J Physiol Biochem, **65** (3), 267-276, 2009

Effects of letrozole on bone biomarkers and femur fracture in female rats

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(Received on May, 2009)

Z. YONDEN, M. AYDIN, E. ALCIN, M.H. KELESTEMUR, S. KUTLU and B. YILMAZ. *Effects of letrozole on bone biomarkers and femur fracture in female rats.* J Physiol Biochem, **65** (3), 267-276, 2009.

We aimed to investigate the effects of the aromatase inhibitor letrozole on femur fracture and serum levels of alkaline phosphatase (ALP), calcium and phosphate in female rats. Intact 32 Sprague-Dawley female rats were divided into four groups (n=8): control, letrozole 0.2 , letrozole 1 (treatment of 0.2 and 1 mg/kg for six weeks) and recovery (letrozole-treated 1 mg/kg for six weeks then allowed to recover for two weeks). Besides, 24 ovariectomized rats were divided into three groups (n=8): ovariectomized+control, ovariectomized+letrozole and ovariectomized+letrozole+ estradiol (10 µg/rat). After experimental period, rats' femur bones were removed for biomechanical studies following decapitation. Serum ALP, calcium and phosphate were measured. Biomechanical values, ALP and phosphate significantly increased by letrozole in a dose-dependent manner ($p<0.05$) while calcium levels and net bone area decreased ($p<0.05$). Ultimate strength was positively correlated with ALP and phosphate and negatively correlated with calcium. The results indicate that letrozole may increase risk of bone fracture and affect bone biomarkers such as ALP, calcium and phosphate in both intact and ovariectomized rats.

Keywords: Letrozole, Biomechanics, Alkaline phosphatase, Calcium, Phosphate.

The continuous process of bone remodeling in the adult skeleton reflects a balance between the bone-forming action of osteoblasts and the bone-resorptive activity of osteoclasts. Estrogen has a protective effect on bones via multiple mechanisms. The net effect of estrogen is to inhibit bone resorption and to stimulate

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new bone formation (1). In estrogen-deficiency states, such as in natural menopause, treatment with gonadotropin- releasing hormone (GnRH) agonists or aromatase inhibitors (AIs), bone resorption predominates and bone loss ensues. Low serum estradiol (E_2) levels are associated with decreased bone mineral density (BMD) and increased fracture risk in women. In menopause, increases in bone turnover markers (approaching 100%) may result from decreased estrogen levels (2).

Chemotherapy-induced ovarian failure and surgical or medical ovarian ablation can cause premature menopause and bone loss in premenopausal women (3). Premenopausal women with hormone-responsive breast cancer may also suffer from bone loss (4). A large cohort study has confirmed that breast cancer treatments in women with nonmetastatic disease are associated with increased rates of osteoporosis/osteopenia due to cancer treatment–induced bone loss (5).

The aromatase enzyme is a microsomal cytochrome P450 hemoprotein-containing enzyme and catalyzes the rate-limiting step in the production of estrogens, *i.e.* the conversion of androstenedione to estrone (E_3) and testosterone to E_2 (6). Aromatase activity is present in many tissues, *e.g.* ovaries, adipose tissue, brain, liver, muscle, breast tissue, and malignant breast tumors. The main source of circulating estrogens is the ovaries in premenopausal women, and the adipose tissue in postmenopausal women (7).

For many years, the selective estrogen-receptor modulator tamoxifen was the standard endocrine adjuvant therapy for early breast cancer. Data on the efficacy and safety of AIs for the treatment of early breast cancer have started to be reported over the last few years. However the data in advanced disease suggest that the AIs are certainly superior to megestrol acetate as second-line therapy and are superior in one or more end points against tamoxifen as first-line therapy—so much so that they are considered to be the treatment of choice in this situation (8). Recent randomized clinical trials have displayed the efficacy of AIs as an adjuvant therapy for the treatment of estrogen-dependent early breast cancer and endometrial cancers in postmenopausal women (9). It is estimated that more than 50% of postmenopausal women with ER^+ breast cancer in the U.S. are currently being treated with an AIs (8).

