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Original article

Evaluation of Antioxidants in Bone Mineral Density of Iranian Osteoporotic Women

[1]Mohammad Reza Oveisi, [1]Naficeh Sadeghi, *[2]Behrooz Jannat, [1]Mannan Hajimahmoodi, [3]Molouk Hadjibabaie, [4]Abdolazim Behfar,

Abstract

Objective(s)
Bone is a dynamic tissue that is continuously renewed throughout life by the process of bone remodeling. Antioxidant system might be involved in the pathogenesis of bone loss, so the aim of this study was to evaluate the total antioxidant capacity (TAC), vitamin C and vitamin E levels of plasma besides measuring enzymatic antioxidants, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) enzymes activity in Iranian osteoporotic women comparing to the control group.

Materials and Methods
Bone mineral density (BMD) of the femoral neck and lumbar spine was measured by dual x-ray absorptiometry. The participants were divided into groups: a) total participants ($-3.9 \leq$ T–score ≤ 3.6) including 192 women, b) the control group (T-score ≥ -1) including 76 women, c) the total patients (T-score < -1) including 76 women. Then, plasma TAC, vitamin C levels, SOD and GR activities, erythrocyte CAT were measured using spectrophotometrical methods separately, and for vitamin E by HPLC analysis.

Results
Comparing the control group and osteoporotic women showed that: a) plasma levels for vitamin C and erythrocyte CAT were markedly lower in the patients than in the controls, but plasma activity of TAC, SOD and GR were significantly higher, respectively. b) the differences were higher between control and patients with severe disease (T-score <-1.7) comparing to patients in the group with milder disease ($-1.7 \leq$ T-score <-1). c) Femoral neck BMD adjusted with age and BMI showed a positive and significant correlation with plasma levels of vitamin C in all subjects, but this relation was reverse or negative for TAC, SOD and GR.

Conclusion
It seems that a physiologic increase in the amount of some antioxidants occurs in osteoporosis; even though this amount may not be sufficient for the human body requirements.

Keywords: Antioxidants, Blood, Bone density, Osteoporosis, Women

1- Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
2- Food and Drug Laboratory Research Center, Ministry of Health and Medical Education, Tehran, Iran
3- Pharmacotherapy Department, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
4- Drug and Food Control Department, Faculty of Pharmacy, Jondishapoor University of Medical Sciences, Ahvaz, Iran
*Corresponding author: Tel: +98-21-66954713; Fax: +98-21-66954707; email: janatbhr@sina.tums.ac.ir

Introduction

Bone is a dynamic tissue that is continuously renewed throughout life by the process of bone remodeling, which involves the coupled events of removal of old bone by osteoclasts and formation of new bone by osteoblasts (1-3). Disturbances in the bone remodeling process can lead to metabolic bone diseases. Osteoporosis, a silent epidemy, is a major metabolic bone disease characterized by low bone mass and microarchitectural deterioration of bone tissue, causing enhanced bone fragility and increased risk of fracture. It is affecting 50% of Iranian women and men over 50 years. According to 1991 WHO report, following heart failure, brain-failure, and cancer, osteoporosis is the fourth factor threatening human's health (4). Some of the risk factors for osteoporosis are race, sex, age, genetics, previous fractures, chronic inactivity, microgravity, excessive sports activity, low body weight, low lifetime calcium intake, hormones, medication, oxidative stress-related factors, smoking, low antioxidant status, excessive lipid intake and nutrition deficiency (5, 6).

The weakening of antioxidant defense or excessive production of reactive oxygen species (ROS) or other free radicals results in oxidative stress (7). It may also result from normal metabolic activity or environmental factors such as diet. Free radicals have an important role in many diseases such as diabetes, degenerative disorders and cancer through lipid peroxidation of membranes, protein cross linkage, mitochondrial, and DNA damage (1).

