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VITAMIN-D DEFICIENCY;

ASSESSMENT OF POTENTIAL RISK FACTOR AND ANTIOXIDATIVE STATUS IN VITAMIN-D DEFICIENT FEMALES.

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ABSTRACT… Background: Deficiency of vitamin D is characterized by the low bone mass which leads to the bone fragility and high risk of fractures. Bone fractures causes the formation of free radicals, generated by the tissue damaged. Uncontrolled production of free radicals accelerates the oxidative stress and increased the bone remodeling process ultimately causes osteoporosis. One of the most damaging effects of free radicals is lipid peroxidation; end product of which is MDA, it also act as major factor in osteoblastic activity. Low level of antioxidative defense system found in osteoporotic patients due to the deficiency of vitamin D. Many important mineral ions removed from bones and risk of bone fragility increases. Current study is aim to check the antioxidative effect produced from excess reactive oxygen species compared with low level of vitamin D which is held responsible for higher or lower activity of bone cells. **Study Design:** Case Control Study. **Setting:** Study was conducted at Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore. **Period of Study:** One year. **Materials and Methods:** Blood samples of 272 post-menopausal osteoporotic women between the age 49-57 were collected from Jinnah hospital Lahore. While the samples of 92 individuals were served as a control. Concentration of both enzymatic and non-enzymatic antioxidant such as CAT, GSH, SOD, GPx and GR, vitamin A, C and E and levels of MDA were estimated spectrophotometrically. While the concentration of IL6, AOPPS, AGEs, TNF-α, MMP9, Isoprostanes, LDH, cholesterol, triglycerides, free fatty acids and phospholipid were measured by using commercially available Elisa kits. **Results:** Blood plasma levels of vitamin D were significantly lower in osteoporosis patients than in normal subjects. In addition, level of stress biomarker such as MDA was found to be higher in patients as compared to control. Due to oxidative stress, level of antioxidants (GSH, CAT, and SOD) was found to be reduced. Blood cells and many other important minerals are also reduces in patient group from their normal amount. **Conclusion:** It concludes that excess production of free radicals over whelms the antioxidative system, thus it may leads to osteoporosis. Further more antioxidant species subjected to body might protect bone loss and also help in acceleration of healing of fractured bones.

Key words: Osteoporosis, vitamin D, CAT, GSH, SOD, GPx and GR, MDA.

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INTRODUCTION

Osteoporosis is the major bone disorder termed as the silent thief. It is the major cause of bone loss due to which bones become brittle and fragile. Excessive decalcification of bones and many other important cellular component leads towards complexities. It is not gender specific, but it mainly affects postmenopausal women due to direct effect of essential hormone called Estrogen. Gradual loss of bones may initially start with no noticeable symptoms until the bones may fracture due to fragility. There are two major cells depending upon function which is osteoblast and osteoclast. Osteoblasts are bone forming cells whereas the osteoclast cells are involved in the removal of old bones. The link between the osteoclast and osteoblast cells regulate the activity of bone through expression of tumor necrosis factor ligand superfamily 11 which is also called as Receptor activator of nuclear factor kappa B ligand (RANKL), and tumor necrosis factor ligand superfamily 11b, called as osteoprotegerin (OPG). RANKL is mostly present in the surface of osteoblasts and stromal cells

and stimulate the activation of the particular receptor. The RANKL that show on osteoclast surface and their precursors promoting the osteoclast formation, activation and suppressing the osteoclastic apoptosis. OBG formed by the action of osteoblast and stromal cells that further bind with RANKL and act as a competitive inhibitor. So maintain balance between RANKL and OPG is necessary which involved in osteoclastic activity of bone resorption.[1]

Osteoprotegerin showed the natural antagonist factor of RANKL. In women, with high level of RANKL is reason of early menopause, the acute phase of estrogen deficiency and it causes the up regulation of bone resorption and it plays role in rapid bone loss. Further more, menopause depends on medical condition in which sex hormone may cease, in addition to this prostate cancer and breast cancer remained associated with RNKL pathway and increase in bone resorption. Many kinds of hormone and inflammatory cytokines activate the osteoclast activity through the RANKL pathway. Several immunological and malignant bone disorder acts as destruction of bone, including rheumatoid arthritis, periodontal disease, and osteocytes bone metastasis.[2]

