Influence of the selective oestrogen receptor modulator (raloxifene hydrochloride) on IL-6, TNF-α, TGF-β1 and bone turnover markers in the treatment of postmenopausal osteoporosis

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ABSTRACT. ***Background*. Osteoporosis that is encountered frequently in postmenopausal women, may cause an increased incidence of vertebral and iliac fractures that are associated with excess morbidity. Raloxifene hydrochloride, a selective oestrogen receptor modulator, has been shown to increase bone mineral density and decrease biochemical markers of bone turnover in postmenopausal women, without stimulatory effects on breast or uterus. Levels of proinflammatory cytokines, including IL-6, and TNF-α and TGF-β1 which are important cytokines involved in remodeling, have been evaluated previously in *in vitro* studies of osteoporosis. However, there seems to be a paucity of *in vivo* research concerned with changes in these cytokines in osteoporosis. *Objective*. In this study, we evaluated the effects of raloxifene (Evista®; Lilly Pharmaceutical Co. USA, 60 mg/day) on biochemical bone turnover markers, serum parathyroid hormone, and 25-OH vitamin D, as well as the serum levels of IL-6, TNF-α and TGF-β1, in 22 postmenopausal, osteoporotic women before and after 12 weeks of raloxifene treatment. *Methods*. Well-matched, postmenopausal, non-osteoporotic control subjects were also enrolled in the study. Serum levels of all the parameters were measured in postmenopausal, osteoporotic women at baseline and end of the study. *Results*. It was found that serum osteocalcin and parathyroid hormone, and urine deoxypyridinoline levels decreased to normal levels with treatment. Serum 25-OH vitamin D levels after treatment in the patient group were higher than those in the control group. Serum IL-6, TNF-α and TGF-β1 levels did not change significantly with treatment. However, serum levels of IL-6 and TGF-β1 in the patient group after treatment, decreased to levels lower than those found in the control group. Serum TNF-α levels in the patient group before and after treatment, were lower than those in the control group. *Conclusion.* Raloxifene treatment reduces bone turnover biochemical markers, parathyroid hormone and induces 25-OH vitamin D in postmenopausal women. Moreover, it also affects some serum cytokine levels in the postmenopausal period.**

Keywords: bone turnover marker, cytokine, osteoporosis, postmenopausal woman, raloxifene

Diseases of the elderly are becoming more common throughout the world as improvements in health care and nutrition enable a greater portion of the population to live to old age. Postmenopausal women have an increased risk of cardiovascular disease and osteoporosis. Osteoporotic fractures are a major cause of increased morbidity in older women. Postmenopausal osteoporosis is a metabolic bone disease characterized by decreased skeletal durability, which leads to increased risk of fractures [1]. Bone mass is lost in postmenopausal osteoporosis due to the loss of relationship between osteoblasts and osteoclasts. Raloxifene is a non-steroidal, benzothiophene-derived, selective oestrogen receptor modulator (SERM) that has agonistic effects on tissue-specific oestrogen receptors in the skeletal system, despite the antagonistic effects on the oestrogen receptors in the breast and uterine tissues. Raloxifene use is associated with increased bone mineral density (BMD), decreased bone turnover, maintenance of normal bone architecture. It is therefore used clinically for prevention and treatment of postmenopausal osteoporosis [2, 3]. Raloxifene modulates bone resorbing-forming cells by two mechanisms: inhibition of osteoclastogenesis and osteoclastic activity and induction of osteoblastic activity [4].

High bone turnover has been claimed to be a risk factor involved in fractures. The equilibrium in bone turnover is maintained by the concerted actions of osteoblasts and osteoclasts. Preclinical data show that osteoclastic differentiation and activity require certain factors such as proinflammatory cytokines, especially IL-6 and TNF-α [5, 6].

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These cytokines and TGF-β1-like growth factors that have important roles in bone metabolisms, originate from osteoblastic cells [7]. The influence of raloxifene on cytokines such as IL-6, TNF- α and TGF-β1 have been shown in *in vitro* studies [4, 7, 8]. However, *in vivo* studies are lacking. The aim of this study was to evaluate *in vivo* the influence of raloxifene on IL-6, TNF-α, TGF-β1 and bone turnover markers in postmenopausal, osteoporotic women.

MATERIALS AND METHODS

Twenty-two postmenopausal, osteoporotic women aged between 46 and 66 (54.14 ± 5.16) years were included in the study after being evaluated for the exclusion criteria. Osteoporosis was diagnosed according to WHO criteria [9]. Osteoporosis was defined as a T-score that is lower than -2.5 SD in measurements performed by Dual Energy Absorptiometry (DEXA) (Hologic). Fifteen postmenopausal women without osteoporosis were included as the control group. Age, menopause age, systemic diseases and medications that they used were recorded.

