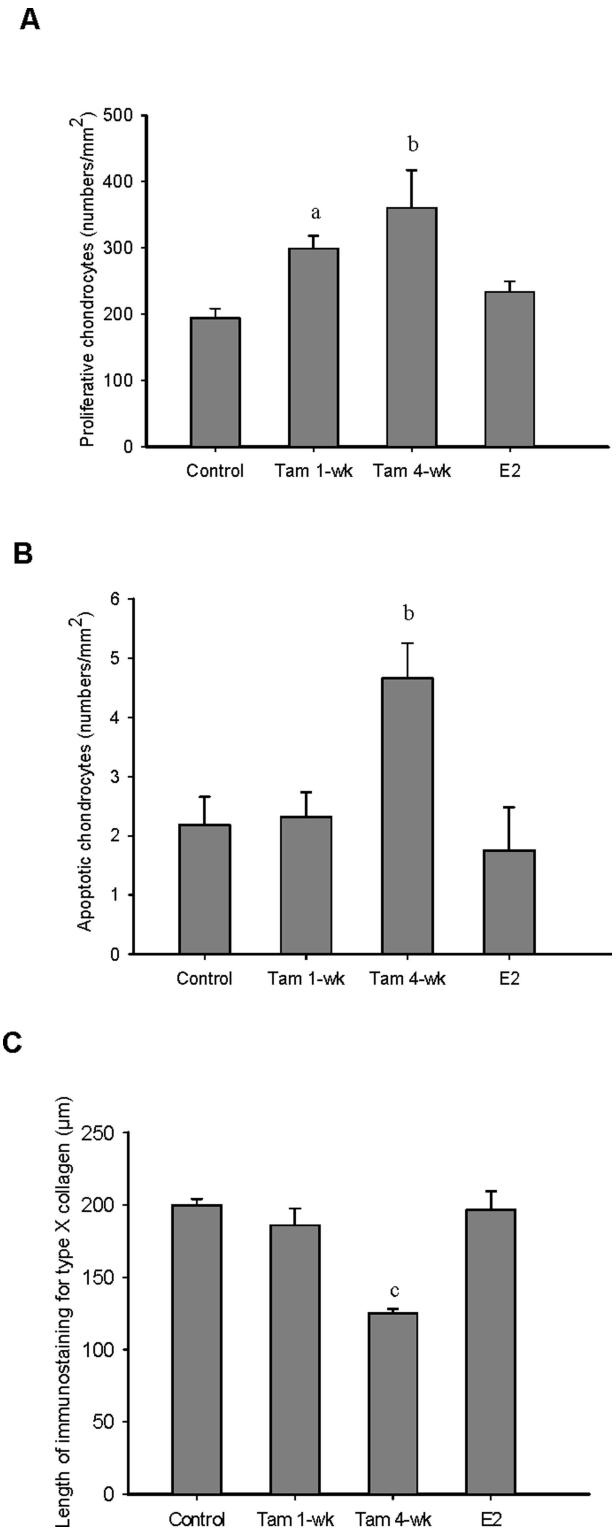

FIG. 4. The frequency of apoptosis (A) and degree of proliferation (B) among chondrocytes, as well as expression of type X collagen (C) in the tibial growth plates of control, Tam 1-wk, Tam 4-wk, and E2 animals killed 4 wk after initiation of the experiment. The values presented are means ± SE. [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ in comparison with the corresponding control values.

5G). Four weeks of Tam treatment considerably decreased the number of PTHrP-positive resting chondrocytes (2.1 ± 0.2 versus 25.1 ± 0.02 positive cells/mm^2 in control, $p < 0.001$; Fig. 5H). Control experiments showed no staining when the primary antibody for PTHrP was replaced by nonspecific rabbit IgG.

