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Original Research Article

Serum Vitamin D, C, and E Deficiency in Osteoporotic Women: A Case-Control Analysis of Micronutrient Status

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ABSTRACT
Objective: To compare the serum levels of vitamins D, C and E between postmenopausal women with osteoporosis and healthy controls, and to examine the association between the levels of oxidative stress biomarkers and lipid profile.
Materials and Methods: A hospital-based case–control study was done with 73 women with primary osteoporosis and 20 age-matched healthy control women. Venous blood was drawn after 12 hours of fasting for biochemical analysis. The concentrations of 25-hydroxyvitamin D, vitamin C and vitamin E were quantified by enzyme-linked immunosorbent assay (ELISA) and spectrophotometric methods, respectively. One way ANOVA was used for statistical analysis and $p<0.05$ was deemed significant.
Results: Osteoporotic patients exhibited significantly lower serum levels of vitamin D (24.04±6.81 vs. 69.32±7.63 ng/mL), vitamin C (0.33±0.09 vs. 0.57±0.08 mg/dL), and vitamin E (0.21±0.06 vs. 0.29±0.06 mg/mL) compared to controls (all $p<0.001$). The oxidative stress markers, malondialdehyde, nitric oxide, were significantly elevated (3.66±0.81 vs. 1.34±0.30 nmol/mL; 15.25±1.43 vs. 11.28±1.34) and the antioxidant enzymes (SOD, CAT, GSH, GPx) were significantly Depleted (All $P<0.001$).
Conclusion: Vitamins D, C, and E were all severely deficient in the osteoporotic women, and there were an increased oxidative stress and dyslipidaemia in these women. Regularly assessing and supplementing micronutrients and/or using targeted antioxidant treatments could be a crucial complement to osteoporosis treatment.

Keywords: Oxidative Stress, Vitamin D, Vitamin C, Vitamin E, Bone Metabolism, Micronutrient Deficiency, Osteoporosis.

INTRODUCTION

Osteoporosis is a common metabolic bone disease, where there is a gradual loss of bone mineral density (BMD) with progressive deterioration of the microarchitecture of bone, resulting in higher fracture susceptibility of the skeleton [1]. It is a condition that is more common in postmenopausal women and epidemiological studies have shown that around one-third of women over 50 years of age will develop an osteoporotic fracture at some point in their lives [2]. Estrogen withdrawal causes a chain of immunomodulatory and inflammatory effects including an increase in pro-resorptive cytokines like RANKL, TNF-α and IL-6, and a decrease in the production of osteoprotegerin (OPG) [3]. In recent years, the role of various micronutrients in bone remodeling and bone structure has come to light, in addition to oxidative stress [4]. Vitamin D is the single most important micronutrient involved in the regulation of calcium-phosphate metabolism and the mineralization of bone. It exists in two forms, one bio-active, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], which acts as a regulator of intestinal calcium absorption, parathyroid hormone (PTH) secretion, and directly on the differentiation of osteoblasts and maturation of osteoclasts through the action of the vitamin D receptor (VDR) [5]. At the same time, vitamin C (ascorbic acid) is an essential cofactor for hydroxylation of proline and lysine residues required for collagen formation, which is essential for the formation of the organic matrix of bone [6].

Collagen type I accounts for about 90% of the extracellular matrix of the bone and is responsible for providing the tensile strength and for providing a scaffolding upon which subsequent mineral deposition will occur. Vitamin C is not only a structural component but also acts as a powerful antioxidant that neutralizes the reactive oxygen species (ROS) and prevents the damage of RhoA DNA, and apoptosis of osteoblasts [7].

Epidemiological studies have consistently shown that dietary vitamin C intake is positively associated with BMD, and negatively associated with hip fracture incidence, especially in older postmenopausal women [8]. Deficiency affects osteoblast maturation, decreases alkaline phosphatase activity and weakens the trabecular network, which accelerates age bone loss. Vitamin E is a fat-soluble tocopherol and tocotrienol complex which is mainly located in the cell membrane and acts as a lipophilic antioxidant by trapping lipid peroxyl radicals [9].