Exclusion criteria were as follows:

– presence of an acute or chronic infection,
– presence of a metabolic bone disease,
– presence of systemic diseases such as chronic renal failure, diabetes mellitus, thyroid function abnormalities, hyperparathyroidism, Cushing syndrome, etc.,
– previous medical treatment for osteoporosis,
– use of corticosteroids, anticonvulsants, anticoagulants or systemic hormone treatments (estrogens, androgens, calcitonine, L-thyroxine) that could influence the parameters to be measured in our study,
– history of thromboembolism or fracture.

All patients included in the study were started on raloxifene hydrochloride 60 mg (Evista®; Lilly Pharmaceutical Co. USA). Serum osteocalcin (OC) and urine deoxypyridinoline (Dpd) were measured by immunoassay (Immulite 1000 analyzer, Diagnostic Product Corp. DPC, Los Angeles, USA), before and twelve weeks after treatment. Serum parathyroid hormone (PTH) was measured using a commercial assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) with inter- and intra-assay coefficient of variations (CVs) of less than 7% across the working range (0.5 pmol/L to 100 pmol/L). Calcium regulating hormones, including intact PTH, were measured with a immunochemiluminometric assay (normal range 10-65 pg/mL; intra-assay and inter-assay CVs 5.0% and 7.3%; respectively); 25-hydroxy vitamin D (25-OHD) was measured with the chemiluminescence assay (normal range 10-68 ng/mL, intra-assay and inter-assay CVs 9.54% and 14.10%; respectively). Total serum calcium was measured with the spectrophotometry assay method (normal range 8.8-10.1 mg/dL; intra-assay and inter-assay CVs 0.65% and 1.25%; respectively), urine calcium was measured with Hitachi spectrophometry (normal range 50-320 mg; intra-assay and inter-assay CVs 2.1% and 2.3%; respectively). Serum alkaline phosphatase (ALP) was measured using the same auto-analyzer (normal range 25-125 IU/L; intra-assay and inter-assay CVs 0.7% and 1.2%; respectively). Serum samples obtained before and after treatment were stored at -80°C, and all the samples were tested for the parameters by ELISA (Dynex DSX full automatic ELISA analyzer, USA) at the end of the study. Levels of cytokines in serum were measured with the commercially available ELISA kits (IL-6, Catalog # KAC1261; TNF-α, Catalog # KAC1751; TGF-β1, Catalog # KAC1688, BioSource International, Inc, Camarillo, CA, USA) according to the manufacturer's instructions. Levels of these mediators below the sensitivity of the assay were 3 pg/mL for IL-6 and TNF-α, 15.6 pg/mL for TGF-β1.

Informed consent for the described procedures was obtained from all patients. Approval for the study was given by the ethics committee of our hospital.

Statistical analysis

T scores, serum bone turnover markers and biochemical parameters were evaluated as mean ± standard deviation (SD). Serum cytokines were evaluated as mean ± standard error of the mean (SEM). The significances of differences between pre- and post-treatment patients group were determined using Student's *t* test. The Mann-Whitney *U* test was used for evaluation of differences between the pre-treatment patients group and the control group, and between the post-treatment patients group and the control group. Differences were considered significant when the p value was less than 0.05.

RESULTS

Demographic characteristics and T scores of the osteoporotic women and control subjects are shown in *table 1*.

Bone turnover biochemical markers for the patient and control groups, pre- and post- treatment, are shown in *table 2*. Serum OC, serum PTH and urine Dpd levels decreased significantly in the post-treatment period (p = 0.052, p =0.001, p = 0.002; respectively). 25-OH vitamin D levels increased non-significantly with treatment

Table 1
Patient and control characteristics and bone mineral density changes after treatment (mean ± SD)

	Pre-treatment Patient group (n = 22)	Post-treatment Patient group (n = 22)	Control Group (n = 15)	P value (within group)	P value (Pre-treatment patients *versus* control)	P value (Post-treatment patients *versus* control)
Age (year)	54.13 ± 5.15		52.66 ± 6.27	-	0.41	
Duration of menopause (year)	5.25 ± 4.25		6.13 ± 3.46	-	0,79	
Lumber spine (T-score)	-2.50 ± 0,46	-2.01 ± 0.31	-1.18 ± 0.41	0.03	0.01	0.11
Femoral neck (T-score)	-2.41 ± 0,47	-1.98 ± 0.39	-1.13 ± 0.29	0.04	0.01	0.26
Trochanter (T-score)	-2.34 ± 0,52	-1.86 ± 0.26	-1.04 ± 0.24	0.02	0.01	0.21

