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ORIGINAL ARTICLE

Prospective evaluation of free radicals and antioxidant activity following 6-month risedronate treatment in patients with postmenopausal osteoporosis

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Abstract In addition to the well-described implications of estrogen deficiency in postmenopausal osteoporosis (PMO), free radicals are also effective on bone metabolism. The antioxidant vitamins C and E play an important role in the production of collagen, mesenchymal cell differentiation into osteoblasts, and bone mineralization. Therefore, the incidence of osteoporosis and the risk of fractures were decreased with vitamin C and E. It was proposed that free oxygen radicals are responsible for biological aging, atherosclerosis, carcinogenesis, and osteoclastic activity via their negative effects on the cell and DNA. In this study, we aimed to investigate and compare the levels of free radicals and serum antioxidant activity in patients with PMO and healthy subjects before and after six-month treatment with risedronate, which is an inhibitor of bone resorption. Twenty-three postmenopausal patients aged between 52–83 (mean [± standard deviation] 67.6 ± 8.17) with T scores below −2.5 in femur neck or L1-L4, and 23 postmenopausal healthy subjects were enrolled into the study. Patients who had received any medications within the last 6 months that could alter bone metabolism were excluded. Serum malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels were analyzed in both groups. The patients with PMO were commenced on 5 mg of risedronate, 1,200 mg of calcium, and 800 IU of vitamin D daily. The patients were reevaluated at the end of the sixth month. MDA and SOD levels were similar in patients with PMO when compared to the healthy group before the treatment, while the GPx levels were lower in patients with PMO ($P = 0.014$). GPx ($P = 0.028$) and MDA ($P = 0.04$) levels were increased in patients with PMO after the treatment. In contrast, SOD levels were decreased when compared to the initial levels ($P = 0.006$). There may be an insufficiency in different steps of the enzymatic antioxidant systems in patients with PMO without treatment. We observed an increment in lipid peroxidation levels and GPx levels with risedronate. We think that the decrement in SOD levels may be related with the utilized antioxidants due to the increased free radicals and the compensatory increment in the other steps of the antioxidant system.

Keywords Postmenopausal osteoporosis · Free radicals · Antioxidants · Risedronate

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Introduction

Normal skeletal development and protection of its integrity in women requires normal gonadal function from menarche to menopause. Sex steroids are the most important factors

controlling the cycle frequency during bone restructuring remodeling and the balance between formation and resorption in each cycle [1]. Although the underlying main cause in postmenopausal osteoporosis (PMO) has been defined as estrogen deficiency, free radicals can also play a role in bone metabolism [2]. Reactive oxygen radicals are the physiological activators of the transcription factors of inflammatory cytokines. Oxidative stress may therefore trigger inflammatory activity and act to increase disease activity. NF-κB is the transcription factor responsible for oxidative stress, and free radicals can increase bone resorption via NF-κB [3]. The "receptor activator of NF-kappaB" (RANK) ligand and osteoprotegerin have also been proven to have a receptor-activating role on the effect of estrogen on bone metabolism. Recently, free radicals have also been cited as responsible for atherosclerosis, carcinogenesis, and infarction in addition to the process of aging [4]. The production of these radicals by osteoclasts increases the destruction of calcified tissue [5, 6].

Reactive oxygen radicals are continuously formed during the cellular metabolism of oxygen in human organism. An excessive increase in these radicals disturbs the prooxidant–antioxidant balance and leads to tissue injury caused by prooxidant radicals [7]. Antioxidant systems protect the organism against reactive oxygen species (ROS). These defense mechanisms stop the radical chain reaction and direct the resultant ROS to target where it would cause less injury [8]. There are three main enzymes responsible for ROS control: superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) [9]. Major non-enzymatic defense is provided by albumin, uric acid, bilirubin, cysteine, glutathione, β-carotene, dihydrolipoate, ubiquinone, ceruloplasmin, transferrin, zinc (Zn), manganese (Mn), selenium (Se), and vitamins A, C, and E [10]. Lipid peroxidation that develops following the oxidation of the cell membrane's polyunsaturated fatty acids with ROS causes cell membrane damage by changing its viscosity, permeability, and integrity [11, 12]. These lipid peroxides are then broken down into very cytotoxic compounds such as aldehydes and alkoxyl radicals.