The major antioxidant defense systems in the body are superoxide dismutase, glutathione-S-transferases(GSTs), glutathione-S peroxidase (GPX), and catalase (8). Antioxidants in the diet, notably vitamin C, vitamin E, ß-carotene, zinc, selenium, lycopene, and polyphenols provide additional defense against oxidative stress. The stress biomarkers have been surveyed in different tissues for assessing age related diseases such as osteoporosis. Therefore, the use of biomarkers to identify patients with oxidative stress may be helpful in managing osteoporosis (9).

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 (1). Epidemiological studies suggest that certain antioxidants (e.g. vitamin C, E, and ß-carotene) may reduce the risk of osteoporosis (6, 10, 11). Recent *in vitro* studies or animal models showed that oxidative stress has an important impact on osteoclast differentiation and function. ROS and antioxidant system might be involved in the pathogenesis of bone loss. In one study, it is demonstrated that oxidative stress markers are important indicators for bone loss in postmenopausal women (12). It is investigated that oxidative stress induced in differentiated cells by a number of different oxidative stimuli and oxidative stress may contribute to the pathology of a number of bone diseases, such as osteoporosis (13).

The aim of this study was to evaluate the total antioxidant capacity (TAC), vitamin C (acid ascorbic), and vitamin E levels of plasma and also to measure enzymatic antioxidants, superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) enzymes activity in Iranian osteoporotic women comparing to the control group. This research will find out the relation between plasma antioxidants status and bone mineral density (BMD) in a part of Iranian society.

Materials and Methods

Participants

In this cross-sectional study, participants were screened and selected among a total of approximately 1000 women that were referred to bone mineral densitometery divison of Jami Clinic in Tehran, Iran, between April to December 2006. Exclusion criteria included secondary osteoporosis, diseases of oxidative stress (diabetes, renal or hepatic insufficiency, cardio and cerebrovascular diseases, dementia and inflammatory diseases), malnutrition, hormone replacement therapy, use of antioxidant vitamins, and antiresorptive drugs. Finally, 192 women gave their information and enrolled. The project was approved by Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran).

On the basis of WHO criteria, the participants were divided into four groups: a) the control group with T-score≥ -1 including 76 women (39.5%), b) the total patients (mild osteopenia + severe osteopenia and osteoporosis) with T-score < -1 including 76 women (39.5%), c) women with -1.7 ≤ T-score < -1 as mild osteopenia (25 women), and finally d) participants with T-score < -1.7 which were considered as severe osteopenia +osteoporosis and including 51 (26%) participants.

Questionnaires included demographic variables (self reported age, body mass index (BMI)), past medical history, nutritional status, smoking habit, functional status and disabilities, self reported fractures and usage of medicines. All participants were on a free diet.

Methods

BMD of the femoral neck and lumbar spine were measured by dual x-ray absorptiometry (QDR 4500R, Holcic, AcclaimR.series). The participants underwent a fasting blood withdrawal in 10-ml heparinized tubes on the day of the bone scan and after centrifugation; erythrocytes (washed 3 times with isotonic NaCl) and plasma were divided into special vials, then temporarily were stored in liquid nitrogen and transferred to faculty of pharmacy, Tehran Universtiy of Medical Sciences, and were kept at -70 ˚C for analysis. The activity of plasma total antioxidants capacity (TAC) was determined by FRAP method. FRAP is a direct method that has been used to examine the entire power of antioxidants in a sample (14).

Plasma vitamin E was measured, after extraction with methanol, by HPLC with UV detection at 280 nm. Methanol, deionized water, and butanol (90:4:6) were used as a mobile phase with a C_8 column. The flow rate was 1.0 ml. min^{-1} and the acetate ester of vitamin E was used as an internal standard (15).

FRASC, a spectrophotometric assay, is an acceptable alternative to HPLC method for measurement of plasma ascorbic acid (16).

Catalase activity was determined according to Aebi method (17) spectrophotometrically at wavelength 240 nm.