The third-generation nonsteroidal AIs such as anastrozole and letrozole bind reversibly to the cytochrome P450 domain of aromatase. Letrozole produces an estrogen-deficient environment by blocking the conversion of androgen to estrogen. The estrogen-deficient environment does not persist for long because these aromatase inhibitors have a short half-life (45 h) and are cleared from the body mainly by the liver (10).

In the short-term, the substantial benefits documented for AIs are associated with a significant, but manageable, increase in adverse events related to bone health (11). The long-term cumulative incidence of AI-associated bone loss is not known in patients with breast cancer. The key to understanding cancer treatment associated bone loss in women is estrogen. The risk of important long-term skeletal problems, including osteoporosis, may increase with AI treatment (12, 13). AIs reduce estrogen levels by about 90% and so it might be expected that bone turnover would increase and that bone loss would be accelerated. Postmenopausal patients with breast cancer who are treated with AIs have significantly higher levels of the

urinary bone resorption markers deoxypyridinoline and pyridinoline (14). AIs reduce bone demineralization and increase fracture by lowering circulating estrogen levels (11).

Because there are not enough data about long-term use of AIs' adverse effects in cancer treatment, particularly letrozole, (8) we aimed to investigate the possible side effects of letrozole on serum bone turnover markers and bone fractures in pre-and postmenauposal physiologic environments.

Material and Methods

Adult female Sprague-Dawley rats (Firat University Experimental Research Center, Elazig-Turkey) weighing between 220-250 g were used in this study. They were kept in constant temperature (21± 1 ^{o}C) and lighting conditions (lights on from 07.00 to 19.00 hrs). Food and water were supplied *ad libitum*. This study was approved by the Experimental Animals Ethics Committe at Firat University Medical School.

Intact model.– This study with intact animals aimed to reveal the effects of letrozole on bone biomarkers and fracture in a dose dependent manner. A daily vaginal smear was performed on the rats and only those showing three consecutive 4-day oestrous cycles were selected for the study. A total of 32 animals were divided into four groups (n=8). The first group served as control and received saline alone by oral gavage. Letrozole was administered to the animals in the second and third groups by daily oral gavage at 0.2 and 1 mg/kg doses, respectively, for a period of six weeks. Additionally we designed a recovery period (two weeks after the last high dose of letrozole) in order to see the post-treatment status. Thus letrozole (1 mg/kg) was also given to the fourth group of rats for six weeks which were then allowed to recover for a period of 15 days. The treatments lasted 42 days and were carried out daily between 09:00 and 10:00 hours.

OVX model.– The second study design with overectomized (OVX) animals aimed to mimic the postmenauposal physiologic environment. A total of 24 adult female rats were OVX and allowed to recover for a period of at least two weeks. A group of OVX animals served as controls and received the vehicle alone. The second group was treated with letrozole (1 mg/kg) for a period of 6 weeks. And in the third group, were given letrozole (1 mg/kg) plus E_2 (10 µg/rat) for a period of 6 weeks.

Letrozole was generously provided by Novartis (Istanbul, Turkey). All other chemicals and reagents were obtained from Sigma Co. (Dorset, UK) unless otherwise indicated.

At the end of the experiments, all animals were decapitated and blood samples were collected. The blood samples were centrifuged at 4 °C and 2500 rpm for 10 min. Serum samples were separated and stored at -20 °C until assayed. Right femurs were isolated and frozen on dry ice and were subsequently processed for biomechanics studies. Serum alkaline phosphatase (ALP) activities and calcium and phosphate levels were determined by spectrophotometric methods using an autoanalyzer, Beckman Coulter, Synchron LX20 (Beckman Coulter. Inc., USA).

Biomechanics studies.– For biomechanical studies, a universal testing machine, Hounsfield H50KM, was used for the

determination of mechanical properties. Three point-bending tests were conducted on the specimens. All specimens were positioned horizontally in the testing machine. A single load down to the catastrophic failure point was applied on the specimens. The load and displacement was recorded and graphed using an X-Y recorder. The strength of the specimens was obtained and observations of the fracture characteristics were done. During the tests, samples were subjected to a preload of 2N and then deformed at a rate of 2 mm/min until failure. A similar calculation procedure given in the literature (15) has been done obtaining the Ultimate Strength and Elastic Modules data.

The net cross sectional areas have been tried to determine in an unusual but much more reliable way in this study and its results have been used in the calculations. Femur contours and areas were measured by the Image-J program.