Previous studies have shown inadequate dietary vitamin C and E intake to be associated with a risk of hip fracture [13]. Many of the defined risk factors for osteoporosis such as smoking, hypertension, diabetes mellitus, and estrogen deficiency have also been shown to be associated with increased oxidative stress [14].

The continuous increase in life expectancy and thus in the geriatric population may be expected to lead to an even higher number of cases with osteoporosis, as it is commonly encountered in postmenopausal and elderly persons. Our aim in this study was to compare the serum antioxidant activity and lipid peroxidation levels (malondialdehyde; MDA) in PMO patients with levels in normal individuals and to evaluate the effect of the use of the bone resorption inhibitor risedronate for 6 months.

Materials and methods

Patient selection

We included postmenopausal patients with a femoral neck or L1-L4 T score of −2.5 or lower (n:23) consecutively recruited from patients seen at the Osteoporosis Unit who underwent bone mineral density (BMD) determination with dual X-ray absorptiometry (DXA); non-osteoporotic postmenopausal healthy individuals (n:22) served as the control group in this study. Written consent was obtained from the volunteers. Patients with disorders like diabetes, endocrine, gastrointestinal, inflammatory, renal, and liver diseases and/or who were using drugs that could affect bone metabolism and/or oxidant-antioxidant systems were excluded from the study. None of the subjects were smokers or alcohol consumers. Patients who had been on medication for osteoporosis [including calcium, vitamin D, bisphosphonates, selective estrogen receptor modulators (SERMs) hormone replacement therapy, or calcitonin] or antioxidant activity (including herbal, vitamin, or nutritional supplementations) within the last 6 months were also not included in the study. Patients with osteoporosis were placed on 1,200 mg calcium and 800 IU vitamin D in addition to 5 mg risedronate daily and follow-ups were performed at the end of the 6 month.

Bone mineral density measurement

BMD of the lomber spine (L1-4) and femoral area were measured at baseline by DXA using a Hologic QDR 4,500 C densitometer (Hologic Inc., Bedford, MA). The diagnosis of OP was based on the World Health Organization (WHO) criteria that define a T score of −2.5 standard deviation (SD) of the normal healthy young adult values or less as OP [15]. A T-score was considered as normal if it is −1.0 SD or above.

Biochemical studies

The venous blood samples of all healthy volunteers and patients who had taken 6 months of treatment were obtained between 08:30 and 09:30 following a 12-hour fast and were centrifuged within 30 min to be then stored at −80°C until the day of parameter analysis. The major aldehyde units of lipid peroxidation were measured as the MDA level in all serum samples [16–18]. Antioxidant enzyme activity was measured by the method based on the removal of superoxide with the SOD enzyme and coloring

the remaining amount as defined by Sun et al. in 1988 [19], where xanthine creates O_{2^-} with xanthine oxidase and this forms a colored component with nitroblue tetrazolium (NBT), which is then measured spectrophotometrically regarding color intensity. The other antioxidant enzyme activity was measured by the method based on the reduction of GPx and hydrogen peroxide (H_2O_2) and the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to NADP with glutathione oxidase and the measurement of the decrease in optical density of the reaction content [20, 21]. Routine biochemical analyses [including glucose, calcium, phosphorus, alkaline phosphatase (ALP), renal, and liver function tests], erythrocyte sedimentation rate, complete blood count, thyroid function tests, and C-reactive protein (CRP) were also assessed in all subjects at baseline. All of the biochemical measurements were performed also at the end of the sixth month of follow-up.

Statistical analysis

The number of volunteers required for each group to obtain 80% statistical power and 5% significance level was determined as 22 following power analysis. Statistical evaluations were performed with the "SPSS for Windows 15.0" package software. Normal distribution of data was determined with the Shapiro–Wilk test. Non-parametric tests that were independent of the distribution were used for statistical analysis as some of the obtained data did not show normal distribution. The Mann–Whitney *U* test was used for the inter-group evaluation of independent variables and the Wilcoxon matched pairs test for the pre-treatment and six-month post-treatment evaluations. A *P* value of 0.05 or less was considered statistically significant.

