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Effect of Free Radicals and Antioxidants on Postmenopausal Osteoporosis

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In the present work, the effect of free radicals and antioxidant enzymes on postmenopausal osteoporosis and the relationship between free radicals, bone mineral density and estrogen levels in postmenopausal women has been investigated. The study population consisted of 30 osteoporotic female patients and 30 control subjects. Venous blood samples of patients and controls were collected and analyzed for femoral and lumbar bone mineral densities (BMD), nitric oxide (NO), malondialdehyde (MDA) and estrogen levels and glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities. In the osteoporotic group, increased NO and MDA levels and decreased GPx activities and BMD values were observed. No significant differences in estrogen levels and SOD activities were obtained between control and osteoporotic group. Although significant negative correlations between the lumbar BMD and MDA ($r = -0.300$; $p = 0.040$) and lumbar BMD and NO ($r = -0.303$; $p = 0.038$) levels were observed, a significant positive correlation between the lumbar BMD and GPx ($r = 0.382$; $p = 0.008$) was observed. There was no correlation between the estrogen levels, BMD, free radicals and antioxidants. Based on our findings it may be suggested that the balance between oxidant and antioxidant defense mechanisms is impaired in postmenopausal osteoporosis and the negative correlation between BMD and NO and MDA values might demonstrate that free radicals cause increase of osteoclastic activity.

Key Words: Osteoporosis; Free oxygen radicals; Anti- oxidants; Estrogen.

INTRODUCTION

Free radicals are produced as a consequence of redox reactions. They include hydroxyl radicals (•OH), superoxide anion radical ($•O_2^-$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) and lead to the specific oxidation of some enzymes, protein oxidation and degradation. The biological effects of free radicals are controlled by antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase[1-3]. Antioxidants are compounds that hinder the oxidative processes and thereby delay or prevent oxidative stress. Under normal conditions the rate and magnitude of oxidant formation is balanced by the rate of oxidant elimination. However, an imbalance between prooxidants and antioxidants results in oxidative stress, which is the pathogenic outcome of oxidant overproduction that

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overwhelms the cellular antioxidant capacity. Oxidative stress has been related to cardiovascular disease, cancer and other chronic diseases that account for a major portion of deaths[1, 4, 5]. Alone or in combination with primary ethiological factors, free radicals are involved in the pathogenesis of more than a hundred diseases.

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration and increase of fracture risk. However, estrogen deficiency is a major etiological factor for the postmenopausal osteoporosis in women while the ethiopathogenesis of osteoporosis is still unknown[6]. Recently, it was suggested that free radicals and oxidative stress have effect on bone mineral density and bone metabolism[7–9]. Nitric oxide is the most investigated and known free radical in osteoporosis[10–12]. The effect of nitric oxide on bone is related to proinflammatory cytokines, estrogen and mechanical strain[13–16]. NO metabolites decrease in postmenopausal period and increased by estrogen replacement therapy[17, 18]. But there is no sufficient evidence about the effect of other free radicals and antioxidant status, such as GPx and SOD on bone mineral density and estrogen level.

Recently, our team has focussed on the effect of some free radicals and antioxidants in human and rat osteoporosis[19]. In this study, we investigated the effect of free radicals and antioxidant enzymes on postmenopausal osteoporosis and the relationship between investigated parameters in postmenopausal women.

EXPERIMENTAL

The study consisted of 30 women (patient group) with postmenopausal osteoporosis and 30 healthy volunteer postmenopausal women (control group) with similar age, weight and height. The women with secondary osteoporosis, hypogonadism, diabetes mellitus, renal disease, hepatic disease, thyroid- parathyroid function disorders, inflammatory disease and taking any medication that affects bone metabolism were excluded from the study. All subjects were informed about the consent of the protocol prior to the study. All *in vivo* studies were conducted upon the approval of the Ethical Committee for Clinical Studies at the Mersin University Hospital.

Bone mineral density (BMD) was measured at the lumbar vertebrae (L2-4) and femoral neck region using dual energy X-ray absorptiometry (DEXA) (Norland XR 46). T score values < -2.5 was accepted as osteoporosis indicator based on the criteria set by WHO[20].