SOD activity was determined spectrophotometrically at 540 nm (18), (UV visible spectrophotometer, GBC Cintra 40, Victoria, Australia). The principle of the described method 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. Superoxide dismutase enzyme based on inactivation by cyanide (CN^-) individes to KCN-sensitive (CNsen SOD) and KCN-nonsensitive SOD (CNnonsen SOD). The catalytic activity of the enzyme is defined by the reduction of the color formation by 50%.

GR activity was determined spectrophotometrically at 339 nm (19). In this method, generally, imidazole as buffer (pH; 6.9), EDTA, oxidized glutathione(GSSG) and the sample (plasma) were mixed thoroughly. Five minutes later, NADPH was added, mixed thoroughly, and the absorbance was recorded immediately (less than 5 seconds) with a spectrophotometer.

Statistical analysis

Statistical analysis was performed using SPSS software version 12. Data are expressed as the mean±SD or as percentage. Descriptive statistics were conducted on all variables to evaluate range, variance, frequencies and normality of data. Demographic and clinical variables were compared using the t- test and X^2 test. Correlation analysis was carried out by means of the Pearson correlation. Analysis of covariance was performed to compare femoral T-score as well as plasma levels of vitamin C between groups, with age and BMI as covariates. Statistical significance was defined as $P< 0.05$.

Results

WHO (World Health Organization) has considered T-score ≥ -1 as normal, -1.7 ≤ T-score <-1 as mild osteopenia, -2.5≤ T-score <-1.7 as severe osteopenia, and T-score < -2.5 as osteoporosis. The T-score of both lumbar spine (L_1-L_4) and femoral neck were measured in all participants.

Control group was selected based on both femur and spine T-scores, so control participants, 76 (39.6%), were normal in both lumbar spine (L_1-L_4) and femoral neck

(T-score ≥ -1). Only femur T-score was considered as criterion in selection of patient group, so 76 subjects (39.6%) with T-score < -1 were grouped as total patient (TP). TP were divided into mild patients (M), -1.7 ≤ T-score < -1, and severe patients (S), T-score< -1.7 (Table 1). 40 persons (20.8%) entered neither patient group, because of femoral T-score ≥ -1, nor control because of spinal T-score < -1. The demographic and clinical characteristics of groups are shown in Table 1.

All participants had normal plasma albumin level due to sufficient protein intake and good nutrition status. No difference in menopause, smoking, nutritional habit, drugs and functional activities was found between groups, but the difference was significant for age and BMI ($P< 0.05$).

The total antioxidant capacity (TAC), ascorbic acid and vitamin E levels of plasma besides measure of enzymatic antioxidants, superoxide dismutase (SOD), catalase, and glutathione reductase enzymes activity were compared between control and patient groups. Furthermore, Pearson correlation was used to examine the associations of total antioxidant capacity (TAC), ascorbic acid and vitamin E levels of plasma besides superoxide dismutase (SOD), catalase and glutathione reductase enzymes activity with BMD in all participants. In this way, the above mentioned 40 participants were included in the study, too.

All of the data (total antioxidant capacity (TAC), ascorbic acid and vitamin E levels of plasma besides superoxide dismutase (SOD), catalase and glutathione reductase enzymes activity) and femur Tscore were entered into partial correlation model as continuous data with adjustment and controlling for age and BMI.

None of these interactive factors (age and BMI) had statistically significant associations with plasma antioxidants levels. BMI was associated with femur mineral density (r= +0.388, $P< 0.01$), but age was inversely associated with femur mineral density (r= -0.310, $P< 0.01$).

Mean±SD levels for antioxidants in control group (T-score ≥ -1), TP (T-score <-1), M patient group (-1.7 ≤ T-score < -1) and S patient group (T-score <-1.7) are shown in Table 2.

T-score was directly examined with plasma levels of antioxidants. The results are shown in Table 3. Therefore, in this way, all participants (n= 192) were included in the study. It is worth saying that in all participants (no attention to control or patient group), there were some differences in antioxidants levels between smokers and non-smokers (Table 4), also pre- and post- menopausal women (Table 5). Femur mineral density were lower in smokers than non-smokers ($P< 0.05$, Table 4) and post- menopausal than pre–menopausal women, too ($P< 0.01$, Table 5).