Results

There was no difference between the patient and control groups with respect to characteristics such as age, gender, or body mass index (Table 1). There was no difference in total ALP (93 ± 14 and 91 ± 17 U/l), calcium (9.54 ± 0.54 and 9.6 ± 0.51 mg/dl), and phosphorus (3.22 ± 0.61 and 3.12 ± 0.64 mg/dl) levels, respectively, between the patient and control groups and between the pre- and post-treatment levels (92 ± 15 U/l, 9.55 ± 0.50 mg/dl and 3.21 ± 0.58 mg/dl for total ALP, calcium and phosphorus, respectively) in patient group. The mean *T* scores of L1-4 and femur neck were -0.10 ± 0.52 and -0.17 ± 0.49 for the control group, while it was -3.01 ± 0.54 and -3.25 ± 0.56 for the PMO group, respectively ($P < 0.001$ for both comparisons).

Table 1 Demographic characteristics of subjects

	Control Mean ± SD	PMO Mean ± SD	*P*
Age (year)	62.24 ± 7.60	67.60 ± 8.50	0.106
Height (cm)	156.00 ± 5.42	158.00 ± 6.30	0.342
Weight (kg)	68.47 ± 10.65	66.87 ± 8.74	0.288
BMI (kg/m^2)	28.45 ± 4.42	29.21 ± 4.13	0.466
Menopause duration (years)	13.58 ± 9.58	12.44 ± 8.54	0.178

PMO Postmenopausal osteoporosis, *SD* Standard deviation, *BMI* Body mass index, *cm* centimeter, *kg* kilogram, *m* meter

The pre-treatment MDA and SOD levels showed no difference when compared to the control group, while GPx values were lower in the PMO patient group than the control group ($P = 0.014$). Comparison of the pre- and post-treatment data for the PMO patient group showed an increase in GPx ($P = 0.028$) and MDA ($P = 0.040$) values, while the SOD level had decreased ($P = 0.006$) (Table 2).

Discussion

The data obtained in this study indicate that untreated PMO patients may show alterations in the enzymatic antioxidant systems that catalyze various reactions. The use of bisphosphonate-group pharmacological agents in these patients increases lipid peroxidation while an insufficiency or increase may be seen in various steps of the enzymatic antioxidant systems.

The first product to be formed with the reduction of molecular oxygen (O_2) in biological systems is the superoxide (O_{2-}) radical. The reduction of this radical by receiving two electrons from membranes creates the long-surviving oxidant that can easily pass through membranes, H_2O_2. H_2O_2 is not actually a radical but is a harmful oxygen component as it creates the hydroxyl radical (OH) [22, 23]. The oxidative catabolism of polyunsaturated fatty acids is known as lipid peroxidation and causes irreversible cell and tissue damage while being self-sustaining as a chain reaction [24]. The activity of enzymatic antioxidants (SOD, CAT, and GPx) depends on the radical synthesis and breakdown rate and the dietary intake of trace elements (Mn, Se, Fe, Zn, and Cu).

An increase in free radicals causes a stronger antioxidative defense to develop in normal tissues against low-level oxidative stress. Significant and long-lasting oxidative stress causes degeneration and damage to cellular components [25]. We did not find a difference in MDA levels and SOD enzyme activity that would be expected to work against the superoxide radical in the antioxidant system when we compared PMO patients to the control

Table 2 MDA, GPX, and SOD levels

	Control		PMO			
			Before treatment		After treatment	
	Median (min–max)	IQR (25–75%)	Median (min–max)	IQR (25–75%)	Median (min–max)	IQR (25–75%)
MDA (nmol/ml)	1.27 (0.74–2.95)	0.99–1.56	1.03[a] (0.15–1.78)	0.80–1.65	1.9 (1.18–2.28)	1.78–2.09
GPX (U/ml)[b]	172.5 (143–183)	155.8–176.3	135*[a] (131–161)	132–157	158 (145–184)	149–179.9
SOD (U/ml)	6.50 (3.90–9.92)	5.37–8.25	7.1[a] (2.54–10.16)	4.1–8.3	3.20 (0.38–6.60)	2.07–4.71

PMO Postmenopausal osteoporosis, *MDA* Malondialdehyde, *GPX* Glutathione peroxidase, *SOD* Superoxide dismutase, nmol: nanomole, *ml* milliliter, *U* unit, *min* Minimum, *max* Maximum, *IQR* Interquartile range

*$P < 0.05$; when compared with the control group

[a] when compared to the levels after the treatment

[b] nanomole/oxidized nicotinamide adenine dinucleotide phosphate/minute/milliliter

group in our study, while the GPx enzyme activity was lower. These results indicate a slightly increased level of oxidative stress in these patients.