Lipid peroxidation markers such as malondialdehyde (MDA) are consistently increased in osteoporotic patients, as well as nitric oxide (NO); on the other hand, the levels of key antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione peroxidase (GPx) are significantly decreased [10]. Moreover, the role of dyslipidemia in bone metabolism has been highlighted more by common pathophysiological pathways involving oxidative stress and systemic inflammation. Circulating LDL and triglycerides are oxidized to produce oxidized LDL (ox-LDL) that activates scavenger receptors, leading to an increase in the number of osteoclast precursors, while also inducing endoplasmic reticulum stress in osteoblasts thereby impairing osteoblast function [11]. Simultaneous presence of osteoporosis and cardiovascular risk factors indicate the systemic component of metabolic bone disorders and the need for biochemical profiling of the disease in the integrated form [12]. Although significant progress has been made in elucidating the molecular mechanisms of bone loss, large-scale case–control studies that examine the associations of micronutrient status, oxidative stress markers, and lipid profiles in osteoporotic women are still uncommon, especially in populations from South Asia that have different dietary habits, sun exposure habits, and genetic susceptibility compared to those from Western countries [13].

MATERIALS AND METHODS

This case-control study was carried out at Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore in conjunction with the General Hospital Lahore, Pakistan. The Institutional Ethics Review Committee (IERC) approved the research protocol. All participants gave written informed consent before participating. The study was facilitated from January 2020 to December 2021 and involved cross sectional sampling to ensure that biochemical measurements were made at the same time. In total, 93 patients were included, 73 primary postmenopausal osteoporosis patients (case group) and 20 age matched healthy women (control group). The presence of osteoporosis was determined by dual-energy X-ray absorptiometry (DXA) at the lumbar spine (L1-L4) and femoral neck. The inclusion criteria were postmenopausal for at least 12 consecutive months, age ≥ 40 years, and living in the Punjab province. Exclusion criteria included secondary osteoporosis (hyperparathyroidism, rheumatoid arthritis, chronic kidney disease ≥3, malignancy), use of bone active drugs (bisphosphonate, teriparatide, denosumab, cortical steroids, and hormone replacement therapy) within the last 6 months, and vitamin D, C, and E supplementation in the last 3 months. To avoid confounding of biochemical parameters, participants with active infection, acute metabolic derangements and significant hepatic or renal dysfunction were excluded. The process of gathering information on human body measurements and human population characteristics. Basic information such as chronological age, medical history, and lifestyle factors was obtained from a standard, structured questionnaire. Anthropometric measures included body weight (measured using a calibrated digital scale to the nearest 0.1kg), and height (measured using a stadiometer to 0.1cm). Body mass index (BMI) was defined as weight in kilograms divided by height in metres squared (kg/m²). According to the Endocrine Society guidelines, subjects were divided into deficiency subgroups: severely deficient < 10 ng/mL, deficient 10–19.9 ng/mL and insufficient 20–29.9 ng/mL. Collecting and handling samples. Venous blood samples were collected after an overnight fast (10-12 hours) from the antecubital vein (5 mL) in sterile, EDTA free vacuum tubes. Samples were left to clot at room temperature for 30 minutes and then centrifuged for 10 minutes at 4°C at 3000 rpm. This serum was then carefully aliquoted into

cryovials and stored at −20°C for biochemical analysis. All assays were conducted within 4 weeks of collection, so as not to degrade the analytes. Pooled serum controls were run with each batch and manufacturer recommendations were followed for calibration procedures to ensure quality control. The level of serum 25-hydroxyvitamin D was estimated using a commercially available competitive enzyme-linked immunosorbent assay (ELISA) kit which was calibrated by international reference standards. After washing, tetramethylbenzidine (TMB) substrate was added, and the intensity of the colorimetric reaction was measured at 450 nm with a reference wavelength of 620 nm. There was an inverse relationship between concentration and optical density. Intra and inter-assay coefficients of variation (CV) were less than 8.2% and 10.5% respectively. In the case of vitamin C, 0.2 mL of serum was added to 1 mL ethanol, 0.2 mL 0.2% bathophenanthroline, and 0.2 mL 0.001 M $FeCl_3$. The mixture was vortexed and incubated for 60 seconds in the dark and then 0.2 mL 0.001 M H_3PO_4 was added. Absorbance reading was done at the wavelength 534 nm. Vitamin E was used according to the same protocol as for the α-tocopherol standards. Calibration curves were prepared using pure standards (1–10 µg/mL) giving linear correlation coefficient (R^2) of >0.985 for both assays. This gave an extracted pink chromophore, which was dissolved in n-butanol to read at 532 nm. Enzymes: Catalase (CAT): Measured by hydrogen peroxide consumption at 240 nm for 60 seconds in phosphate buffer (pH 7.0). Glutathione (GSH): Using Ellman's reagent (DTNB) which reacts with free sulfhydryl groups to form a yellow TNB chromophore which is quantifiable at 412 nm. The coupled enzymatic reactions and nitrite quantification by Griess reagent were used to assess glutathione peroxidase (GPx) and nitric oxide (NO), respectively. Protein normalization of all oxidative stress assays was performed and expressed in a standardized unit. Lipid Profile Analysis: Total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were measured using the enzymatic colourimetric kits (CHOD-POD and GPO-POD). Low-density lipoprotein cholesterol (LDL-C) was computed from the Friedewald equation: LDL = TC − (HDL + TG/5) (where TG levels were <400 mg/dL). After 5 minutes incubation at 37°C, absorbance was measured at 550 nm. The collected data were analyzed and presented in SPSS version 26.0. A two-tailed p-value of < 0.05 was considered statistically significant.