Measurement of bone parameters by pQCT

Four weeks after initial administration of the drug, the cortical BMC was reduced in both the Tam 1-wk and Tam 4-wk animals ($p < 0.05$ and $p < 0.001$, respectively; Fig. 6A). These reductions reflected decreases in the cortical bone area ($p < 0.05$ and $p < 0.001$, respectively), whereas cortical volumetric BMD was unaffected (Table 2). The decreases in cortical bone area in both the Tam 1-wk and Tam 4-wk groups were associated with attenuations in cortical thickness ($p < 0.05$ and $p < 0.001$, respectively; Table 2), as well as in the periosteal ($p < 0.001$ and $p < 0.01$, respectively; Fig. 6B) and endosteal circumferences at the tibia diaphysis ($p < 0.01$ and $p < 0.01$, respectively; Fig. 6C). Both 1- and 4-wk treatment with Tam reduced the area and thickness of cortical bones, as well as the polar moment of inertia (mm^4) and the moment of resistance (mm^3) ($p < 0.05$ and $p < 0.001$, respectively; Table 2). In addition, the trabecular BMD (measured in the proximal metaphyseal area of the tibia) was markedly diminished after 4 wk of Tam treatment (Fig. 6D).

After the 14-wk recovery period, no significant differences in either trabecular BMD or cortical BMC remained (Figs. 6A and 6D). In contrast, both the cortical periosteal and endosteal circumferences were still significantly reduced in all rats treated with Tam (Figs. 6B and 6C). Moreover, the polar moment of inertia was still significantly lower compared with control in both Tam 1-wk and Tam 4-wk animals (Table 2). Furthermore, the cortical volumetric BMD, which was normal immediately after 4 wk of treatment, was significantly elevated in both groups that received Tam after the 14-wk recovery period (Table 2).

With the exception of an increase in cortical volumetric BMD after 18 wk, treatment with estradiol had no influence on any of these bone parameters at either time point (Fig. 6; Table 2).

Effects of Tam on the mechanical properties of the femur

After 4 wk, the structural toughness decreased in both the Tam 1-wk ($p < 0.05$) and Tam 4-wk ($p < 0.01$) groups compared with controls (30.6 ± 5.4 and 22.5 ± 2.05 versus 53.3 ± 11.1 Nm × 10E[minus]3, respectively). The ultimate strength of the femur was significantly reduced in Tam 4-wk animals ($p < 0.01$) compared with controls (59.8 ± 3.05 versus 88.7 ± 7.9 N).

Testis and brain weights

In Tam 4-wk animals, the testis weight was reduced in animals killed immediately after the treatment period (0.6 ± 0.15 versus 1.4 ± 0.1 g/per testis for control rats; $p < 0.001$). However, when the testis weight was expressed as a percentage of total body weight, it was not significantly