Table 2
Bone turnover and biochemical markers in patients (pre-treatment and post-treatment periods) and control individuals (mean ± SD). See text for p values

	Pre-treatment	Post-treatment	Control
Osteocalcin (ng/mL)	15.12 ± 6.45	12.59 ± 5.92	9.67 ± 2.49
Deoxypyridinoline (nM/nM creatine)	6.17 ± 2.92	4.09 ± 1.17	3.85 ± 1.09
Parathyroid hormone (pg/mL)	66.39 ± 11.57	45.86 ± 12.58	42.90 ± 9.37
25-hydroxy vitamin D (ng/mL)	30.71 ± 22.76	36.76 ± 17.67	20.46 ± 7.29
Calcium (mg/dL)	9.67 ± 0.36	9.55 ± 0.31	9.43 ± 0.41
Phosphorus (mg/dL)	3.74 ± 0.55	3.63 ± 0.48	3.76 ± 0.56
Alkaline phosphatase (IU/L)	78.72 ± 18.42	77.50 ± 15.95	72.05 ± 12.91
Urine calcium/creatinine ratio	0.17 ± 0.16	0.16 ± 0.10	0.16 ± 0.09

($p = 0.128$). Serum Ca^{++} and serum phosphorus levels did not change significantly ($p = 0.277$, $p = 0.362$; respectively). Additionally, 24-hour urine Ca^{++}, the 24 hour urine Ca^{++}/creatinine ratio and serum ALP levels did not change significantly after treatment ($p = 0.586$, $p = 0.838$, $p = 0.527$; respectively). Serum OC, serum PTH and urine Dpd levels that were significantly higher in osteoporotic women compared to the controls in the pre-treatment period ($p = 0.003$, $p = 0.001$, $p = 0.001$; respectively) were not found to be significantly different between the two groups in the post-treatment period ($p = 0.118$, $p = 0.485$, $p = 0.547$; respectively). Serum 25-OH vitamin D levels were not significantly different between the groups pre-treatment, although they increased significantly in the patient group compared to the control group in the post-treatment period ($p = 0.226$, $p = 0.001$; respectively).
IL-6 (23.41 ± 3.87 pg/mL *versus* 15.55 ± 1.86 pg/mL, $p = 0.108$), TNF-α (26.65 ± 0.66 pg/mL *versus* 26.93 ± 0.67 pg/mL, $p = 0.682$), TGF-β1 (33.18 ± 4.0 ng/mL *versus* 24.73 ± 6.77 ng/mL, $p = 0.225$) levels did not change significantly in the patient group after treatment. However, interesting results were obtained when pre- and post-treatment values were compared with the control group. Pre-treatment serum IL-6 levels that were not significantly different from the controls (23.41 ± 3.87 pg/mL *versus* 26.47 ± 2.65 pg/mL, $p = 0.112$) were found to be significantly lower than in the control during the post-treatment period (15.55 ± 1.86 pg/mL *versus* 26.47 ± 2.65 pg/mL, $p = 0.002$) (*figure 1*A). Serum TNF-α levels in the patient group were significantly lower than in the control group, in both the pre- and post-treatment period (26.65 ± 0.66 pg/mL *versus* 31.11 ± 0.95 pg/mL, $p = 0.001$; 26.93 ± 0.67 pg/mL *versus* 31.11 ± 0.95 pg/mL, $p = 0.002$; respectively) (*figure 1*B). Pre-treatment serum TGF-β1 levels were not significantly different from the controls (33.18 ± 4.0 ng/mL versus 35.13 ± 4.89 ng/mL, $p = 0.748$), but decreased to values lower than the controls with treatment (24.73 ± 6.77 ng/mL *versus* 35.13 ± 4.89 ng/mL, p=0.017) (*figure 1*C).

DISCUSSION

Postmenopausal osteoporosis is a metabolic bone disease characterized by decreased skeletal strength, which leads to increased fracture risk [1]. Although a number of factors have been implicated in the etiology of postmenopausal osteoporosis, the lack of estradiol –a systemic regulator of bone remodeling- appears to play a significant role [10]. Oestrogen replacement therapy has long been an important therapeutic modality for the prevention and treatment of postmenopausal osteoporosis. However, long-term compliance with oestrogen therapy is limited, and there are increasing concerns regarding its safety [11]. Recently, raloxifene, a SERM has been used as an anti-resorptive drug for the prevention and treatment of osteoporosis [12]. Although it is mainly used for the treatment and prevention of osteoporosis, the mechanisms by which this compound modulates the activity of bone cells are still poorly understood. It is of interest to note that, *in vivo*, raloxifene has been hypothesized to have a role not only as an anti-resorptive drug but also as a stimulator of osteogenic cells [4]. Additionally, the MORE study demonstrated only a moderate reduction in the markers of bone turnover in long-term-treated patients, suggesting a potential effect of this drug on osteoblastic cell activity [12]. However, *in vivo* studies evaluating the effects of raloxifene on levels of cytokines that can influence osteogenic turnover and anti-resorptive effects, are not very numerous. In this study, we aimed to evaluate the response of cytokines that have been shown to be indicators of osteoblastic and osteoclastic activity to raloxifene treatment *in vivo*.