Statistics.– All results are expressed as Mean ± SEM. A one-way ANOVA test was performed on the findings using SPSS 12.0 for Windows. Correlations between variables were analyzed using the Pearson's correlation coefficient. $P<0.05$ was considered to be statistically significant.

Results

Biomechanics Studies.– Fig. 1 shows the biomechanics parameters of the femoral mid-diaphysis as determined by the three-point bending test for all groups. A slight, increase in the ultimate strength values of both the high dose letrozole-treated intact groups and OVX animals compared to the controls was observed ($p<0.05$, Fig. 1). In the OVX animals, the treatment with letrozole or letrozole+E_2 did not show significant change in the ultimate tensile strength and elastic module. Additionally, a considerable increase in the elastic module of the samples taken from letrozole-treated intact and OVX animals was observed compared to the control group ($p<0.01$, Fig. 1). Decreased displacement values were obtained although they were not statistically significant (data not shown).

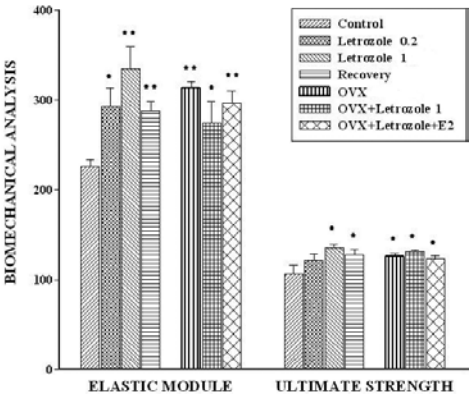

Fig. 1. *Elastic modules (EM= Stiffness X Length / Area, MPa) and Ultimate strength (UTS=Load (P) /Area, N/mm^2) of three-point bending test of femoral mid diaphysis.*
(Mean±SEM; $^*p<0.05$ and $^{**}p<0.01$ compared to control group, using ANOVA).

When the strength calculations is taken into account, hallow structure of bone is considered. The wall thickness has been determined by using image J technique (Fig. 2). It is a common observation from the examination that although the outer boundaries do not show any considerable change, the inner boundaries show a considerable change. In other words, the thickness of the cortical side or the thickness having net bone section which is the main load carrying area gets thinner. The whole cross-sectional area of femur diaphysis of the high-dose letrozole animals was higher than the controls ($p<0.05$) and the net area was lower than the control and recovery groups ($p<0.05$) (Fig. 2).

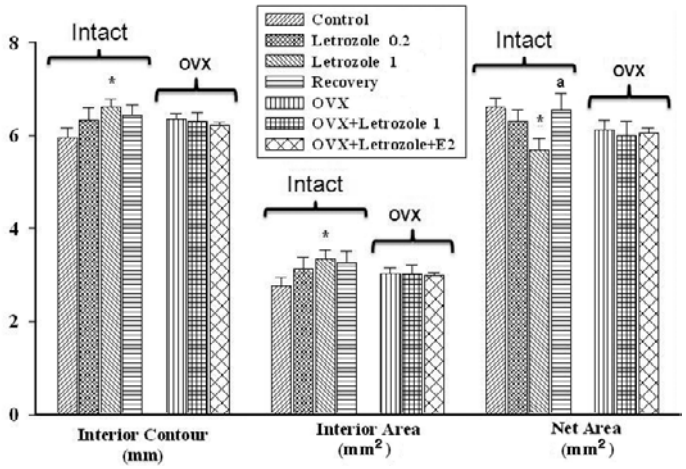

Fig. 2. *Measures of femur contours and areas.*
Results are Mean ± SEM; *p < 0.05 compared to corresponding control group; ᵃp < 0.05 compared to Letrozole (1mg) group, using ANOVA.