Previous assessments show that lack of Vitamin D signifies the clinical importance of rickets as well as it because metabolic diseases that raise with respect to age. About sixty percent of old people and 70 to 100 percent of healthy people prevented low level of 25-hydroxyvitamin D and instant increase in ALP and parathyroid hormone of serum.[3] UVB deficiency directly leads to the absence of an enzyme in skin which is 7-dehydrocholestrol, that is major initiator of Vitamin D and reduce risk factor of osteoporosis.[4] Greater complexion of skin occurs due to higher levels of melanin. Recent study revealed that the people of dark complexion need 10-15 times more exposure of sun to produce vitamin D.[5] Condition of atmosphere and time whether provided its day or night effect the concentration of Vitamin D. Bone is a dynamic organ that undergoes continuous remodeling by the coordinated, and balanced, resorption and formation activities of bones cells. Increase rate of bone turn over due to the over expression of RANKL exceeded more bone resorption.[6]

Menopause is a state directly linked to aging. It brings physiological changes in female body. Decrease of steroids sex hormone during menopause in women directly leads to much critical disorder and also affects the normal metabolism of body. The chances of Osteoporosis, cardiovascular diseases, impairment in glucose metabolism and breast cancer increased during menopause. Several kind of trace elements, particularly Ca, Mg, Cu, Mn play vital role in bone remodeling. Estrogen deficiency in postmenopausal women effects the change activity of these trace elements which enhanced the oxidative stress condition and leads to tissue damage after menopause.[7] Gradual loss of bones in menopause condition typically starts between 45-55 years of age. This change in reproductive potential is the direct result of a decline in production of hormones by the ovaries, which causes physical manifestations that negatively impact the quality of life of menopausal women. Release of estrogen mediated by FSH produced from anterior pituitary gland, which in turn mediates the granulose cells of ovary to synthesize estrogen.[8]

Oxidative stress play important role in aging process and as a result excessive production of free radicals such as reactive oxygen species which overcome the body's antioxidant defense system. Loss activity of estrogen in the female reproductive system is highly associated with the critical disorder such as osteoporosis. Oxidative stress results weakening of antioxidant defense or an over excessive formation of ROS in the body. ROS contain one or more unpaired electrons; it is state that makes them highly toxic that fills orbital and stabilizes their electron balance, Many free radicals, including hydroxyl (OH –), superoxide radicals (O2 –), hydrogen peroxide (H2O2), singlet oxygen, and lipid peroxides produced. These type of ROS responsible for damage protein, lipids and DNA that is manifested in many chronic diseases including osteoporosis. Under normal conditions, the cell has ability to fight

against free radicals. A number of endogenous antioxidant present in the body which used to suppress down the function of free radicals. Free radicals stimulate more osteoclastic cells through up regulation of RANKL and inhibiting the activity of osteoblastic cells. More minerals released from the bone cells and ultimately bones become brittle and weak. BMD and age are not the only factors that affect the frequency of fractures, other factors usually linked to the low level of bone mineral density include biochemical indices removal of old bones and clinical factors such as age, preceding fragility fracture, early menopause, family history, and use of oral corticosteroids. Guidelines for finding osteoporosis or those at high risk of increasing the disease, based on an analysis of risk factors, have been proposed by the International Committee for Osteoporosis Clinical Guidelines.[9]

MATERIAL AND METHOD

Blood samples were collected from randomly selected postmenopausal women aged 49 up to 57 years from Punjab-Pakistan. Demographic distribution of data is shown in Table-I. The women with hysterectomy or bilateral oophorectomy, acute infections, diabetes mellitus, diseases of the kidneys, lungs and liver, malignancies, and those consuming hormonal medications in the last three years were excluded from study. The experimental protocol was approved by the Research Ethical Committee of The Institute of molecular biology and biotechnology, The University of Lahore. Five ml of venous blood sample were taken from the anticubital vein of each participant. The sample bottles were centrifuged within one hour of collection, after which the serum were separated and stored at -70°C until assayed.

