THE PLASMA ANTIOXIDANT ACTIVITY OF SUPEROXIDE DISMUTASE ENZYME IN OSTEOPOROSIS

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Abstract- Osteoporosis is a metabolic disease characterized by reduction in bone density and susceptibility to deformity and fracture. Some studies show that osteoblasts can create inter-cellular free radicals that lead to cellular death. Superoxide dismutase (SOD) plays an essential role in cell defense against reactive oxygen metabolites. The purpose of this study was to measure the plasma SOD activities in Iranian women with osteoporosis compared to the control group. SOD activity was measured spectrophotometrically at 540 nm in 192 women. Plasma activity of SOD (mean ± SD) was 1.72 ± 0.79 μg protein in the control group, 2.05 ± 0.87 μg protein in patients as a whole [(mild osteopenia + severe osteopenia and osteoporosis) (T-score < -1)] and 2.32 ± 0.91 μg protein in patients with severe osteopenia and osteoporosis (T-score < -1.7). In this study, that plasma activity of SOD was significantly higher in patients than in controls. Furthermore, this difference was more prominent between the controls and patients with severe disease (T-score < -1.7) than patients as a whole. T-score of femur adjusted for age and body mass index (BMI) showed negative significant correlation with plasma activity of SOD.

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INTRODUCTION

Osteoporosis is a metabolic disease causing reduction in bone density and susceptibility to deformity and fracture. It is a silent and prevalent disease affecting 50% of Iranian women and men over 50. According to 1991 WHO report, after heart-failure, brain-failure and cancer, osteoporoses is the fourth factor threatening human's health (1).

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Osteoporosis is more prevalent in western countries, especially in white women. The risk of osteoporotic fracture in white women's lifetime is 30%, compared to 9 percent risk for breast cancer. Probably the reason of high prevalence of osteoporosis in these women is the insufficient bone concentration in this race. The risk of osteoporotic fracture in black people is less than Indian and Japanese women. Apart from race, sexual hormones are the most important factors to determine the bone mass in women (2).

Reduction of bone density is the result of imbalance between bone formation and destruction, which depends to different factors, the most important one is increase in age. By reaching the age

of around 80, about 30% of the bone mass have been lost. Reduced level of physical activity in adults is another important factor (3). Smoking; low-weight; use of alcohol and caffeine; and low calcium, protein and vitamin D intake are other causes of osteoporosis (2).

Concerning treatment of osteoporosis, we are unable to revert back the osteoporoses to its original form; it is just possible to prevent deterioration. For relief the pain, the patient can use analgesic drugs. Heat, massage and rest are other options (3). Using calcium, estrogen, calcitonin and vitamin D has been recommended too.

Recently, some limited studies concerning role of antioxidants in osteoporosis have been done and results show that there is a correlation between antioxidants and osteoporosis (4). Free radicals have an important role in many diseases such as diabetes, degenerative disorders and cancer (4). In normal conditions, there is a balance between free radicals and antioxidants defensive system; sometimes this balance is lost which is called oxidative stress. Oxidative stress has been center of attention in recent studies of osteoporosis pathogenesis (4). Some studies show that osteoblasts can create inter-cellular free radicals that lead to cellular death.

Antioxidant is a chemical that in low concentration prevents oxidation and production of oxygen free radical species. There are some antioxidants that change the oxygen free radicals to some compounds that are less harmful. These include superoxide dismutase (SOD), catalase and glutathione peroxidase enzymes (5). There are three forms of superoxide dismutase in human; SOD_1 in cytoplasm, SOD_2 in mitochondria and SOD_3 is outer-cellular space. SOD_1 activity has a role in some disease including Down syndrome, gingival inflammation and bronchopulmonary dysplasia.

The purpose of this study was to measure the plasma SOD activity in Iranian women with osteoporosis and comparing it to the control group. This research will find out the relation between antioxidant superoxide dismutase enzyme and osteoporosis in a part of Iranian society.

MATERIALS AND METHODS

In this study, subjects were screened among a total of approximately 1000 women referred to Bone Mineral Densitometry Division of Jami Clinic in Tehran, Iran. The main exclusion criteria that could interact with interpretation of the results were secondary osteoporosis, diseases caused by oxidative stress, malnutrition, hormone replacement therapy and use of antioxidant vitamins and antiresorptive drugs. Accordingly, 220 women were selected and asked to participate in the study and finally 200 women gave their information and enrolled. We obtained informed consent from all participants. The project was approved by Ethics Committee of Medical Sciences/University of Tehran.

The participants were divided into three groups; 1) the control group (T-score ≥ -1) including 76 women (39.5%), 2) the total patients [(mild osteopenia + severe osteopenia and osteoporosis) (T-score < -1)] including 76 women (39.5%) and 3) women with femur T-score < -1.7, which were considered as severe osteopenia and osteoporosis and including 51 (26%) participants.

The questionnaire included demographic variables [self reported age, body mass index (BMI), history of diseases, nutritional status, smoking habit, functional status and disabilities, self reported fractures, and use of medicines]. The questionnaire was administered by a trained interviewer. All subjects were on free diet.