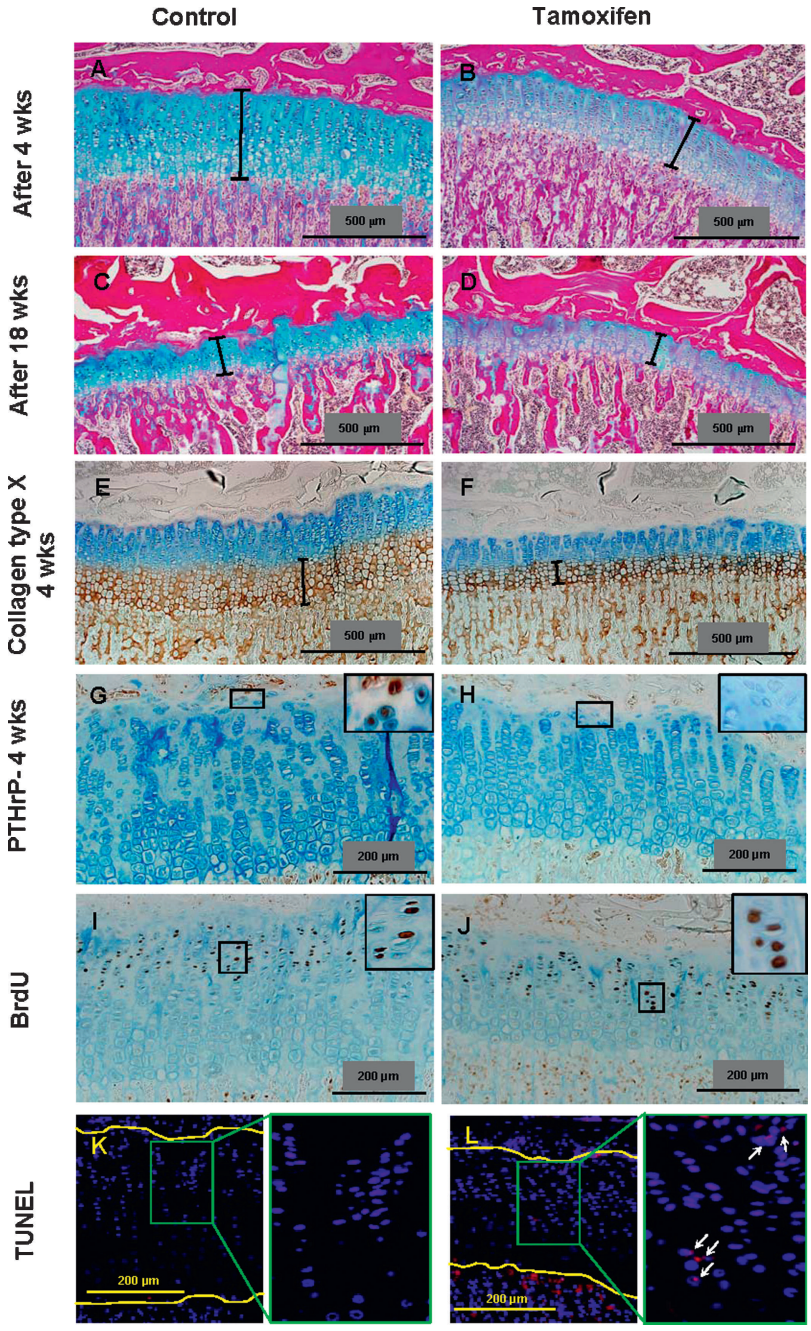

FIG. 5. Representative stained sections of the tibial growth plate from rats receiving peanut oil (A, C, E, G, I, and K) or Tam dissolved in peanut oil (B, D, F, H, J, and L) for 4 wk and killed (A, B, and E–L) or allowed to recover for 14 wk (C and D). These sections were stained with either Alcian blue/Van Gieson (A–D) or antibodies directed toward type X collagen (E and F) or PTHrP (G and H). The vertical lines indicate the height of the growth plate (A–D) or width of the band staining positively (brown color) for type X collagen (E and F). The horizontal bars depict a distance of 200 (G–L) or 500 μm (A–F). Proliferative BrdU positive chondrocytes in control (I) and Tam 4-wk group (J). TUNEL$^+$ chondrocytes were detected by Alexa-546 (red color), whereas the total number of chondrocytes was detected by DAPI counterstaining (blue colors) in control (K) and Tam 4-wk animals (L). The area between the yellow lines is the growth plate (K and L). High-power magnifications of indicated areas are also included (G–L). Arrows indicate apoptotic cells (L).

affected by Tam treatment (0.39 ± 0.1% in Tam 4-wk versus 0.48 ± 0.01% in control; $p = 0.44$). After 14 wk of recovery, the mean absolute testis weight was 1.88 ± 0.06 g in Tam 4-wk animals, which was not significantly different from controls (1.87 ± 0.03 g/testis). Brain weight was not influenced by treatment with either Tam or E2 (data not shown).

DISCUSSION

We report here the novel finding that Tam causes permanent retardation of longitudinal and cortical growth of the tibia in young male rats. These effects are associated with a transient reduction in the serum levels of IGF-I, suggesting that impairment of bone growth by Tam may occur systemically through the growth hormone (GH)/IGF-I axis. In addition, Tam enhances the frequency of both proliferation and apoptosis and suppresses the differentiation of growth plate chondrocytes, indicating a possible local inhibition of longitudinal bone growth as well. The existence of such a local effect would be consistent with the pronounced accumulation of Tam observed in the growth plate cartilage of treated animals.