BIOCHEMICAL ANALYSIS

MDA was measured by spectrophotometric method of Okawaet al.,[10] while SOD was estimated by spectrophotometric method of Kakkaret al.[11] Reduced glutathione was determined by the method of Moron et al.[12] Glutathione peroxidase activity was measured by methods of Leventet al.[13] CAT was measured by spectrophotometric method of Abebi, (1984).[14] Advanced oxidation protein products (AOPPs) were determined according to the method of Witko-Sarsatet al.[15] while grease's reagent are used for the estimation of NO.[16] Fasting Blood Glucose (FBG), GGT, ALB, BUN, HCO_3, Uric Acid and Creatinine were determined by Merck kit method. C-Reactive protein (CRP) was determining by CRP Latex test kit. The levels of tumor necrosis alpha (TNF-α), Lactate dehydrogenase,cholesterol, triglycerides, free fatty acids and phospholipid were determined by using commercial kit of BioAssay Systems.

Age (year)	n
18-34	90
35-49	140
Above 50	42
Gender	**n**
Male	80
Female	192
Ethnicity	**n**
Caucasian	190
Non-caucasian	82
Year of education	**n**
< 12 year	90
>12 year	182
Yearly income	**n**
Under 10,000	100
10,001-30,000	100
More then 30,000	72
Diagnosis of bone loss	**n**
No	82
Yes	190
History of fracture	**n**
No	140
Yes	132
Family history of osteoporosis	**n**
Don't know	60
No	70
Yes	142
Taking Ca+ supplement	**n**
No	82
Yes	190

Table-I. Demographic data distribution in osteoporotic patients

RESULTS

The data presented in Table-II in vitamin-D deficient women represents a clear image regarding the role of elevated oxidative stress in the progression of osteoporosis. Mean age of the subjects was 42.52±15.02 years, with age

range of 49-57 years. Mean BMI was 35.27±6.57 kg/m^2. The majority of postmenopausal women (70%) were overweight and 30% were in the normal category. Mean estradiol concentration in age matched control was 39.33±2.66 differed significantly from study group having mean estradiol concentration 6.61±1.33 pg./ml. Lumbar T-score was -1.93±0.021, mean femoral neck T-score was -0.91±0.013, and mean distal radial T-score was -1.83±0.017. The results of present study shows that plasma concentration of both enzymatic and non-enzymatic anti-oxidants such as SOD, CAT, GPx, GSH, Vit-A, Vit-C, Vit-E and D were lowered significantly in osteoporotic patients (0.09±0.08, 2.21±1.18, 6.62±0.38, 4.23±1.64, 432.16±94.99, 0.36±0.23, 0.24±0.093 and 5.45±1.20) in comparison to the healthy control (0.50±0.13, 3.91±0.80, 8.23±0.68, 9.80±1.23, 613.48±44.45, 0.569±0.087, 0.294±0.050 and 13.17±0.81). Levels of oxidation products such as MDA, AOPPs, AGEs, isoprostanes, NO, lipoperoxides, protein carbonyl and total thiols were elevated significantly in osteoporotic women (3.81±1.13, 1.45±1.09, 2.77±0.29, 385.19±19.21, 57.91±8.93, 41.66±7.67, 5.58±0.99, 0.59±0.15 and 0.37±0.22) in contrast to normal subjects (1.44±0.37, 0.85±0.040, 2.56±0.104, 70.08±11.23, 19.46±1.38, 26.55±4.77 and 2.18±0.92). Hematology profile revealed different concentrations of blood cells in osteoporotic vitamin-D deficient women. Neutrophils, RBCs, lymphocytes, platelets, Hct and Hb found to be reduced in osteoporosis (38.17±8.12,4.30±0.31,9.27±1.63,179.75±9.12, 34.93±3.50, 10.57±1.87) as compared to healthy control (56.11±3.47, 4.64±0.13, 307.86±5.31, 41.26±1.53 and 14.13±0.89) while monocytes and WBC were elevated in diseased patients (7.39±3.75 and 9.27±1.63) relative to normal (3.65±5.24 and 7.58±0.40).As far as the levels of inflammatory markers IL-7, TNF-α and MMP-9 was concerned, increased concentration was observed in osteoporotic patients (31.62±4.46, 6.74±0.84 and 5.63±1.27) as compared to normal subjects (29.97±1.11, 5.65±0.52 and 1.59±0.61) while concentration of homocysteine was increased (9.03±1.98/2.78±0.033) and arginine (40±3.09/65±1.99) is decreased in diseased group. Markers that indicates hepatic and renal functioning were also differed significantly in both groups. Levels of circulating minerals Ca, Mg, Sod, Pot and Cl and metals including Zn, Cu, S Fe and Se were also differed significantly in both groups.