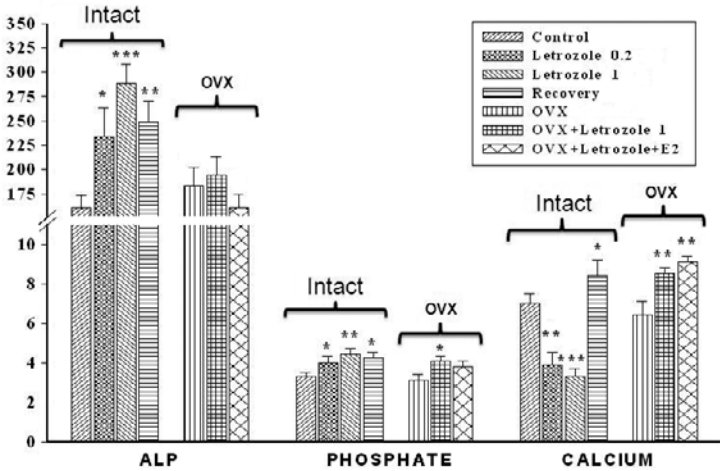

Fig. 3. *Levels of serum bone turnover markers.*
Alkaline Phosphatase (ALP; IU/L), Phosphate (mg/dl) and Calcium (mg/dl). (Mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 compared to corresponding control group using ANOVA).

Biochemical markers.– The levels of ALP and phosphate (P) and calcium (Ca^{2+}) in serum can be compared in Fig. 3. In the intact animals, ALP and P levels were significantly increased by letrozole in a dose-dependent manner while Ca^{2+} levels decreased (Fig. 3). In the animals treated with letrozole and then given a recovery period (2 weeks), ALP and P levels were lower than that of the letrozole-

treated groups without recovery but higher than the controls. Although letrozole plus recovery reduced Ca^{2+} levels compared to letrozole alone, the levels were still higher than controls ($p<0.05$). OVX didn't affect serum ALP, P and Ca^{2+} levels. Both P and Ca^{2+} levels increased in the 0VX plus letrozole animals ($p<0.05$). In the E_2-treated group, Ca^{2+} levels increased ($p<0.05$), but ALP and P levels were not changed compared to the control levels ($p<0.05$).

Correlation analysis.– The correlation among particular values was also tested. The serum ALP levels showed a positive correlation with the serum P levels ($r=0.35$, $p<0.05$) and showed a negative correlation with the serum Ca^{2+} levels in total group ($r=-0.29$, $p<0.05$). In addition, the serum ALP levels were negatively correlated with net bone area in total, intact and OVX groups ($r=-0.29$, $r=-0.37$ and $r=-0.47$ respectively, $p<0.05$). Serum P levels were positively correlated with serum Ca^{2+} levels in OVX rats ($r=0.60$, $p<0.01$).

In intact rats, elastic modules and ultimate strength were negatively correlated with net bone area ($r=-0.42$, $p<0.05$ and $r=-0.58$, $p<0.01$ respectively). Also, only ultimate strength was negatively correlated with net bone area ($r=-0.52$, $p<0.001$) in total groups. Ultimate strength was positively correlated with serum ALP and P levels in intact animals ($r=0.46$, $p<0.05$ and $r=0.44$, $p<0.01$ respectively) while negatively correlated with serum Ca levels in total groups ($r=-0.29$, $p<0.05$). Elastic modules were positively correlated with ultimate strength in total and intact groups ($r=0.40$, $p<0.01$ and $r=0.51$, $p<0.05$ respectively) and negatively correlated with displacement in total, intact and OVX groups ($r=-0.73$, $p<0.001$; $r=-0.62$, $p<0.01$ and $r=-0.85$, $p<0.001$ respectively).

Discussion

In this study, intact rats treated with letrozole in different doses (0.2 and 1 mg) had significant increases in serum ALP levels at six weeks; compared with the control group in a dose-dependent manner. Also, there were significant decreases in serum Ca^{2+} levels and significant increases in serum P levels. On the other hand, both P and Ca^{2+} levels increased in the OVX plus letrozole animals. The absence of any differences in bone markers in OVX without letrozole group compared to the intact control group may suggest estrogen released from tissues other than ovaries could play a role in maintenance of bone mineral balance.