Table1. Descriptive characteristics (age, BMI, smoking, number of children, menopause condition and femur T-score) of the study participants (control, total, mild, and severe patients).

	Controls	Total patients (TP)	Mild patients (M)	Severe patients (S)
Femoral T-score	> -1	≤ -1	-1.7< & ≤-1	≤ -1.7
Number of Participants	76	76	25	51
Age (yr)	47.62±10.43	55.24±11.19$^{\alpha}$	49.64±8.23	57.98±11.49$^{\alpha}$
BMI (Kg/m^2)	27.64±4.53	25.46±3.40$^{\alpha}$	26.29±3.55	25.05±3.28$^{\alpha}$
Number of smokers	5	13	5	8
Number of children	2±2	3±2	3±3	3±2
Number of menopause	21	38	5	33

$^{\alpha}$ $P< 0.001$

Table 2. Antioxidants levels of Plasma [total antioxidant capacity (TAC), vit E, vit C, total Superoxide dismutase (SOD), cyanide non sensitive SOD, cyanide sensitive SOD, glutathione reductase (GR)] and erythrocyte[catalase (CAT)] for the study participants (control, total, mild, and severe patients).

	Severe patients (S)	Mild patients (M)	Total patients (TP)	Controls
Femoral T-score (BMD)	≤ -1.7	-1.7< & ≤-1	≤ -1	> -1
Number of participants	51	25	76	76
Plasma TAC (total antioxidant capacity) (μ M)	972.20±232.04 [a]	878.52±176.85	941.93±218.86 [a]	851.65±262.41
Plasma vit E (μg/ml)	8.23±2.09	8.58±2.33	8.34±2.15	8.38±2.38
Plasma vit C (μ M)	47.31 ± 36.07 [c]	70.28 ± 61.58	54.73 ± 46.65 [a]	74.55 ± 67.60
Plasma total SOD (μg protein)	2.31±0.91 [c]	1.50±0.40	2.05±0.87 [b]	1.72±0.76
Plasma CNnonsen SOD (μg protein)	1.28±0.76 [a]	1.05±0.33	1.20±0.66	1.04±0.57
Plasma CNsen SOD (μg protein)	1.04±0.60 [c]	0.44±0.34 [a]	0.84±0.60	0.68±0.52
Plasma GR (U/L)	90.01±58.57 [c]	66.72±19.80	82.35±50.33 [b]	64.71±31.26
Erythrocyte CAT (k/gHb)	363.00±55.78 [b]	269.66±53.03	369.62±63.10 [a]	390.62±60.78

[a] $P< 0.05$, [b] $P< 0.01$, [c] $P< 0.001$

Table 3. Correlation between antioxidants (total antioxidant capacity (TAC), vit E, vit C, total superoxide dismutase (SOD), cyanide non sensitive SOD, cyanide sensitive SOD, glutathione reductase (GR), catalase (CAT) levels and T- score values for total participants (n= 192, -3.9 ≤Femoral neck T–score≤3.6).

Antioxidants	Femoral neck T–score	
	P	Correlation (r)
Plasma TAC (μ M)	<0.01	-0.195
Plasma Vit E (μg/ml)	0.36	+0.079
Plasma vit C (μ M)	<0.01	+0.192
Plasma total SOD (μg protein)	<0.001	-0.216
Plasma CNnonsen SOD (μg protein)	<0.05	-0.162
Plasma CNsen SOD (μg protein)	<0.05	-0.160
Plasma GR (U/L)	<0.001	-0.278
Erythrocyte CAT (k/gHb)	0.16	+0.100

Table 4. Antioxidants levels of Plasma [total antioxidant capacity (TAC), vit E, vit C, total superoxide dismutase (SOD), cyanide non sensitive SOD, cyanide sensitive SOD, glutathione reductase (GR)] and erythrocyte [catalase (CAT)] and femoral neck T-score values for total smokers and non–smokers (n= 192, -3.9 ≤ Femoral neck T–score≤ 3.6).

