

Raloxifen Prevents Bone Loss in Castrated Male Mice

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Summary

Raloxifen is a selective estrogen receptor modulator which prevents bone loss in ovariectomized female mice in a fashion similar to estrogens. Since testosterone-deficient male mice also lose bone mass, we were interested in testing the effects of raloxifen on bones in intact and castrated male mice. Bone density was significantly reduced in castrated animals (1.36 ± 0.04 g/ml) as compared to intact animals (1.42 ± 0.03 g/ml) ($p < 0.01$). When castrated mice with extraordinarily low concentrations of testosterone and with reduced weight of seminal vesicles were treated with raloxifen, the changes in bone density and bone minerals resulting from castration (1.36 ± 0.04 g/ml) were entirely prevented (1.40 ± 0.01 g/ml). Cortical bone was lost in orchidectomized mice, and this decrease in cortical thickness of the femur was prevented by raloxifen administration. Raloxifen in a dose used in humans for treatment of osteoporosis decreased the weight of seminal vesicles, an organ which is highly sensitive to the androgenic effect, decreased the concentration of testosterone (12.5 ± 2.8 $\mu\text{mol/l}$) ($p < 0.01$) but not to the same level as in the case of castrated animals (0.6 ± 0.3 $\mu\text{mol/l}$), and did not have any effect on bone density or mineral content in intact mice. The results of the present study may thus be interpreted as supporting the hypothesis that raloxifen is an effective agent against the deleterious effects of castration-induced osteopenia in male mice and also support the hypothesis that estrogens may have physiological skeletal effects in male mice.

Key words

Orchidectomy • Bone density • Raloxifene • Male mice

Introduction

It is well established that androgens exert marked effects on bone homeostasis. Orchidectomy in mature male mice results in a rapid development of osteopenia in the femur. The surgically castrated animals are widely used as a model of bone loss due to androgen deficiency (Wink and Fetts 1980). Recent findings of the role of estrogen receptors in the bone as demonstrated in experimental and clinical observations in estrogen receptor deficiency favor the hypothesis that androgens may influence the bone tissue predominantly after

aromatization to estrogens (Vanderschuren *et al.* 1997). Several lines of evidence implicate estrogen deficiency as a cause of bone loss in elderly men. Raloxifen is a selective estrogen receptor modulator from the benzothiophene class that binds to the estrogen receptor with a high affinity. Raloxifen, a selective estrogen receptor modulator (SERM), has estrogen-agonist effects on bone but is not feminizing (Doran *et al.* 2001). Since the effect of testosterone on bone in men may partly be mediated by estradiol the skeletal actions of raloxifen in men are of interest (Amin 2003).

As far as we are aware, there is no previous

report on long-term effects of raloxifen on adult male mice. In order to test the hypothesis that raloxifen may protect against bone loss in castrated male mice, we have used the orchidectomized mouse model.

Materials and Methods

Adult male mice of strain H (Velaz Prague) weighing approximately 30 g were used for the experiments. They were fed a standard laboratory diet containing 23 % protein, 1.2 % calcium and 0.6 % phosphorus with water *ad libitum*, and were kept in an indirectly illuminated room with controlled temperature (24 ± 2 °C). The animals were divided into four groups of 8 animals each: 1) intact controls, 2) intact animals fed raloxifene for 3 months, 3) animals 3 months after castration, and 4) castrated mice fed raloxifene for 3 months. Raloxifene (Elli Lilly Evista) was mixed to the diet and consumed in a dose 5.0 mg/kg/day for 3 months. We used the dose, which is considered to be comparable to a dose of raloxifen used for treatment of osteoporosis in women. Such a dose has also been used by Al Jamal and Dubin (2000). The animals were weighed before and after the experiment and their food consumption was measured daily, including the drug in a dose indicated above. When the animals were killed, blood was withdrawn from the heart, seminal vesicles and femurs were removed, cleaned and weighed on a torsion balance.

The femurs were isolated, cleaned of soft tissues, the marrow elements were flushed out with cold saline through a needle inserted into the marrow cavity and finally the bone was weighed and placed in an unstoppered glass vial filled with deionized water and then was placed into a desiccator. The bones were suspended on a fine wire mesh and weighed in air and in water to an accuracy of 0.1 mg. The volume and density of the femurs were calculated from the mass in air and water by Archimedes principle (Kalu *et al.* 1994). The bones were then dried to constant weight and incinerated for 24 h at 600 °C to white ash which was weighed. The ash weight was expressed per milliliter of volume of unashed femur. Bone ashes were then dissolved in 3 mol/l hydrochloric acid before the determination of calcium and phosphorus content.