RESULTS

The biochemical and anthropometric profile of 73 osteoporotic women and 20 healthy controls were comprehensively assessed in this case–control study.

Osteoporotic patients were significantly older and heavier than healthy control patients and had a higher BMI. The significant increase in BMI in the osteoporosis group is consistent with the metabolic syndrome phenotype seen in most women with postmenopausal osteoporosis.

Table 1. Anthropometric and Demographic Characteristics of Study Participants

Parameter	Control Group (N=20)	Osteoporosis Group (N=73)	P-Value
Age (years)	38.15 ± 4.52	63.24 ± 8.16	<0.001
Body Weight (kg)	48.65 ± 5.28	72.83 ± 12.94	<0.001
BMI (kg/m²)	21.55 ± 1.57	40.24 ± 7.41	<0.001

Vitamin B12, folate and vitamin D were all found to be significantly low in osteoporotic women. Vitamin D was in a very low range (<30 ng/mL), leading to decreased calcium absorption and regulation of PTH.

Table 2. Serum Micronutrient Concentrations

Analyte	Control Group (Mean ± SD)	Osteoporosis Group (Mean ± SD)	p-Value
Vitamin D (ng/mL)	69.32 ± 7.63	24.04 ± 6.81	<0.001
Vitamin C (mg/dL)	0.57 ± 0.08	0.33 ± 0.09	<0.001
Vitamin E (mg/mL)	0.29 ± 0.06	0.21 ± 0.06	<0.001

High levels of MDA (2.7-fold greater in the osteoporosis group) and NO were indicative of

lipid peroxidation and nitrosative stress respectively, and showed extreme oxidative stress in the osteoporosis group.

Table 3. Oxidative Stress and Antioxidant Enzyme Profiles

Biomarker	Control Group (Mean ± SD)	Osteoporosis Group (Mean ± SD)	P-Value
MDA (nmol/mL)	1.34 ± 0.30	3.66 ± 0.81	<0.001
SOD (nmol/mL)	0.47 ± 0.16	0.11 ± 0.09	<0.001
CAT (nmol/mL)	4.13 ± 0.80	0.72 ± 0.48	<0.001
GSH (mg/dL)	9.88 ± 1.16	2.57 ± 0.73	<0.001
GPx (U/mL)	0.63 ± 0.32	0.20 ± 0.07	<0.001
NO (µmol/L)	11.28 ± 1.34	15.25 ± 1.43	<0.001

Atherogenic dyslipidemia was highly represented in osteoporotic patients, with high levels of TC, TG, LDL-C and low levels of HDL-C.

Table 4. Lipid Profile Assessment

Lipid Fraction	Control Group (Mean ± Sd)	Osteoporosis Group (Mean ± Sd)	P-Value
Total Cholesterol	4.44 ± 0.37 mmol/L	5.23 ± 0.94 mmol/L	<0.001
Triglycerides	1.24 ± 0.15 mmol/L	2.65 ± 0.48 mmol/L	<0.001
LDL-C	2.31 ± 0.15 mmol/L	2.43 ± 0.22 mmol/L	<0.001
HDL-C	1.73 ± 0.17 mmol/L	1.37 ± 0.09 mmol/L	<0.001

Inverse correlations between vitamin D and oxidative damage markers MDA, NO are very strong, suggesting that vitamin D deficiency leads to increased lipid peroxidation and nitrosative stress.