T-scores of the femoral neck and lumbar spine were measured for subjects, using dual energy X-ray absorptiometry (QDR 4500^R, Holcic, Acclaim^R series).

The study subjects underwent a fasting blood withdrawal in 10 ml heparinized tubes on the day of the bone densitometry, and after centrifugation, the plasma was distributed into special vials. The vials were temporarily stored in liquid nitrogen, and then transferred to Faculty of Pharmacy, Medical Sciences/University of Tehran for analysis. The chemicals and reagents were purchased from the Merck (Darmstadt, Germany) and Fluka (Steinheim, Germany) companies and of the purity required for this SOD test and all the solutions were prepared with distilled water. The pH of the solutions was

measured with a model 713-pH meter (Metrohm, Heisau, Switzerland).

SOD activity was determined spectrophotometrically at 540 nm (6), (UV visible spectrophotometer, GBC Cintra 40, Victoria, Australia). The principle of the method described is based on the enzymatic generation of superoxide by a microbial NADH diaphorase and the detection of O^-_2 by oxidation of hydroxylamine, which is measured by a colorimetric reaction. The incubation and color reaction procedures are shown in Table 1 and 2, respectively. In this method, total SOD, KCN-sensitive and KCN-insensitive SOD were measured. The catalytic activity of the enzyme is defined by the reduction of the color formation by 50%.

Statistical analysis was performed using SPSS. The data are expressed as the mean ± SD or as percentage. Descriptive statistics were conducted on all the variables to evaluate range, the variance, frequencies and normality of the obtained data. Demographic and clinical variables were compared by the X^2 test. Correlation analysis was carried out by means of the Spearman test. Analysis of covariance was performed to compare femoral T-score as well as plasma activity of SOD among the groups, with age and BMI as covariates. Statistical significance was defined as $P < 0.05$.

RESULTS

Eight out of 200 enrolled subjects were excluded from the study because of missing adequate data; so total number of subjects was 192.

In present study, the T-scores of both lumbar spine (L1-L4) and femoral neck were measured in all participants. Three groups were compared according the values suggested by the WHO's Division for T-score and osteoporosis. Participants with normal femur and spine T-score (T-score ≥ -1) were considered as control group (76 women). Participants with T-score < -1 were considered as patient [(mild osteopenia + severe osteopenia and osteoporosis), 76 women], and group with femur T-score < -1.7 were considered as having severe osteopenia and osteoporosis which included 51 participants.

We found no differences in the number of diseases, drugs and functional activities between these groups, but the differences were significant for age and BMI ($P < 0.05$).

Plasma SOD activity were compared between the controls and the patients. SOD activity and femur T-scores were entered into multivariate models as continuous data and then adjusted for age and BMI. Plasma activity of total SOD, KCN-sensitive and KCN-insensitive SOD in control and patients groups are shown in Table 3.

Table 1. Incubation procedure*

Pipette successively into 10 ml test tubes:	Incubation blank ml	Maximal absorbance (0% inhibition ml)	Sample ml	Concentration in assay mixture
Water	0.70	0.60/0.5	0.50/0.40	-
Buffer	1.00	1.00	1.00	Phosphate 100 mmol/l
Air saturated hydroxylamine	0.10	0.10	0.10	Hydroxylamine 0.5 mmol/l
AQ	0.10	0.10	0.10	AQ 0.2 mmol/l
Diaphorase	-	0.10	0.10	Diaphorase 750 U/ll
KCN	-	0.10	0.10	KCN 1.5 mmol/l
		Mix by shaking		
Sample or	-	-	0.10	Dil. 1+1 to 1+99 *e.g.* volume fraction 0.05
SOD resp.	-	-	0.10	SOD 125-25 units/l
NADH	0.10	0.10	0.10	NADH 10 mmol/l

Mix by shaking, incubate for 15 min at room temperature (apporx. 22° C). 0.5 ml aliquots are used for color reaction

* For the determination of CN^- insensitive SOD maintain the correct total volume by adding 0.40 ml water instead of 0.50 ml.

Table 2. Color reaction procedure

Pipette successively into 10 ml test tubes:	Reagent blank	Calibration standards	Sample
Sulphanilamide	0.5 ml	0.5 ml	0.5 ml
Buffer	1.0 ml	0.5 ml	0.5 ml
Incubation mixture	-	-	0.5 ml
Naphtyl ethylenediamine	0.5 ml	0.5 ml	0.5 ml
Shake; after 20 min read absorbance at 540 nm against reagent blank			

None of the interactive factors (age, BMI) had statistically significant associations with the plasma SOD activity (though r values were negative for both). BMI was associated with femur mineral density (r = +0.388, $P < 0.01$), while age was inversely associated with femur mineral density (r = -0.31, $P < 0.01$). It is worth saying that there were more plasma SOD activities in the smokers than in the non smokers. Smoking habit was not associated with femur T-score but femur mineral density was lower in the smokers compared to the non smokers.