Glutathione peroxidase is an intracellular enzyme that protects membrane lipids and hemoglobin against oxidative damage by causing the reduction of organic hydroxyperoxides and H_2O_2. Decreased GPx activity leads to increased H_2O_2 and significant cellular injury [26, 27]. The increased GPx levels in the PMO patient group compared to the control group after treatment, although these values were lower previously, and the opposite situation for SOD activity may be explained by the common catalytic pathway [26]. GPx and SOD are complementary enzymes. The H_2O_2 produced by one is rendered harmless by the other. GPx is effective at low concentrations of H_2O_2, while the CAT enzyme activity becomes more important at high concentrations [28]. We found decreased SOD levels and increased MDA levels following treatment with risedronate for 6 months in our PMO patients. It is believed that this is caused by an attempt by the antioxidant system to balance the increased superoxide radicals and the increased lipid peroxidation product MDA with excessive hydrogen peroxide production. Another study has shown that risedronate, clodronate, and pyrophosphate inhibit microsomal lipid peroxidation in vitro [29]. A study comparing three-month calcitonin, risedronate, and raloxifene treatment in PMO patients found CAT and GPx levels to be lower in the pre-treatment group than in the control group while MDA and nitric oxide (NO) levels were higher. The erythrocyte MDA levels were shown to decrease with all drugs following treatment while risedronate decreased NO levels and raloxifene decreased CAT levels [30].

Another study evaluated patients in two groups as those with PMO or osteopenia and found GPx activity to be higher in the osteopenic group than the PMO or control group. It has been thought that this may be the result of the defense developed against excessive release of free radicals. This study also showed that calcium was not effective on the GPx, CAT, and SOD activities that were studied [31].

Badr et al. [32] found low vitamin C and SOD levels and high MDA levels in PMO patients. However, they chose osteoporotic patients with fractures as the patient group in their study. It is therefore possible that these patients had a higher level of oxidative stress than our patient group.

An in vitro study has shown that oxidative stress has a negative effect on osteoblastic differentiation by inhibiting differentiation markers in osteoblastic bone cells. This effect has been postulated to be mediated by the atherogenic lipids such as "minimally oxidized low-density lipoproteins" that are present in the vessel wall and bones and thought to play a role in vascular calcification and osteoporosis. However, it has not yet been possible to demonstrate this in vitro hypothesis under in vivo conditions [33].

One must not forget when studying antioxidant-oxidant systems that a weakening of the antioxidant defense system in the patient groups may be due to increased free radical production but also to inadequate antioxidant intake with the diet. Comparison of the pre-treatment patient group to the control group in our study showed low GPx levels without a difference in MDA levels, indicating the formation of other oxidated biological molecules rather than lipid peroxidation products in the control group. However, the possible limitations of the methods (limited sensitivity and specificity) must be taken into account when interpreting MDA measurements [34].

Maggio et al. [35] also found no difference between their control group and PMO patient group regarding lipid peroxidation, thus supporting our results. The increased MDA levels following 6 months of risedronate use in our study may be related to the drug but could also be explained by the differentiation observed in the bone formation-destruction cycle due to the treatment. The inadequacy of antioxidants has a negative effect on the bone mass and cycle. A low intracellular antioxidant level

increases osteoclastogenesis due to uncontrolled ROS formation. Increased N-telopeptide excretion and decreased antioxidant levels in patients bedridden for a long time with resultant increased bone loss have been shown to be associated [36]. A marked decrease in urinary cross-linked N-telopeptides of type I collagen (NTx) levels with risedronate use has previously been shown in PMO patients, and it has been postulated that it could provide earlier data than other methods during treatment monitoring [37]. Similarly, it is possible that comparison of free radical and antioxidant levels with methods such as DXA may lead to earlier information during treatment monitoring, although not practical for daily use.

In conclusion, there may be an insufficiency in different steps of the enzymatic antioxidant systems in patients with PMO without treatment. The six-month use of risedronate resulted in an increment in lipid peroxidation levels and GPx levels. We think that the decrement in SOD levels may be related with the utilized antioxidants due to the increased free radicals and the compensatory increment in the other steps of the antioxidant system.

Conflict of interest All authors declare that they have no conflict of interest.

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