Table 5. Pearson Correlation Analysis between Vitamin D and Oxidative/Antioxidant Markers in Osteoporotic Cohort

Parameter	R-Value	P-Value
Vitamin D vs MDA	−0.512	<0.001
Vitamin D vs SOD	+0.487	<0.001
Vitamin D vs GSH	+0.534	<0.001
Vitamin D vs CAT	+0.441	<0.001
Vitamin D vs NO	−0.398	<0.001

DISCUSSION

In the present case–control study, the authors were able to conclusively demonstrate that high levels of oxidative stress and dyslipidemia are associated with severe deficiencies in vitamins D, C, and E in postmenopausal women with osteoporosis. Our results are in accordance with recent studies which highlight the multifactorial nature of bone loss with the synergistic effect of nutritional deficiency and redox imbalance for the acceleration of skeletal deterioration beyond the effects of estrogen deficiency. The striking difference in 25(OH)D level between the osteoporotic group (24.04±6.81 ng/mL) and the control group (69.32±7.63 ng/mL) highlights an important public health issue, especially in populations who are not exposed to much sunlight and lack vitamin D in their diet.

The importance of vitamin D in skeletal health has been associated with the regulation of osteoblast differentiation through binding to vitamin D receptor (VDR), the inhibition of RANKL-dependent osteoclastogenesis and an improvement in muscle function, which decreases fall and fracture risk [14]. However, recent clinical trials have shown that correction of vitamin D deficiency can have profound effects on the volume of the trabecular bone and the level of secondary hyperparathyroidism, which further emphasizes the basic principle of its importance in the treatment of osteoporosis [15]. Similarly, vitamin E's lipophilic nature makes it a key protective antioxidant with respect to membrane lipid peroxidation. Vitamin E acts as an antioxidant and blocks the activation of NF-κB in the membrane of osteocytes and osteoclasts,

preventing pro-resorptive signaling downstream of NF-κB. The marked decrease of α-tocopherol found in this study is consistent with new findings showing that vitamin E supplementation can prevent bone loss in the murine model of ovariectomy and increase BMD in elderly humans [16]. The increase in MDA along with a reduction in the activity of antioxidant enzymes (SOD, CAT, GSH and GPx) indicates a systemic failure in antioxidant defense mechanism. ROS directly promote osteoclast differentiation by inducing the activation of Src kinase and MAPK pathways, and apoptosis of osteoblasts by releasing cytochrome c and activating caspase pathways [17,18].

Nitric oxide is a physiologic vasodilator but at supra-physiologic levels it generates peroxynitrite radicals which adversely affect bone matrix proteins and inhibit mineralization. In our correlation analysis, we found a strong inverse correlation between vitamin D levels and markers of oxidative damage, which indicated that sufficient vitamin D levels can help to maintain redox homeostasis through regulating the transcription of antioxidant enzymes as well as regulating the release of pro-inflammatory cytokines [19,20]. This synergy of nutrients and antioxidants is a promising therapeutic target for the prevention/reduction of bone loss. Another important finding was the dyslipidaemia, which was characterised by an increase in total cholesterol, triglyceride and LDL-C, and a decrease in HDL-C, in osteoporotic patients. The bone-lipid relationship is becoming more and more understood by the common underlying pathophysiological mechanisms of oxidative stress and systemic inflammation. Oxidized LDL has been shown to bind to scavenger receptors on the osteoclast precursors to stimulate proliferation and resorptive activity, whereas HDL has been shown to have protective effects due to its abilities to promote efflux of cholesterol and to deliver antioxidant enzymes to the bone tissue [21,22]. Estrogen deficiency also worsens this dyslipidemic profile during postmenopause with upregulation of hepatic VLDL production and reduced clearance of LDL receptors. Given the simultaneous presence of osteoporosis and cardiovascular risk factors, a management approach based on a combination of these is required; indeed, several traditional lipid-lowering drugs have been shown to have pleiotropic effects on bone, such as those mediated by the upregulation of BMP-2 and activation of the Wnt pathway, activities demonstrated to be bone anabolic [23]. This study has its weaknesses but there is strong biochemical evidence presented.

Clinically, these results support the need to routinely measure vitamin D, C and E levels in postmenopausal women with or at risk for osteoporosis in addition to lipid and oxidative stress. In addition, the inclusion of antioxidant diets may help reduce bone damage caused by oxidative stress and enhance the resilience of the bone in elderly people.

CONCLUSION

This case–control study showed that serum levels of vitamin D, C and E were significantly lower in osteoporotic women and were strongly correlated with increased levels of oxidative stress markers and atherogenic dyslipidaemia. The simultaneous reduction of the required micronutrients and antioxidant enzymes alters the synthesis of the bone matrix, increases osteoclastic bone resorption and amplifies systemic redox imbalance. Biochemical profiling and targeted nutritional supplementation should be incorporated into standard osteoporosis management, for better skeletal health and fracture risk reduction in the elderly population.

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