Calcium was measured by the method of Gitelman (1967) and phosphorus according to Kraml (1966). Testosterone in the blood plasma was determined by conventional radioimmunoassays (CIBA-Corning Diagnostic Corp.).

For the bone morphology we used the method presented by Beall *et al.* (1984) and (Vanderschueren *et al.* (1992). The left femurs were also removed and cleaned of tissue for X-ray examination. Standardized roentgenographs of the femur were made using Philips Mamo Diagnost 3000 X-ray machine at controlled exposures of 26 kV at 5.5 mA. Morphometric measurements were performed directly on the X rays after magnification with a fine caliper. On the roentgenographs at 40 % of the total length starting from the distal end, the outer and inner bone diameters and cortical width were measured after magnification with the fine caliper. Seminal vesicles were cleaned and weighed and the weight was expressed in relative values (mg/100 g b. wt).

Differences between groups were determined statistically by analysis of variance followed by Duncan's multiple range test (Duncan 1955).

Results

The effects of castration and raloxifen administration on all groups of animals are summarized in Table 1. We did not find any differences in body weight after castration and raloxifen had also no significant effect on body weight. Serum calcium and phosphate concentration were normal and did not vary between the groups. Significant changes were observed in the weight of seminal vesicles, namely a striking decrease of seminal vesicles weight was found in orchidectomized mice (49.7 ± 3.2 mg/100 g b.wt). The administration of raloxifen to castrated mice did not produce any changes in the weight of seminal vesicles.

As expected plasma testosterone was decreased in castrated mice (0.5 ± 0.2 µmol/l). Seminal vesicles weight (406 ± 72 mg/100 g b.wt) and plasma testosterone levels (12.5 ± 2.8 µmol/l) were significantly lower in intact animals treated with raloxifen, but remained higher than the seminal vesicles weight and plasma testosterone levels of castrated animals (49.7 ± 3.2 mg/100 g b.wt; 0.5 ± 0.2 µmol/l) ($p < 0.01$)

Bone density, ash and mineral content of the femur were significantly decreased in orchidectomized mice (1.36 ± 0.04 g/ml) compared with untreated animals (1.42 ± 0.03 g/ml). The decrease in bone density and mineral content in the orchidectomized mice was completely prevented by treatment with raloxifen (1.40 ± 0.01 g/ml). The administration of raloxifen to intact mice did not cause a significant increase in bone

Table 1. Variables of body weight, bone density, bone mineral content, plasma testosterone and seminal vesicles weight in individual groups of animals.

	Intact	Castrated	Intact + Raloxifene	Castrated + Raloxifene
<i>Initial body weight (g)</i>	30.2 ± 1.0	31.3 ± 1.0	31.5 ± 0.9	30.6 ± 1.2
<i>Final body weight (g)</i>	48.2 ± 4.0	42.1 ± 2.5	45.8 ± 3.0	42.3 ± 2.5
<i>Volume of femur (μl)</i>	64.0 ± 7	53.5 ± 5	53.8 ± 5	55.3 ± 3
<i>Density of femur (g/ml)</i>	1.422 ± 0.03	1.361 ± 0.04*	1.419 ± 0.03	1.400 ± 0.01
<i>Ash content (g/ml)</i>	0.532 ± 0.04	0.484 ± 0.04 *	0.518 ± 0.03	0.552 ± 0.05
<i>Calcium (mg/ml)</i>	202.2 ± 6.0	184.2 ± 5.0 *	195.0 ± 4.0	206 ± 5.0
<i>Phosphate (mg/ml)</i>	91.4 ± 3.0	82.3 ± 3.7 *	87.2 ± 3.0	92.1 ± 4.0
<i>Seminal vesicle (mg/100 g b.wt)</i>	558 ± 124	49.7 ± 3.2 *	406 ± 72 *	52.3 ± 1.1*
<i>Plasma testosterone (μmol/l)</i>	24.0 ± 3.8	0.5 ± 0.2 *	12.5 ± 2.8 *	0.6 ± 0.3 *
<i>Plasma calcium (mmol/l)</i>	2.3 ± 0.08	2.2 ± 0.06	2.2 ± 0.05	2.3 ± 0.08
<i>Plasma phosphate (mmol/l)</i>	4.1 ± 0.1	4.2 ± 0.2	4.2 ± 0.1	4.2 ± 0.1

Data are means ± S.D. (n = 8). * p<0.01 vs. intact animals

Table 2. Morphometric variables (mm) determined on femur in individual groups of animals.