Bone turnover markers have been associated with fracture risk in many previous studies. Higher bone turnover markers such as serum OC, serum PTH and urine Dpd levels in postmenopausal women before treatment when compared to the controls indicate osteoporosis and a probable risk of fracture in our patients. It has been shown that raloxifene decreases osteoporotic fracture risk by slowing down bone turnover. Decreases in serum OC, serum PTH and urine Dpd levels after raloxifene treatment shows that this therapy is beneficial in osteoporotic patients [12-18]. Moreover, although statistically non-significant, increases in serum 25-OH vitamin D levels with treatment and higher post-treatment 25-OH vitamin D levels in the patient group compared to the control group confirms the positive influence of raloxifene on bone formation. Hansdottir *et al*. have also detected an increase in 25-OH vitamin D and a decrease in serum PTH after raloxifene treatment [17]. These results are similar to our results.

It has been proposed that raloxifene has this effect as a recult of its influence on cytokines such as IL-6, TNF-α, TGF-β and other factors such as growth factors [14, 19, 20]. In our study, IL-6, TNF-α and TGF-β1 levels did not change significantly after three months treatment with raloxifene. Although, it has been indicated that IL-1, IL-6 and TNF-α levels are increased in postmenopausal women and that IL-1 beta and TNF might cause FAS-mediated osteoblastic apoptosis and therefore osteoporosis [21, 22],

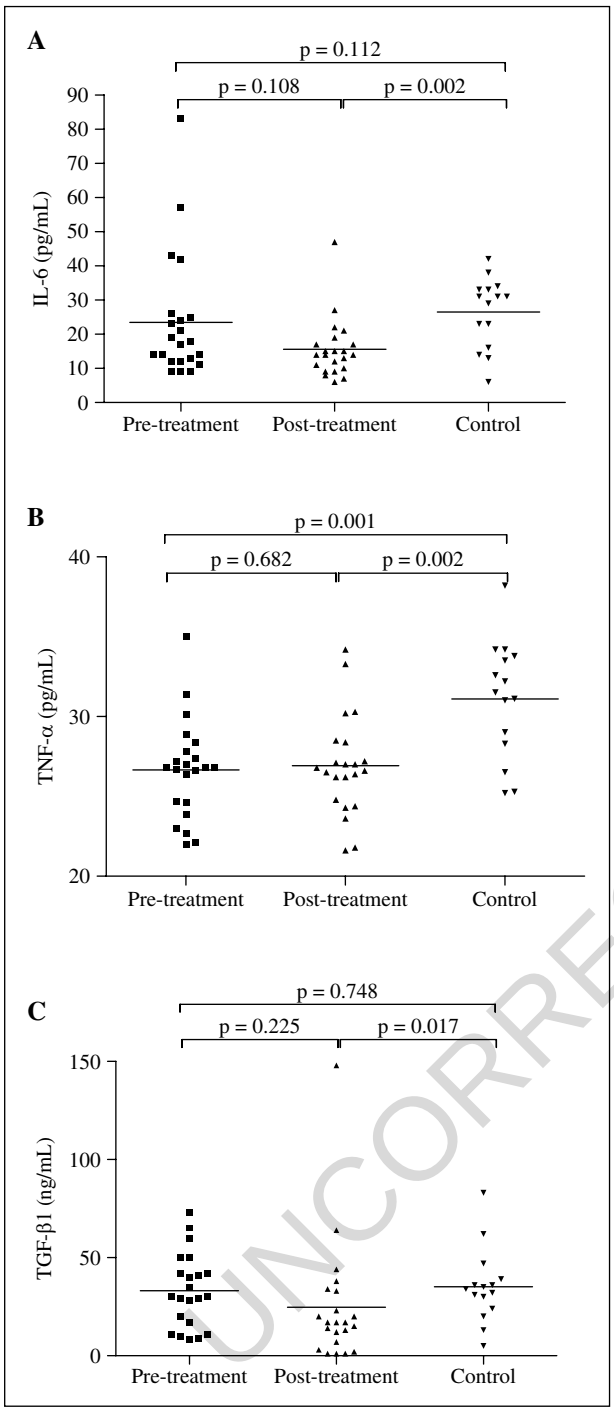

Figure 1
Serum IL-6 levels in patients with osteoporosis in pre- and post-treatment periods and control subjects (**A**). Serum TNF-α levels in patients with osteoporosis in pre- and post-treatment periods and control subjects (**B**). Serum TGF-β1 levels in patients with osteoporosis in pre- and post-treatment periods and control subjects (**C**) (mean ± SEM).