	Non–smokers	Smokers
Number of Participants	172	20
Femoral T–score (BMD)	-0.62±1.32	-1.22±1.07 [a]
Plasma TAC (μ M)	897.73±252.39	928.47±225.07
Plasma Vit E (μg/ml)	8.67±2.40	8.11±2.40
Plasma vit C (μ M)	62.73±56.73	53.74±26.58
Plasma total SOD (μg protein)	1.82±0.87	2.32±1.11[a]
Plasma CNnonsen SOD (μg protein)	1.09±0.57	1.35±0.96
Plasma CNsen SOD (μg protein)	0.73±0.59	0.97±0.59
Plasma GR (U/L)	72.71±42.13	59.58±25.58 [a]
Erythrocyte CAT (k/gHb)	265.88±43.18	268.18±40.49

[a] $P< 0.05$

Table 5. Plasma or erythrocyte antioxidants (total antioxidant capacity (TAC), vit E, vit C, total superoxide dismutase (SOD), cyanide non sensitive SOD, cyanide sensitive SOD, glutathione reductase (GR), catalase (CAT)) levels and femoral neck T-score values for total pre- and post - menopausal women (n= 192, -3.9 ≤Femoral neck T–score≤3.6).

	pre- menopausal	post - menopausal
Number of Participants	109	83
Femoral Tscore (BMD)	-0.44±1.12	-0.98±1.45[b]
Plasma TAC (μ M)	823.81±223.72	998.64±247.49[c]
Plasma Vit E (μg/ml)	8.11±2.15	9.29±2.56[b]
Plasma vit C (μ M)	69.80±66.94	50.82±26.41[a]
Plasma total SOD (μg protein)	1.82±0.85	1.95±0.99
Plasma CNnonsen SOD (μg protein)	1.09±0.64	1.16±0.62
Plasma CNsen SOD (μg protein)	0.73±0.55	0.79±0.65
Plasma GR (U/L)	64.26±28.35	80.64±51.75 [a]
Erythrocyte CAT (k/gHb)	268.17±44.17	263.42±41.06

[a] $P< 0.05$, [b] $P< 0.01$, [c] $P< 0.001$

Discussion

In this study, the plasma levels for the total antioxidant capacity (TAC), vitamin C and vitamin E besides measuring of enzymatic antioxidants, superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) enzymes activity comparing between control and Iranian osteoporotic women showed that: a) plasma levels for vitamin C and erythrocyte CAT were markedly lower in the patients than the controls, but plasma activity of TAC, SOD and GR were significantly higher, respectively. b) Furthermore, these differences were higher between control and S patients with severe disease (T-score <-1.7), than M patients group (-1.7 ≤ T-score <-1) with milder disease. c) femural neck BMD, T-score for femur adjusted with age and BMI, showed a positive and significant correlation with plasma levels of vitamin C in all subjects (r= +0.192, $P< 0.01$), but this relation was reverse or negative for TAC, SOD, CNnonsen SOD, CNsen SOD and GR .

Epidemiological studies suggest that certain antioxidants (e.g. vitamin C, E, and ß-carotene) may reduce the risk of osteoporosis (10, 11) and counteract the adverse effects of oxidative stress on bone. Some investigations have indicated that osteoporosis is associated with biochemical markers of oxidative stress, such as H_2O_2, superoxide and urinary excretion of isoprostanes, 8-iso-prostaglandin F alpha, lactic acid (20, 21), and also plasma antioxidants (22-24). The hydrogen peroxide (H_2O_2) that is produced by endothelial cells, increases osteoclastic formation, activity and bone resorption (25-27). H_2O_2 also modulates intracellular calcium (Ca^{2+}) activity in osteoblasts by increasing Ca^{2+} release from the intracellular Ca^{2+} stores (28). Removal of the H_2O_2 from cells by catalase provides protection against oxidative damage to the cell and stimulates the mineralized bone nodule formation. Superoxide has been localized both intracellularly and at the osteoclast-bone interface using nitroblue tetrazolium (NBT), which is reduced to purple-coloured formazan by ROS, suggesting the participation of superoxides in bone resorption. Osteoclastic superoxide is produced by NADPH oxidase (29). Glutathione reductase (GR) belongs to a family of flavin-containing pyridine nucleotide disulphide oxidoreductases and the major function of these homologous dimeric proteins are to catalyze the conversion of GSSG to the reduced glutathione (GSH) using NADPH as a coenzyme. Accordingly, GR plays a key role in antioxidant capacity of the cells through maintaining high ratio of GSH/GSSG (30). Ascorbic acid is a potent antioxidant and protects body against free radicals (31), indeed it is known as the terminal small-molecule antioxidant (32). It is necessary in collagen biosynthesis and is the main protein of bone matrix (33-35). Other mechanisms may be related to the role of ascorbic acid in osteoblastic growth or in promoting calcium absorption.