Our goal was to use an oral dosage of Tam that resulted

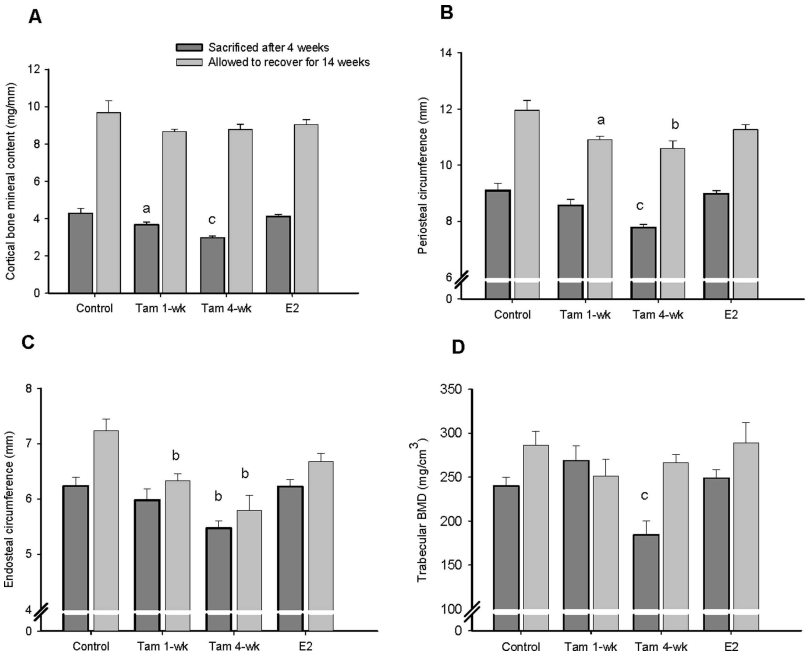

FIG. 6. Effects of tamoxifen and estrogen treatment on bone parameters in the tibia as determined by pQCT. Cortical BMC (A), periosteal (B) and endosteal circumferences (C), and trabecular BMD (D) were analyzed 4 (dark gray bars) and 18 (light gray bars) wk after initiation of the experiment. $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ in comparison with the corresponding control values.

TABLE 2. BONE PARAMETERS IN RATS TREATED WITH TAM OR E2

		Experimental group of rats			
Parameter	*Time elapsed (wk)*	*Control*	*E2*	*Tam 1-wk*	*Tam 4-wk*
Cortical area (mm^2)	4	3.5 ± 0.2	3.3 ± 0.08	3.0 ± 0.1*	2.4 ± 0.1†
	18	7.2 ± 0.4	6.6 ± 0.2	6.3 ± 0.1	6.3 ± 0.2
Cortical thickness (mm)	4	0.46 ± 0.02	0.44 ± 0.01	0.41 ± 0.005*	0.37 ± 0.008†
	18	0.75 ± 0.02	0.73 ± 0.01	0.73 ± 0.001	0.75 ± 0.001
Cortical volumetric BMD (mg/cm^3)	4	1225 ± 9	1236 ± 7	1234 ± 4	1229 ± 8
	18	1344 ± 8	1382 ± 4†	1379 ± 6‡	1401 ± 4†
IP_CM_W (mm^4)	4	5.4 ± 0.7	5.1 ± 0.2	4.0 ± 0.4*	2.7 ± 0.1†
	18	21.3 ± 2.5	18.1 ± 1.1	15.6 ± 0.7‡	14.1 ± 1.2‡
RP_CM_W (mm^3)	4	2.9 ± 0.3	2.9 ± 0.1	2.4 ± 0.1*	1.8 ± 0.1†
	18	7.5 ± 0.8	6.8 ± 0.3	6.6 ± 0.3	6.56 ± 0.3

pQCT was used to determine cortical area, thickness, and volumetric BMD in the diaphyseal region of the left tibia immediately after the treatment period (week 4) and after 14 wk of recovery (week 18). In addition, the calculated polar moment of inertia (IP_CM_W) and moment of resistance (RP_CM_W) are detailed. The treatment regimen is detailed in the legend to Fig. 1. The values are means ± SE ($n = 5$ except for the E2 group at week 4 where $n = 4$).