By adjustment for BMI and age, T-score was directly examined with the plasma activity of SOD. It revealed that there was no significant relation between plasma activity of SOD and femur T-score in the all participants (r = -0.083, $P = 0.405$), in controls (r = +0.153, $P = 0.337$) or in patients with severe osteopenia and osteoporosis (r = -0.169, $P = 0.389$), but it was significant for the patients as a whole (r = -0.389, $P = 0.012$).

DISCUSSION

In this study, the plasma levels of SOD activity among Iranian osteoporotic women showed that plasma activity of SOD was significantly higher in the patients than in the controls. Furthermore, this difference was more significant between the controls and patients with severe disease (T-score < -1.7) than in the total patients. T-score of femur adjusted with age and BMI showed negative significant correlation with plasma activity of SOD in patients as a whole. After adjustment of plasma activity of SOD for BMI and age, no significant relation was observed between the plasma activity of SOD and femur T-score in all participants, the control group and severe osteopenia patients, but it was reverse and significant for the total patients.

Some investigations have indicated that osteoporosis is associated with biochemical markers of oxidative stress, such as urinary excretion of isoprostanes and plasma antioxidants (7-11). Maggio *et al.* reported that exogenic and endogenic plasma antioxidants level, such as SOD in osteoporotic patients, are less than control group (11). Study of antioxidant enzymes in synovial fluid of the patients with primary and secondary osteoarthritis showed that glutathione reductase and other antioxidant synovial activities were higher in the patients than in the controls; also the difference was more between the controls and the secondary osteoarthritis patients (12). Ozcogmen reported that SOD activity in patients with postmenopausal osteoporosis is more than the control (13).

Antioxidant enzymes increase with oxidative stress and exercise training, too. However, the increase in antioxidant defenses might not be physiologically proportionate to the needs created by the increase in prooxidant events and thus might

Table 3. The plasma SOD activity*

Group	N	Total SOD (μg protein)	Cyanide sensitive SOD (μg protein)	Cyanide insensitive SOD (μg protein)
Control (T-score > -1)	76	1.72 ± 0.79	1.04 ± 0.57	0.68 ± 0.52
Total patient (T-score < -1)	76	2.05 ± 0.87	1.20 ± 0.66	0.84 ± 0.60
Sever osteopenia (T-score < -1.7)	51	2.32 ± 0.91	1.28 ± 0.76	1.04 ± 0.60
Osteoporosis (T-score < -2.5)	14	2.83 ± 0.26	1.55 ± 0.43	1.27 ± 0.49

*Data are given as mean ± SD.

affect the requirements for dietary antioxidants (14). On the other hand, Wolf *et al.*, by investigating more than 10,000 women between ages of 50 and 80 years, found that total plasma antioxidant enzymes such as glutathione peroxidase and SOD in the osteoporotic women is not lower than the non osteoporotic women (15).

Cellular-molecular happenings in osteoporosis with upsetting of balance between osteoblasts and osteoclasts activities cause increase in the production of free radicals in bone cells and intracellular space. Thus bone formation decreases due to the increase in bone loss because of inhibitory effects of free radicals on osteoblastic differentiation.

It seems that a decrease in the amount of antioxidants does not always occur in oxidative stress disease, and sometimes it will increase by physiological ways; for example, sports and physical activities increase the amount of antioxidants, malondialdehyde, creatinine kinase and uric acid in 4 days. In heart attack, the amount of vitamin C and SOD was the same in patient and control groups, but in the patients these amounts was more in red globulin than control group (16, 17).

Kimura *et al.* determined serum extracellular SOD (EC-SOD) concentrations in 222 patients with type II diabetes and 75 healthy control subjects (18). The serum EC-SOD concentration was significantly higher in patients with type 2 diabetes (99.3 ± 1.3 ng/ml) compared with the control subjects (68.4 ± 2.3 ng/ml). Stepwise multiple regression analysis of the data from the diabetic common phenotype group showed a significant relationship between serum EC-SOD concentration and duration of diabetes. So a strong relationship between the serum concentration of EC-SOD and the severity of both micro and macrovascular diabetic complications was observed. These findings suggest that serum EC-SOD concentration levels may be a marker of vascular injury possibly reflecting hyperglycemia-induct oxidative injury to the vascular endothelium and decreased binding of EC-SOD to the vascular wall (18).

The patients with neuroblastoma exhibited a transient increase in Mn-SOD following chemotherapy, but after 1 week the levels decreased markedly to the control levels (19). Mn-SOD was intensely stained in bone marrow cells of patient, whose cancer cells had moved into the bone marrow. High levels of Mn-SOD were also found in cultured human neuroblastoma cells (19).

In present study, significant association between the plasma activity of SOD and bone marrow density was observed; and mean plasma activity of SOD in the patients was higher than in the control group. We also observed increasing amount of plasma antioxidant in the smokers compared to the non-smokers. According to the different studies, it seems that oxidative stress can cause osteoporosis and a physiologic increase in the amount of antioxidants; even though this amount may be not sufficient for the human body desires.

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Conflict of interests

The authors declare that they have no competing interests.

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