DISCUSSION

Osteoporosis is a major health problem related to bone disorder that affect the hundreds of people but mainly common in postmenopausal women. It creates the significant burden on individual and society. A healthy skeleton is tightly regulated by the balanced activities of bone resorbing osteoclasts and bone forming osteoblast to maintain the normal physiological structure and mineral contents.[17] It has been well reported that there is equality between bone resorption and bone formation. In women, the hormonal changes that occur throughout per menopause and the immediate postmenopausal years stimulate RANKL production (both directly and indirectly), resulting into accelerated bone loss. Level of oxidants has been associated with aging process. Higheractivity of osteoclastic cells causes low estrogen level that result in the elevation of reactive oxygen species formation. There is higher accumulation of oxidative damage to biomolecules which is the major initiating factor of menopause related estrogen decline. Several mechanisms showed that sexual hormone mainly in women estrogen play important role in the bone remodeling process. There is decline in antioxidative system, which is associated to impairment of systemic oxidative balance.[18] According to the data low level of this antioxidant defense system in osteoporotic patients has been observed. It has been detected that High concentrations of ROS can damage osteoblast cells that cause prevention of normal growth and development. There is decrease amount of catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD) in osteoporosis which showed excessive amount of reactive oxygen species with accelerating osteoclastic activity. Present finding suggests marked reduction in the level of GPx which is accountable for more lipid peroxidation and oxidative damage to DNA. This type of disturbance results in inflammation, apoptosis and destruction of the cells.