* $p < 0.05$, † $p < 0.001$, and ‡ $p < 0.01$ compared with the corresponding control value.

in serum levels of Tam and its metabolites in our experimental animals that were close to those found in patients receiving this drug. In studies where Tam has been used to treat pubertal gynecomastia in boys or to limit estrogenic action in patients with McCune-Albright syndrome, the doses administered range between 20 and 40 mg/d,[12,16] which are doses that have been reported to give serum concentrations between 10 and 150 ng/ml.[27] Thus, the serum concentrations of Tam achieved in our rats were comparable to the lowest concentrations observed in patients receiving this drug. Also serum concentrations of Tam metabolites were similar to the lowest levels reported in patients treated with Tam.[28]

It is important to emphasize that rats metabolize Tam in a manner similar to humans[20] but only after oral administration,[20] whereas the metabolism of this drug by mice is quite different. This may explain the striking difference between the responses of our rats to Tam documented here and the stimulatory effect of this drug on bone growth observed in mice.[29] Despite this resemblance in Tam metabolism in rats and humans,[20,28] we chose not to use a higher dose, because this might induce loss of body weight, malaise, and even death in treated rats.[28]

Even at the dose used in this study, Tam caused a slower increment in body weight and a reduction in the relative BMI of our rats. The possible influence of Tam on human body weight remains controversial, although it was recently shown that Tam reduces the BMI of healthy subjects.[30] Furthermore, it has been reported that Tam induces anorexia and weight loss in rats.[31,32] Therefore, it is possible that the retardation of growth observed in this study was secondary to weight loss induced by Tam. However, the

fact that the lengths of the femur, tibia, and total body remained shorter while body weight was completely normalized after 14 wk of recovery indicated the existence of some other underlying mechanism(s).

In an attempt to elucidate the mechanisms involved in Tam-induced growth retardation, we performed histomorphometrical analyses and examined cell proliferation, differentiation, and apoptosis in sections prepared from the tibial growth plate. The findings clearly showed that the growth retardation caused by Tam is associated with both elimination of chondrocytes through apoptosis and inhibition of chondrocyte differentiation. These results are consistent with our previous observation that Tam induces apoptosis of stem-like chondrocytes in cultured fetal rat metatarsal bones, an effect mediated through activation of both caspase-8 and -9 through the FasL/Fas pathway.[19] Moreover, our present in vivo demonstration that Tam treatment downregulates PTHrP expression in resting zone chondrocytes further supports our previous finding that Tam primarily targets stem-like chondrocytes in cultured rat metatarsal bones.[19] It is worth to emphasize that we could not detect any PTHrP staining in prehypertrophic chondrocytes as previously reported,[33] which most likely is because of methodological differences. PTHrP is well known to delay chondrocyte differentiation and ablation of the PTHrP gene in mice results in expansion of the hypertrophic layer.[34] In accordance, we observed an increased number of hypertrophic chondrocytes in Tam-treated rats. The fact that the size of hypertrophic chondrocytes was decreased in Tam-treated rats could be linked to lower circulating levels of IGF-I, a well-known stimulator of chondrocyte hypertrophy.[35]

