Antioxidant status in patients with osteoporosis: A controlled study

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Abstract

Objective: We aimed to investigate serum antioxidant enzymes and nitric oxide (NO) levels in postmenopausal women with osteoporosis (OP) and in healthy controls; and to determine the relationship between these enzymes, NO and clinical parameters in this present study.

Methods: Forty-five postmenopausal women fulfilling OP diagnostic criteria of World Health Organization (WHO) and 42 postmenopausal healthy women without OP were enrolled. Patients in the study population were selected among individuals that were not pre-diagnosed or pre-treated for OP. Patients with metabolic bone diseases, fracture history, which were smokers, alcohol users and taking antioxidant drug treatment, were excluded from the study. Dual Energy X-ray Absorptiometry (DXA) results, body mass indices and demographic data were recorded. Erythrocyte catalases (CAT), glutathione reductase (GR) enzyme activities and erythrocyte glutathione (GSH) levels, plasma malondialdehyde (MDA) levels were measured by spectrophotometer whereas plasma nitrite-nitrate (NOx) levels were measured by ELISA microplate-reader.

Results: Patients had significantly lower GR (P< 0.01) enzyme activity and higher levels of MDA (P< 0.01) and NO (P< 0.01) than non osteoporotic healthy controls. There was no significant difference between both groups in erythrocyte GSH levels and CAT activities. Total femoral BMD measurements significantly correlated with MDA levels (P = 0.001). There was no significant relationship between other antioxidants and lumbar or femoral BMD.

Conclusion: Oxidative stress may play an important role in postmenopausal bone loss and therefore it might be considered when pathogenesis of postmenopausal OP has been investigated.

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Keywords: Osteoporosis; Antioxidant; Nitric oxide

1. Introduction

Osteoporosis (OP), which is characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased bone fragility, can result in an increased risk of fractures. The pathogenesis of OP is poorly understood. Aging and genetic factors are important, moreover, many factors such as smoking, immobilization, inefficient calcium intake, thyroid and parathyroid function disorders, gastrointestinal and kidney diseases cause OP [1,2]. In the literature, limited numbers of previous reports have shown the relationship between bone mineral density (BMD) and oxidative stress. Antioxidant systems play important roles in the development of OP [3–10]. On the other hand, some authors as Wolf et al. have noted that serum concentrations of antioxidants were not related to BMD and supportive intake of antioxidants had no effect on BMD [11]. Additionally, role of nitric oxide (NO), which has a biphasic effect on bone, in the pathogenesis of OP, is still being debated [12–17]. As a result of these studies, catabolic process on bone cells is believed to be accelerated by increased serum NO, which has arisen from serum antioxidant enzyme deficiency.

In order to clarify these conflicting results, we aimed to define and match the levels of serum antioxidants and NO in postmenopausal OP patients and healthy control groups, and make a decision about whether there is relationship of these antioxidants and NO with clinical parameters or not.

2. Methods

Forty-five postmenopausal women fulfilling OP diagnostic criteria of World Health Organization (WHO) [18] and 42 postmenopausal healthy women without OP were enrolled in this
study. Patients in the study population were selected among individuals that were not pre-diagnosed or pre-treated for OP. Patients with metabolic bone diseases, fracture history, and who were smokers, taking alcohol and antioxidant drug treatment were excluded from the study. Demographic data of study population were recorded. The study protocol was approved by the local ethics committee of our institution and all participants gave written informed consents. BMD of patients was measured by Dual Energy X-ray Absorptiometry (DXA).

2.1. BMD measurements

BMD was measured at the lumbar spine (L2-4) and femoral area by DXA using a LUNAR DPX densitometer (GE Lunar Corporation, Madison, WI, USA). The diagnosis of OP was based on the WHO criteria [18]. So OP is defined as a T score of −2.5 or less, indicating a BMD that is at least 2.5 S.D.s less than the mean for young adults.

In order to investigate the presence of compression fractures, thoracic and the lumbar spine radiographs were performed.

2.2. Biochemical measurements

For laboratory investigations, following 12 hours of fasting, blood samples of all patients were collected at eight o’clock in the morning. Blood samples were collected into tubes containing sodium citrate as anticoagulant in the early morning after an all night fasting and were separated immediately by centrifugation at 3000 rpm 10 min at +4 °C. The plasma samples were frozen at −80 °C until for nitrite + nitrate (NOx) and MDA assays. The buffy coat on the erythrocyte sediment was separated carefully after the plasma was removed. The erythrocyte sediment washed three times with 0.9% NaCl solution to remove leftover leukocytes and plasma components. After each procedure, erythrocyte-saline mixture was centrifuged at 3000 rpm 10 min at +4 °C. Erythrocyte sediments were treated with 4-fold ice-cold deionized water to obtain stock hemolysate containing ∼5 g hb/100 ml for the erythrocyte CAT and Glutathione reductase (GR) activity assays. Hemolysate hemoglobin content was determined using Drabkin reagent.