Clinically, the use of AIs was reported to be associated with an increase in fracture risk. Most of the clinical trials are inadequate in size to examine the effect of AIs on fracture risk. The numbers of fractures were 577, 125, 384 and 352 in the trials of anastozole (13), exemestane (16) and letrozole (17, 18) respectively. Letrozole increases bone loss at the rate of 2%–3% per year (19). It also causes increases in bone fractures (18). In another report, there was a small increase in new-onset osteoporosis rates in letrozole-treated group compared to the placebo. However, there was not a significant difference in the incidence of fractures between the two groups (12). There appear to be consistent findings of high bone turnover and accelerated bone loss with all licensed AIs (20). In a study which investigated the effect of letrozole on 32 women with inactive breast disease and 42 healthy women, it was reported that there was an increase in bone resorption markers (21). PÉREZ *et al.*

reported that letrozole therapy significantly increased urinary excretion of the bone turnover marker *N*-telopeptide (19). Our findings are similar to those reported in previous studies that chronic estrogen suppression already vulnerable to osteoporosis is the effect of the AIs on bone health.

Estrogen depletion may increase receptor activator of nuclear factor-kappa beta ligand (RANKL) (22) which binds to the receptor on osteoclasts. Increased RANKL production increases serum ALP activities and stimulate osteoclastogenesis. Our earlier results showed that letrozole decreases serum estradiol levels (23). With the help of these data we can explain the molecular mechanisms of bone degradation related to AIs treatment.

ALP in the circulation is produced by bone and liver. Serum ALP levels of letrozole-treated rats were found to be increased compared to the control group in the present study. The positive correlation between ALP and P, negative correlation between ALP and Ca^{2+} and ALP and net bone area suggested that the source of ALP could be bone rather than liver. Fracture risk is more consistently related with bone resoption markers rather than markers of bone formation. However, ALP was also found to be a good predictor of fracture risk in the Hawaii Osteoporosis Study (24).

The negative correlation between ALP and net bone area supports both the accuracy of our bone field measurement method and the idea that ALP is a bone resorption marker. Currently, examination of bone turnover biomarkers is not recommended for routine screening or diagnosis of bone loss (25). However, our results suggested that measuring bone biomarkers such as ALP, Ca^{2+} and P is a cheaper and easier method for screening and diagnosis of bone loss in patients prescribed with AIs.

Clinical practice guidelines recommend baseline and annual follow-up bone density monitoring for all patients initiating AI therapy (11). The resulting decrease in bone mass leads to an increased incidence of fractures. Some authors did not report any significant changes in BMD despite letrozole and anastrozole treatment for 16 weeks (26). Studies have shown that substantial deterioration of bone microarchitecture can occur before bone density is affected (27). Bone quality is increasingly identified as an important determinant of overall bone health. In the present study, we performed an easier and practical test, the three-point bending test, to evaluate the risk of fracture rather than BMD in the animal experiments.

Although serum Ca^{2+} and P are not widely used in fracture risk estimation, their combination with ALP and some additional morphometric methods provide a good correlation between biochemical parameters and mechanical tests. Serum Ca^{2+}, P and ALP levels were found to be related with the three-point bending test, which gave relavant values compatible with different non-invasive techniques (28).

Some authors found that there were no changes in the elastic module, stiffness and ultimate strength in letrozole treated animals (15). In the present study, we found that high-dose letrozole (1 mg/kg) caused significant increases in elastic module and ultimate strength and insignificant decrease in stiffness. These differences could be due to the method of bone field measurement. The net cross-sectional areas were calculated with a new method using the Image J programm. This program does not take into account cancellous parts or voids in the cross-sectional

area of the bone. In previous studies (15), the whole cross-sectional area, including the cancellous parts, is considered. In our study, although the outer boundaries did not show any considerable change, the inner boundaries exhibited a significant change. In other words, the thickness of the cortical side of the bone, which is the main load-carrying area, got thinner. The thinning of the cortical side of the bone increases the risk of fractures.

In conclusion, the biochemical values and biomechanical findings in this study suggest that long-term use of letrozole may increase the risk of osteoporosis and fractures. However, our biochemical parameters are not commonly used in fracture risk estimation. Nonetheless, the correlation between simple biochemical measurements and biochemical results leads us to propose that Ca^{2+}, P and ALP levels could be valuable to measure during the clinical follow-up evaluating the risks for osteoporosis, bone deformation and fracture in certain patients who take AIs treatment. Current clinical practice should include the measurement of bone mineral markers such as ALP, P and Ca^{2+} in post-menopausal women taking AIs and in osteoporosis patients on antiresorptive therapy.

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