In contrast to our previous findings in cultured metatarsal bones,[19] we observed here a partial catch-up of longitudinal bone growth after the 14-wk recovery of animals that were previously treated with Tam, a phenomenon associated with enhanced chondrocyte proliferation. The occurrence of catch-up growth after exposure to Tam in vivo, but not in vitro, suggests that there exists compensatory upregulation of systemic factors that promote longitudinal bone growth in the intact animal. The normalization of serum levels of IGF-I after withdrawal of Tam indicates that the IGF-I axis may play an important role in connection with this compensatory growth. In contrast, catch-up growth of cortical bone does not occur, not even after short-term (1 wk) treatment with Tam, suggesting that bone is more susceptible than growth plate cartilage to the deleterious effects of this drug. An important observation in this context is that our Tam-treated rats showed a bone phenotype very similar to that described in male knockout mice lacking the estrogen receptor (ER)α,[25] which strongly indicates that Tam inhibits the action of ERα on bone. Furthermore, the presence of decreased serum levels of IGF-I in these knockout mice,[25] as in our rats exposed to Tam, suggests that this drug also acts as an antagonist of the effects of ERα in the liver or on the GH/IGF-I axis. However, we have recently shown that Tam induces apoptosis in a human chondrocytic cell line through nongenomic mechanisms, and the nonselective ER antagonist ICI 182,780 was not able to prevent Tam-induced apoptosis.[19] Thereby, we believe that the skeletal effects observed after Tam treatment most likely is caused by both genomic and nongenomic mechanisms.

Although not definitive, our findings do suggest that changes in the GH/IGF-I axis, at least in part, explain the skeletal alteration observed in young male rats exposed to Tam. Such a proposal is consistent with the observations that Tam decreases serum levels of GH in adolescent boys,[36] as well as in male and female rats.[37] However, it has also been reported that Tam treatment decreases serum IGF-I levels in humans independent of GH secretory status.[38] Such an effect could be secondary to decreased liver production of IGF-I secondary to a hepototoxic effect caused by Tam. Indeed, liver toxic effects have been reported in Tam-treated breast cancer patients.[39,40] However, in this study performed in male rats, we found serum albumin and ALT levels to be unaffected, excluding any significant Tam-induced hepatotoxicity. Our findings are consistent with a very recent paper showing that female rats are more sensitive to Tam-induced toxic and metabolic changes than males.[41] Overall, these observations support the suitability of our male rat model for studies of Tam effects on bone growth.

Our finding that both the cortical area and cortical thickness of the tibia is significantly decreased after 4 wk of Tam treatment is well reflected by the results of the three-point bending experiments showing impaired mechanical properties of the femur shaft. Furthermore, trabecular BMD decreased during Tam treatment but almost completely recovered 14 wk later, which probably reflects attenuation in ER stimulation during the treatment period, followed by normalization of ER activation during recovery. In contrast, and as was also the case for the length of the long bones, the reduction in cortical bone size caused by treatment of Tam during the period of sexual maturation was not reversed after 14 wk of recovery. Because cortical bone size and moment of inertia are important indicators of bone strength, it is possible that administration of Tam to young individuals might increase the risk for fractures.

Finally, our finding that administration of E2 to rats results in significant elevation of their serum levels of IGF-I is consistent with numerous clinical observations of a positive correlation between serum levels of estrogen and IGF-I.[42–44] After 14-wk recovery from E2 treatment, a significant reduction in the height of the growth plate was detected in our animals, an observation that is consistent with the senescence hypothesis.[45] However, bone length was not affected in these animals, which is in contrast to a previous report of reduced bone growth in mature intact female rats treated with E2.[46] The discrepancy could be explained by the fact that our rats were younger, of the opposite sex, and given E2 by a different route of administration.

We conclude that Tam acutely inhibits longitudinal bone growth in young male rats, an effect associated with an enhanced frequency of chondrocyte apoptosis, narrowing of the tibial growth plate, and a reduction of serum IGF-I levels caused by this drug. Our observations indicate that, if administered to individuals who are still growing, Tam might potentially cause premature fusion of the growth

plate and thereby compromise final height, an effect that is usually undesirable. Furthermore, we report here that, at least in rats, exposure to Tam results in persistent reduction of cortical bone size as a result of the attenuated periosteal and endosteal growth of the cortical bone. Taken together, these findings suggest that administration of Tam to individuals who are still growing might decrease both their final height and cortical bone size.

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Address reprint requests to:
Elham Karimian, MD
Pediatric Endocrinology Unit Q2:08
Karolinska University Hospital
171 76 Stockholm, Sweden
E-mail: Elham.Karimian@ki.se

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