CAT activities were measured in a fresh suspension of hemolysates. 1:1000 dilution of this concentrated homolysate was prepared with phosphate buffer immediately before the assay CAT activity was determined by the method of Aebi. The principle of the assay is based on the determination of the rate constant (s−1, k) of hydrogen peroxide decomposition by catalase enzyme. The rate constant was calculated from the following formula: 

\[ k = \frac{2.3}{\Delta A} \log(A_1/A_2) \]

GR detection was performed according to Carlberg and Mannervik, modified by Kazim Husain [19,20]. GR activity of samples was calculated by observing absorbance change in first and third minutes at 340 nm by Shimadzu UV 160 A spectrophotometer.

MDA concentration was measured in terms of thiobarbituric acid reactive substances, spectrophotometrically by the method of Yoshioka T and the values expressed as μmoles of malondialdehyde (MDA) formed per liter plasma [21]. Direct quantitative measurement of NO level in the biological samples is very difficult, because it is a very labile molecule. In aqueous solution, NO reacts with molecular oxygen and accumulates in the plasma as nitrite (NO2−) and nitrate (NO3−) ions. Therefore, NOx, stable oxidation end products of NO, can be readily measured in biological fluids and have been used in vitro and in vivo as indicators of tissue NO production. In this study, the levels of NO metabolites in serum samples were analyzed using a modification of the cadmium-reduction method as described by Navarro-Gonzalves et al. This reaction using pre-treatment of samples to reduce nitrate to nitrite which can be accomplished by catalytic reactions using Cd. The samples were analyzed spectrophotometrically using a microplate reader and quantified automatically against NaNO2 standard curve and the results were expressed as μmol/L. Blood was collected into tubes containing EDTA as anticoagulant in the early morning after an all night fasting for total GSH levels were analyzed using method as described by Teitze [22].

2.3. Statistical analyses

Software Statistical Package Sciences for windows version 14.0 was used for all calculations. For the discrepancies among the groups, Student’s t-test was employed. For correlation between results, the Pearson’s correlation coefficient was performed. Chi-square test was used for comparing categorical variables. The level of significance was set at p value less than 0.05.

3. Results

The mean age of patient group and controls were 55.6 (S.D. = 2.9) years and 56.6 (S.D. = 2.4) years, respectively. There was no significant difference between ages of the both groups (p = 0.085). The demographic characteristics of patients and controls were shown in Table 1.

No compression deformities were encountered in the thoracic and lumbar spine of OP patients.

Plasma MDA and NO levels were significantly higher in study population than those of in the control group (Table 2). However, erythrocyte GR activity was significantly lower in study group than that of the control group, there was no significant difference between the both groups in erythrocyte GSH level and CAT activity.

While there was a significant correlation between plasma MDA levels and femoral BMD in the study population (r = −0.464, P = 0.001), there was no significant correlation

Table 1

<table>
<thead>
<tr>
<th>Demographic data of both groups.</th>
<th>Patients (n = 45)</th>
<th>Controls (n = 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [year, mean (S.D.)]</td>
<td>55.6 (2.9)</td>
<td>56.6 (2.4)</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>45/0</td>
<td>42/0</td>
</tr>
<tr>
<td>BMI [kg/m2, mean (S.D.)]</td>
<td>29.4 (4.4)</td>
<td>27.9 (4.3)</td>
</tr>
<tr>
<td>Menopause age [year, mean (S.D.)]</td>
<td>49.7 (1.5)</td>
<td>50.1 (1.5)</td>
</tr>
</tbody>
</table>

F/M: female/male; BMI: body mass index.
between other antioxidants and lumbar or femoral BMD values.

4. Discussion

Oxidative stress has been demonstrated to play a role in pathogenesis of many diseases such as cardiovascular disease [23,24], cerebrovascular disease [25] and Diabetes Mellitus [26]. Nowadays, studies indicating that formation and resorption of dynamic bone tissue all through life have been affected by an increase in oxidative stress and/or antioxidant enzyme deficiency are increasing in number in the literature.

In this present study, there were significant differences between patients and control group in plasma MDA, NOx levels and erythrocyte GR activities. In the literature, there were a few studies investigating antioxidant enzyme conditions in OP patients [4,5,9,10]. In one of these studies, it is recently reported that serum MDA and NO levels have been increased significantly in study group than those of in the control group, which is in line with the results of our study [9]. Altındag et al. reported results of their study that increased osteoclastic activity and decreased osteoblastic activity may be associated with an imbalance between oxidant and antioxidant status in postmenopausal OP [10]. Similarly, Maggio et al. demonstrated that antioxidant defenses were markedly decreased in osteoporotic females [5]. Sontakke and Tare showed elevated levels of MDA and reduced activities of GSH-Px in osteoporotic groups in comparison to healthy controls [4]. A correlation between oxidative stress and reduced bone density was reported in three different studies other than these four studies [3,7,8]. Furthermore, in previous studies, in which NO effects on bone were investigated, NO was reported to be an important regulator on bone metabolism [12–17].