Fasting venous blood samples were collected into test tubes and were centrifuged within 30 min at $1500 \times g$ for 5 min. Serum was separated and analyzed for enzymatic activity of alkaline phosphatase, SOD, GPx and malondialdehyde (MDA) and NO levels on the same day. Serum was stored frozen at –20°C for further analysis. Clinical examinations, complete blood cell counts, routine biochemical tests, serum calcium and phosphorus levels, thyroid function tests, parathormone, sex hormones, prolactin, calcium excretion in 24 hour-age urine, vitamin D_3, erythrocyte sedimentation rates, C reactive protein and rheumatoid factor levels were determined in both control and patient groups. Height and weight of the patients were measured and body mass index (kg/m^2) was calculated before the study. Osteocalcine and C telopeptide (CTx) levels were measured in all subjects for the evaluation of bone metabolism. Serum osteocalcine levels were

measured by using radioimmunassay (RIA-Wallac Gama Counter) and serum C telopeptide levels were measured by electrochemiluminescence (Elecsysis 2010, Roche Diagnostic).

MDA measurement method was carried out spectrophotometrically. The absorbance of the pink colour produced upon the formation of thiobarbituric acid-malondialdehyde complex was measured[21].

The oxidized end products of NO (nitrite and nitrate) were analyzed by a photometric endpoint determination (nitrite/nitrate, colorimetric method; catalogue no. 1-746-081, Roche Diagnostics GmbH, Mannheim, Germany). Nitrate was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase. The resultant nitrite reacted with sulphanilamide and N-(1-naphthyl)-ethylene-diamine dihydrochloride producing red-violet diazo dye. The absorbance of the diazo dye was measured spectrophotometrically at 540 nm.

SOD and GPx enzyme activities were measured according to the methods of Sun *et al.*[22] and Paglia *et al.*, respectively[23]. Activities of all enzymes (except SOD) were expressed as international units. One unit of SOD activity was defined as the amount of the enzyme causing 50% inhibition in the nitroblue tetrazolium reduction rate and the results were expressed as unit/mL. The GPx activity method was carried out based on the reduction in the absorbance at 340 nm due to the consumption of NADPH.

Student t-test and Man Whitney U-test were performed for the statistical analysis (SPSS Version 10.0). Correlations were evaluated by using the Pearson's correlation coefficient. For statistical analysis, p was set at 0.05 throughout the paper.

RESULTS AND DISCUSSION

The effect of free radicals and antioxidant enzymes on bone mineral density and estrogen levels in postmenopausal women was invesigated. Venous blood samples of both patients and healthy volunteers were collected and analyzed for femoral and lumbar BMD, NO, MDA, estrogen, GPx and SOD levels. The characteristic findings and biochemical data of the patient and control groups were presented in Table-1.

The relationship between the free radicals and osteoporosis has not been known clearly to date. There have been a few studies reported on this issue. In early studies, Garrett *et al.*[7] showed the effect of free radicals on osteoclastogenesis and bone resorption in rodents *in vitro*. Later, Mody *et al.*[9] demonstrated that oxidative stress enhances differentiation of bone cells. It has been well-known that NO is one of the most abundant free radicals in the body and there is evidence that NO modulates bone remodelling and bone loss *in vitro* and *in vivo*. It has been demonstrated that bone cells express NOS (nitric oxide synthase) enzymes and can produce NO. Studies clearly indicate that NO plays an important role as paracrine and autocrine mediator of bone cells in response to diverse stimuli such as proinflammatory cytokines, mechanical strain and sex hormones[10]. NO has biphasic effect on bone. Low concentration of NO potentiates IL-1 induced bone resorption whereas high concentration of NO inhibits osteoclast formation and activity[24].

TABLE-1
CHARACTERISTIC FINDINGS OF PATIENT AND CONTROL GROUPS[a, b]

	x ± SD		*p*
	Control group	Patient group	
Age (years)	55.1 ± 6.01	56.17 ± 5.82	0.261
BMI (kg/m^2)	27.62 ± 3.36	26.13 ± 3.51	0.356
Lumbar BMD (g/cm^2)	1.032 ± 0.12	0.768 ± 0.01	0.000
Femur neck BMD (g/cm^2)	0.892 ± 0.01	0.750 ± 0.01	0.000
Estrogen	13.12 ± 6.53	11.56 ± 5.96	0.460
NO (µmol/L)	18.74 ± 4.87	25.91 ± 5.67	0.000
MDA (µmol/L)	6.48 ± 4.74	11.26 ± 8.47	0.027
SOD (U/mL)	2.99 ± 2.01	4.01 ± 1.98	0.086
GPx (U/mL)	3.00 ± 2.23	0.99 ± C.98	0.000
Osteocalcine (ng/mL)	27.17 ± 5.89	26.15 ± 6.21	0.608
CTx (ng/mL)	0.259 ± 0.002	0.309 ± 0.11	0.135

[a]BMI: Body mass index, BMD: Bone mineral density, NO: Nitric oxide, MDA: Malondialdehyde, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CTx : C-telopeptid.
[b]N = 30.