Abrahamsen *et al*. have shown that TNF-α is not related to the extent of bone loss and serum estradiol levels [23]. Moreover, Pratelli *et al*. have shown that TNF-α is low in postmenopausal women [24]. Therefore, lower TNF-α levels in postmenopausal women both before and after treatment compared to the control group is not a determining result in our study. Moreover, although IL-6 levels in postmenopausal women before treatment is not very different from the postmenopausal control women, it is interesting to note that IL-6 levels after treatment are lower than those found in the controls. The most important determinant of the postmenopausal period is oestrogen deficiency. Oestrogen exerts its effect *via* the oestrogen receptor. The inhibitory action of the activated oestrogen receptor on binding of NF-κB to the IL-6 gene is one of the best-examined examples of cross-talk between ligand-activated oestrogen receptors and proinflammatory transcription factors. Expression of IL-6 at inflammatory sites is largely controlled by NF-κB [25]. Antagonism of NF-κB activity has been observed with many nuclear receptors, including the receptors for glucocorticoids [26, 27], androgens [28], progesterone [29], the retinoid X receptor [30], and the peroxisome proliferator-activated receptor γ [31]. There is also ample evidence that the p65 subunit of NF-κB represses oestrogen receptor-α-mediated transactivation [32]. Additionally, raloxifene, a SERM, has been shown to decrease IL-6 levels *via* the oestrogen receptor [8]. These studies indicate that absence of a difference between IL-6 levels in postmenopausal women before treatment and the control women and lower IL-6 levels in the post-treatment group when compared to the controls might be attributed to the oestrogen receptor-mediated IL-6 gene suppression by raloxifene.

Evidence for the role of TGF-β in regulating BMD comes from a number of observations: it is present at high concentrations in bone [32, 33], it promotes osteoblastic proliferation [34] and their subsequent differentiation [35], and it also decreases osteoclastic activity by several mechanisms [36, 37]. The rate of bone formation is altered in TGF-β1-knockout mice [38], and administration of TGF-β corrects the bone density deficiency in elderly mice with osteoporosis [39] and in rats with suspension-induced osteoporosis [40]. TGF-β protein is found in a wide variety of tissues in addition to bone, including blood. There are three closely related TGF-β isoforms (β1, β2 and β) with isoform-specific distribution patterns [41]. To date, only the β1 isoform has been detected at levels in excess of 1 ng/mL in human blood [42, 43]. We therefore measured only the TGF-β1 isoform in serum. In our study, TGF-β1 levels were not significantly different between the postmenopausal control group and pretreatment, postmenopausal patient group. Moreover, although serum TGF-β1 levels tended to decrease after treatment, this was not statistically significant. Additionally, TGF-β1 levels in the post-treatment patient group were lower than in the control group. There are reports in the medical literature that show that TGF-β1 levels do not change in postmenopausal, osteoporotic women [44, 45] Similar to these studies, our results also indicate that TGF-β1 does not have an important role in the pathogenesis of postmenopausal osteoporosis. However, it is an interesting finding that TGF-β1 levels were lower after raloxifene treatment when compared to the control cases. It has been shown in an *in vitro* study that raloxifene has no impact on TGF-β1 synthesis in primary osteoblastic cultures obtained from healthy postmenopausal women and it has been proposed that osteoblastic TGF-β1 synthesis does not have a role in raloxifene or estradiol treatment [7]. The absence of any other study about this issue, makes a better interpretation of this result very difficult.

In conclusion, the absence of any significant change in cytokine and growth factor levels after three months of raloxifene treatment, but presence of some difference from

normal controls may be attributed to the small number of cases and the short duration of treatment. Perhaps the cytokine pattern in bone turnover in osteoporotic patients is different from normal healthy controls. Unfortunately, the absence of sufficient clinical research and the presence of inconclusive *in vitro* results and clinical studies make precise interpretation of our results impossible. Longer term clinical studies, with a larger study population, are needed to allow a fuller explanation of these mechanisms.

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