Maggio *et al* reported that exogenic and endogenic plasma antioxidants level, such as SOD in osteoporotic patients, are lower than

control group (24). Studying the role 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 higher between the controls and the secondary osteoarthritis patients (36). Ozgocmen reported that SOD activity in patients with postmenopausal osteoporosis is higher than the control (12). 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 affect the requirements for dietary antioxidants (37).

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 are not lower than the non osteoporotic women (38).

These studies present mixed results and it is difficult to explain differences and interpretations, because studies varied according to the exposure, outcomes, the site, and the various confusing factors. It seems that antioxidants reduction does not always occur in oxidative stress diseases, and sometimes it is increased by physiological ways, for example, sports and physical activities increase the amount of antioxidants, malondialdehyde, creatinine kinase, and uric acid in four days. In heart attack, the amount of vitamin C and SOD was equal in patient and control groups, but in the patients these amounts were higher in red globulin than control group (39, 40). Kimura *et al* determined serum extracellular SOD (EC-SOD) concentrations in 222 patients with type II diabetes and 75 healthy control subjects. The serum EC-SOD concentration was significantly higher in patients with type 2 diabetes compared with the control subjects. Those findings suggest that serum EC-SOD concentration levels may be a marker for vascular injury possibly reflecting hyperglycemia-induct oxidative injury to the vascular endothelium and decreased binding of EC-SOD to the vascular wall (41).

In current study, a significant association between the plasma levels of vitamin E and BMD was not observed. However, mean plasma levels of vitamin E in S patients were higher than the control group. It seems that more extensive study with larger sample size might supply definite results about this association. However, in the present study, significant association between TAC, vitamin C besides enzymatic antioxidants, SOD, CAT, GR activity, and bone mineral density were observed. Mean plasma activity of TAC, SOD, GR in the patients were higher than in the control group, though it was lower for vitamin C and CAT. We also observed increasing amount of plasma antioxidants in the smokers compared to the nonsmokers.

Some limitations of our study should be acknowledged. Sample size was not large in the study, and there were some large variations in the results. Furthermore, the lack of data about antioxidants levels before osteoporosis onset hinders the possibility of ascertaining whether low antioxidants are a reason or a consequence of osteoporosis. Though it was asked about nutrition, but there was no information about amount of food antioxidants intake that can make some interactions with the results. On the other hand, in this study, age was different between a group, which was handled by adjusting and matching in the statistical analysis.

According to this study, it seems that a physiologic increase in the amount of some antioxidants occurs in osteoporosis even though this amount may not be sufficient for the human body desires and needs. On the other hand, vitamin C and catalase decrease make it possible to depict a relation between osteoporosis occurrence and antioxidants deficiency, but it is too early to suggest that eating antioxidant products prevents osteoporosis. It would be a healthy practice to include antioxidant containing food in the diet, prevention or treatment of oxidative stress-related chronic diseases, including osteoporosis. The final results of our study may indicate that some antioxidants may be useful either as a dietary alternative to drug therapy or as a complement to drugs used in women at risk of osteoporosis.

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