Bone loss is associated with diminished estrogen levels, increased osteoclastic activity and bone resorption in postmenopausal women[9, 25]. Nitric oxide may modulate estrogen's anabolic effect on bone homeostasis by restraining the osteoclast mediated bone resorption and the stimulation of osteoblast activity. Wimalawansa and co-workers have shown that transdermal nitroglycerine (NG) prevented bone loss in a rat model for estrogen depletion induced osteopenia and women after ovariectomy[26-28]. Hukkanen *et al.*[29] suggested that the effects of estrogen on bone metabolism may be, at least in part, NO dependent and, reversely, organic nitrates such as NG may be beneficial in preventing excessive loss of bone in postmenopausal women. Furthermore, Cuzzocrea *et al.*[30] found increased plasma levels of NO in ovariectomized mice and it was suggested that there was a relationship between the estrogen and NO levels. In the present study, NO level was significantly higher in osteoporotic patients than controls ($p < 0.05$); but there was no correlation between the NO and estrogen level in osteoporotic patients. The reason of increase in the NO levels in osteoporosis may be found from specific pathophysiological findings.

Studies investigating the antioxidant status in patients with postmenopausal osteoporosis are very limited. Sontakke and Tare[25] found that MDA level increased and SOD and GPx activities decreased in osteoporosis. In addition, Maggio *et al.*[31] reported that MDA levels do not change in elderly osteoporotic women while SOD and GPx levels are depressed and the same authors suggested that the antioxidant defense markedly decreases in osteoporotic women. In present study, it was found that MDA level significantly increased whereas glutathione peroxidase activity decreased in osteoporotic patients as compared with control

group ($p < 0.05$). GPx levels showed a significant negative correlation with SOD and NO levels ($p = 0.033$, $r = -0.303$; $p = 0.015$, $r = -0.341$, respectively) and a positive correlation with osteocalcine levels ($p = 0.044$, $r = 0.316$). Although SOD activity values were higher than those of controls, the difference was not statistically significant. This insignificant change in SOD activity may show that this enzyme does not play an important role in female osteoporosis.

The increase in MDA level, as a marker of endogenous lipid peroxidation, may be due to the fact that the antioxidant enzymes cannot properly balance an overproduction of reactive oxygen species. Reduction in GPx activity as observed in this study may be due to the increased consumption resulting from an increase in the levels of free radicals such as hydrogen peroxide. A further reduction in patients with osteoporosis may be due to higher magnitude of oxidative stress in these subjects.

The studies investigating the effect of estrogen on antioxidant enzymes are limited and contradictory. Strehlow *et al.*[32] demonstrated that 17 β-estradiol increased manganese (MnSOD) and extracellular superoxide dismutase (ecSOD) transcription rate. Half-life of MnSOD mRNA was not influenced whereas ecSOD mRNA was stabilized by estrogen. Copper-zinc SOD, glutathione-peroxidase and catalase were not affected by estrogen[32]. Azevedo *et al.*[33] showed that estrogen did not affect catalase activity but reduced SOD and GPx activities. Oge *et al.*[34] demonstrated that estradiol had no significant effect on SOD and catalase activities after ovariectomy. In our study, no correlation among the estrogen level, SOD and GPx activities was obtained.

The investigation of the relationship between BMD and NO, MDA, GPx levels, significant negative correlations between the lumbar BMD and MDA ($r = -0.300$; $p = 0.040$) and lumbar BMD and NO levels ($r = -0.303$; $p = 0.038$) were determined in our study. On the contrary, a significant positive correlation between the lumbar BMD and GPx ($r = 0.382$; $p = 0.008$) was observed. BMD values in the patients were lower than those of controls. These results suggest that free radicals have effect on bone metobolism and enhance osteoclastic bone resorption.

In conclusion, the data of this study demonstrate that the pro-oxidant/anti-oxidant balance was in favor of prooxidants and oxidative stress is increased as evidenced by increased levels of serum MDA, NO and decreased level of GPx.

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