	Intact	Castrated	Intact + Raloxifen	Castrated + Raloxifen
<i>Femur length</i>	17.4 ± 0.3	17.2 ± 0.3	17.4 ± 0.4	17.3 ± 0.3
<i>Outer diameter</i>	2.14 ± 0.07	2.11 ± 0.09	2.13 ± 0.05	2.14 ± 0.03
<i>Inner diameter</i>	1.62 ± 0.12	1.84 ± 0.13 *	1.64 ± 0.13	1.65 ± 0.11
<i>Cortical width</i>	0.52 ± 0.02	0.27 ± 0.02 *	0.49 ± 0.03	0.49 ± 0.04

Data are means ± S.E.M. (n = 8). * p < 0.01 vs. intact animals

density, the ash and mineral content of the femur, compared to that in untreated animals. Femoral length and outer diameter were not significantly different between the groups 3 months after orchidectomy. At this time, however, orchidectomized mice had significantly thinner cortical width. The decrease in cortical widths was prevented by the administration of raloxifene.

Discussion

The present results confirm the well-known fact that castration leads to osteopenia in experimental animals. An extraordinary responsiveness to the withdrawal or administration of androgens can be demonstrated in mice. The bones of our castrated mice were characterized by a reduction of bone density and ash as well as by lower calcium and phosphorus content.

When castrated mice with low concentrations of testosterone and weights of seminal vesicles were treated with raloxifene, the changes in bone density resulting

from castration were entirely prevented. The decrease in cortical thickness in orchidectomized mice was prevented by raloxifene treatment. At the concentration studied, raloxifene exerted a potent estrogenic activity on bone resorption and a moderate effect on the weight of seminal vesicles and plasma testosterone in intact mice.

We have used a dose which is considered to be comparable to a dose of 60 mg raloxifene used in the treatment of osteoporosis in women and which was used in our experiments (Al Jamal and Dubin 2000). However, this treatment might not reflect the pharmacological and physiological conditions in humans. All mice in our experiment remained healthy throughout the experiment, consumed their food ration daily and showed no significant weight changes.

Pharmacological therapy with the estrogen agonist raloxifene in castrated mice supports the hypothesis that estrogens may also have physiological skeletal effects in male mice. Many arguments favor the hypothesis that the androgen action on bone could depend

on the aromatization of androgens into estrogens. Treatment with 17-beta estradiol, but not with testosterone, in two men with the aromatase gene mutation resulted in an increase in bone mineral density (Gennari *et al.* 2004), whereas treatment with 17-beta estradiol, or testosterone, was ineffective in a man who had no functional estrogen receptor due to a premature stop codon in the estrogen receptor gene (Smith *et al.* 1994. Herrmann *et al.* 2002). Treatment with 17-beta estradiol decreased the levels of bone turnover markers in healthy elderly men and in patients who had been orchidectomized due to prostatic cancer (Taxel *et al.* 2000). Szulc *et al.* (2001) also suggested that 17-beta estradiol is the most potent determinant of bone mineral density among sexual steroids in men.

Bone cells contain not only estrogen receptors (Nilsson *et al.* 1999) but also enzymes of sex steroid pathways such as aromatase and 17-beta hydroxysteroid dehydrogenase (Scopacasa *et al.* 2000). Raloxifen, which is an estrogen antagonist for the breast, is an estrogen agonist for the skeleton (Riggs and Hartmann 2003).

Several lines of evidence implicate estrogen deficiency as a cause of bone loss in elderly men. In healthy middle-aged men, raloxifene treatment has been associated with increased serum estradiol and with

decreased biochemical markers of bone turnover (Uebelhart *et al.* 2004).

Raloxifene treatment of intact male rats for 14 and 28 days produced a significant dose-dependent regression of ventral prostate and seminal vesicles (Steiner *et al.* 2001). Raloxifene exerts the effects on bone in the mice that are similar to those of estradiol. Raloxifene replacement in androgen-deficient adult male mice prevents bone loss.

These pilot data support the theoretical usefulness of the selective estrogen receptor modulators as a therapeutic regimen for male osteoporosis. Our findings with raloxifene are consistent with the reported effects of another selective estrogen receptor modulator tamoxifen on bone in male mice (Broulík 2000).

The results of the present study may be interpreted as supporting the hypothesis that raloxifene is an effective agent against the deleterious effects of castration-induced osteopenia in male mice. Furthermore, the estrogens may also have physiological skeletal effects in male mice.

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