Variables	Control (n=100) Mean±SD	Patients (n=272) Mean±SD	P≤0.05
MDA (nmol/ml)	1.44±0.037	3.81±1.13	0.000
SOD (IU/ml)	0.50±0.013	0.09±0.008	0.023
GSH (µmol/L)	9.80±1.23	4.23±1.64	0.0325
CAT (IU/L)	3.91±0.80	2.21±0.18	0.0015
Isoprostanes	70.08±11.23	385.19±9.21	0.016
NO (Nitrite/nitrate) (µmol/L)	19.46±1.38	57.91±6.93	0.25
GSH-Px (µmol/ml)	8.23±0.68	6.62±0.38	0.035
Neutrophils %	56.11±3.47	38.17±5.12	0.004
Lymphocytes %	35.49±3.87	25.69±2.367	0.016
Monocytes %	3.65±0.24	7.39±3.75	0.035
RBCs (X10^12/l)	4.64±0.13	4.30±0.31	0.0125
WBCs (X10^9/l)	7.58±0.40	9.27±1.63	0.014
Hb(g/dl)	13.67±0.69	10.41±1.28	0.0235
PLTs (X10^9/l)	307.86±5.31	179.75±9.12	0.025
Hct,%	41.26±1.53	34.93±3.50	0.0014
Hb(g/dl)	14.13±0.89	10.57±1.87	0.016
Creatinine (mg/dl)	0.72±0.03	1.56±0.29	0.0254
GGT IU/L	42.87±6.64	58.07±8.71	0.0032
hs-CRP (mg/dl)	1.04±0.024	1.47±0.301	0.025
IL-7 (pg/ml)	5.65±0.52	6.74±0.84	0.0166
TNF-α (pg/ml)	29.97±1.11	31.62±4.46	0.054
AOPPs (ng/ml)	0.85±0.040	1.45±1.09	0.015
AGEs (ng/ml)	2.56±0.104	2.77±0.29	0.015
TAS (mmol/L)	1.54±0.150	1.38±0.16	0.1658
LDH (U/L)	256.94±7.95	606.09±6.18	0.0154
MMP-9(ng/ml)	1.59±0.061	5.63±1.27	0.0269
Protein carbonyl (IU)	2.18±0.092	5.58±0.99	0.015
Total Thiols (T.SH)	0.37±0.022	0.59±0.15	0.01115
Arginine (µmol/L)	65±1.99	40±3.09	0.0135
Homocysteine(µmol/L)	2.78±0.033	9.03±1.98	0.0165
8-OHDG (ng/ml)	5.98±0.91	11.30±1.78	0.0125
Uric Acid (mg/dl)	41.67±3.78	176.77±4.78	0.0325
Lipoperoxidase	26.55±4.77	41.66±7.67	0.01235
Paraoxonase-1 (PON1) u/ml	77.53±8.98	8.65±1.99	0.0325
Bicarb (mg/L)	28.66±1.59	22.58±4.15	0.0012
Ca(mg/dL)	9.67±0.33	4.16±0.61	0.0235
Na(mEq/L)	4.37±0.35	5.24±0.49	0.0125
Pot (mEq/l)	4.05±0.48	5.80±0.95	0.0326
Mg (mEq/L)	1.71±0.17	1.62±0.44	0.0235
Cl(mEq/L)	103.36±1.32	90.87±6.27	0.0365
Zn (Units/mg)	0.81±0.013	0.61±0.12	0.0156
Cu (µg/dl)	0.68±0.014	1.05±0.02	0.03135
Se (g/mol)	0.054±0.015	0.038±0.014	0.03265
S Fe(µg/dL)	85.28±3.37	138.04±9.20	0.0135
S Ferritin (µg/dL)	105.55±1.94	186.39±6.86	0.0256
Vit A (µg/ml)	613.48±4.45	432.16±4.99	0.0154
Vit C (µg/ml)	0.569±0.087	0.36±0.23	0.0165
Vit E (µg/ml)	0.294±0.050	0.24±0.093	0.0251
Vit D (ng/ml)	13.17±0.81	5.45±1.20	0.0115
TCH (mg/dl)	4.40±0.33	5.15±0.66	0.041
Tg (mg/dl)	1.30±0.13	2.55±0.56	0.0165
LDL (mg/dl)	2.30±0.181	2.91±0.62	0.0165
HDL (mg/dl)	1.69±0.13	1.30±0.14	0.0216

Table-II. Estimation of different variables in women's suffering from osteoporosis

RBCs involved in the transportation of oxygen, white blood cells play role against certain pathogens, platelets play role in blood clotting.[19] It is suggested that low levels of blood cells were found in this bone disorder which accounts for more complexities, thus making the body weaker day by day. The Hb level is also reduced from their normal value which shows the occurrence of anemia. So it can be inculcated that patient of postmenopausal osteoporosis is at a higher risk of anemia.[20] Osteoclasts are known to produce intracellular ROS, which cause decrease in alkaline phosphatase (ALP) activity that is partially inhibited by vitamin E and cause cell death. In osteoclastic activity, H_2O_2 has been shown to decrease cell growth, calcification, mineralization and gene expression of osteogenic markers such as ALP. With the help of GGT test high level of ALP seen in osteoporotic patient according to subjected study that indicated more damaged to bone. Zinc is needed for the body defensive system to work properly. Copper also help keeps the blood vessels, nerves, immune system and bone healthy. Selenium is important in making antioxidant enzymes which plays role in preventing the cell damage. Low level of these important ions has serious damage to bone which increases the fragility of bones and thus more chances of fractures.[21] so in the view of the above context it is suggested that the low level estrogen are more susceptible to osteoporosis.

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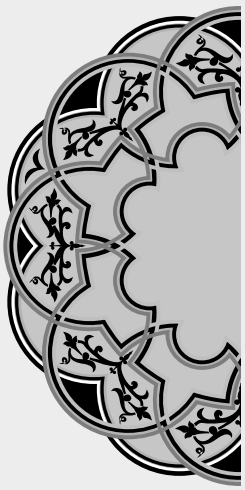

"Nowadays people know the price of everything and the value of nothing."

Oscar Wilde

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