Reactive oxygen species (ROS) are normal by-products of cellular metabolism. In many joint diseases, proinflammatory factors such as cytokines and prostaglandins are released at sites of inflammation, together with ROS [27]. Enhanced osteoclast activity observed in bone disorders may have been responsible for increased production of ROS in superoxide forms, which is evident by increased levels of serum malondialdehyde (MDA) levels. One of the most damaging effects of ROS is lipid peroxidation, the end product of which is MDA [28]. MDA is one of the most frequently used indicators of lipid peroxidation. MDA is known as a potential biomarker for oxidative stress [29]. Sontakki et al. reported that apart from lipid peroxidation function, MDA had an osteoclastic activity [4]. In this present study, serum MDA levels were higher in the study group than in the control group. Compatible results with our results have been reported in the literature [4,7,9]. Besides, in our study, there was a significant relationship between MDA levels and femoral neck BMD values. Contrary to our results, Ozgocmen et al. reported no significant relationship between MDA levels and neither lumbar nor femoral BMD values [9]. All of these results support that increased serum MDA levels may be used as a biochemical marker indicating osteoclastic activity.

NO is an important biological mediator produced from L-arginine through the enzyme nitric oxide synthase (NOS). It plays an important role in vascular relaxation, regulation of blood flow, immune function, stress, neurotransmission, modulation of nociception, induced vasodilatation and in pain modulation [17]. Previous studies conducted in animal models and in humans have shown that NO is an important regulator of bone metabolism [12–16]. In our study, serum NO level was significantly higher than the values in the control group and there was no significant relationship between NO levels and neither lumbar nor femoral BMDs. However, Yalin et al. found significant correlation between serum NO levels and both lumbar and femur neck BMD [7]. Additionally, Ozgocmen et al. reported no significant relationship between NO levels and lumbar BMD, there was a significant relationship between NO levels and femoral BMD [9]. According to these results, we may state that NO levels regulate the bone mass. But more studies investigating the relation between NO and lumbar and femoral BMD are required.

Glutathione is a compound classified as a tripeptide made of three amino acids: cysteine, glutamic acid and glycine. Glutathione is an antioxidant that protects cells from toxins such as free radicals [30]. Decrease in GSH level is a marker for the degree of oxidative stress [31]. In our study, serum GSH level was lower in the study group than in the control group. However, the difference between these groups was not significant.

Reduction of oxidized GSH is provided by glutathione reduc-tase, which is rather important for keeping reduced GSH at high levels within cells [32]. Avitabile et al. reported that there was a strong relationship between decreased bone mineral density and decreased GR levels. Authors proposed the hypothesis that a decrease in antioxidant enzyme activity, like GR, might cause markedly increased bone demineralization and, as a result, may increase destructive free radical levels [33]. Our findings are also in line with their hypothesis. In contrast to the results of this present study, Sontakke et al. reported that there was no significant difference between GR level in postmenopausal OP patients and individuals in the control group [4]. However, numbers of patients and healthy subjects enrolled into this study were fewer than patients and healthy subjects enrolled into our study. Also, in this study mean age of the control group (20–30 years) was younger than our study. We found significant difference between total GSH levels in OP patients compared with the control group. Low levels of GR were also obtained in OP patients; actually it should not be regarded as an inconsistency. Besides the researchers indicating that total GSH is decreased at oxidative stress, some researchers state an unchanged level of
GSH [34–36]. Annuk et al. reported that oxidative stress occurs at chronic renal injury; oxidized glutathione is increased, reduced GSH is decreased. However, total GSH is found unchanged and even had a tendency to decrease in control group [37]. If we had analyzed oxidized and reduced GSH, we would expect increased levels of oxidized GSH and decreased levels of reduced GSH in OP patients.

Catalase is a common enzyme found in living organisms. Its functions include catalyzing the decomposition of hydrogen peroxide to water and oxygen [38]. Catalase has one of the highest turnover rates of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second. Low level of catalase activity could primarily damage the endoplasmic reticulum in the cells [35]. Although serum CAT activity was lower than that of in the control group, the difference was not significant. CAT values in OP patients in Ozgocmen et al. study were also low, but the difference between the groups was significant [9].

It has been reported in the previous studies that the level of NO and other anti-oxidants showed some daily variations [39–43]. We prevent these daily variations of the level of anti-oxidants by collecting the blood samples of patients in both study and control groups at the same time of the day [43].

The important limitation of the present study is that we could not assess any other bone resorption and formation markers. Further studies investigating relationship between antioxidant enzymes, NO levels and these markers should be performed.

Consequently, we believe that oxidative stress may play an important role in postmenopausal bone loss. Therefore it might be considered when pathogenesis of postmenopausal OP has been investigated.

Conflicts of interest

None of the authors has any conflicts of interest to declare.

References