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DIFFERENTIAL EXPRESSION OF PROPHETIC VARIABLES OF MEDICAL IMPORTANCE AND THEIR INTERPLAY TO DEVELOP OSTEOPOROSIS

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ABSTRACT

INTRODUCTION: Osteoporosis, also known as silent thief, is a noteworthy condition in which mineral ions that are important for bone structure loose gradually. Deficiency of active vitamin D and down-regulation of osteoprotegrin leads to the bone mineralization through maintaining the activity of osteoclastic and osteoblastic cells. **AIM:** To evaluate the differential expression of prophetic variables of medical importance and their interplay in the development of osteoporosis. **MATERIAL AND METHOD:** Thirty healthy females and fifty osteoporotic females screened at Ganga Ram hospital Lahore were included in the present study. Serum was evaluated for the levels of 8-hydroxy-2-deoxyguanosine, isoprostanes, vitamin D, Malondialdehyde, nitric oxide, superoxide dismutase, catalase and glutathione via their respective spectrophotometric and ELISA methods. **RESULTS:** The present study has reported that the levels of stress markers

were significantly higher in the osteoporotic females. Levels of MDA, 8-OHdG, isoprostanes and NO were significantly higher in the females with osteoporosis (2.59±0.15 nmoles/ml, 0.326±0.01 pg/ml, 22.06±2.26 pg/ml and 57.91±3.93 μmol/L) as compared to healthy females (0.95±0.13 nmoles/ml, 0.02±0.001 pg/ml, 0.99±0.13 pg/ml and 19.46±1.38 μmol/L), while the levels of antioxidants such as SOD, CAT, GSH and vitamin D were significantly lower in osteoporotic femalesin comparison to control group. **CONCLUSION:** the current study suggests a significant role of calcium under the influence of vitamin D as well as several oxidative markers and inflammatory cytokines i.e., IL-7, sRANKL and OPG in the progression of osteoporosis. Up-regulation of sRANKL and down-regulation of OPG are involved in the bone resorption by the activation of osteoclast cells.

Keywords: Oxidative stress biomarkers, Osteoporosis, serum Receptor Activator of Nuclear factor k-B ligand (sRANKL), Osteoprotegrin (OPG), Antioxidants, Vitamin D

INTRODUCTION

Osteoporosis is often known as a silent thief, because in this condition minerals of bone gradually loss and bone turnover for longer period, without any symptoms bones become fragile and is prone to have fractures. Anyhow, epidemiological ratio executes that occurrence of osteoporotic fractures are two to three times more common in females as compared to males (Manolagas and Stavros, 2010). Osteoporotic fractures or bone loss due to the osteoporosis is considered asone of the alarming issue of the early future projected exist almost 8.1 billion people up to 2050. The basic risk factors of osteoporosis are following including, increased oxidative stress, hypo-secretion of several hormones such as luteinizing hormone (LH), follicle stimulating hormone (FSH) and parathyroid hormone (PTH) etc. Sufficiency ratio of vitamin D and down-regulation of Osteoprotegrin (OPG) prevent to bone mineralization through maintaining the activity of osteoclast and osteoblast cells (Colaianni *et al.,* 2013). Women age at 50 years get more chance to osteoporosis, due to decrease production of estrogen because deficiency of estrogen leads to excess bone resorption mainly in postmenopausal women. It was well-established that the lack of Vitamin D directly relation with bone disorder in children called rickets, whose ratio were raised within the passage of time (Cotte *et al.,*2010; Sara *et al.,*2011). Estrogen has the prominent role to maintain the skeletal growth and balance between bone

metabolisms. Low levels of estrogen directly influenced at bone resorption and trigger to up-regulate activity of osteoclastic cells. Estrogen regulates the bone balance with the help of many unexpected effects of immune system and also oxidative stress has directly effect on bone cells. At tissue level osteocytes regulates bone remodeling (Nabipour*et al.,* 2011). The processes in which the old bones cells were exchange their place with new bones cells are called "Bone Remodeling". The bone remodeling process start by the interaction of several multicellular molecular agents includes transmitter chemical messenger hormones, cytokines and growth factors. There are three types of cells that play role in bone remodeling including osteoclast for bone resorption activity, osteoblast for formation of bones and osteocytes cells which were embedded on surface of bones, originate from the bone forming cells. Osteoclastic cells are nucleated from blood cells origins that further proceeded in the precursors of monocyte/macrophage lineage whereas bone forming cells are the cells of mesenchyme origin further discriminate from bone marrow cells (Rumpler *et al.,* 2012; Baron and Hesse, 2012).

During the process of bone remolding the specific type of proteolytic enzymes were secreted and initiate the exchange process between new bone cells and old or damaged bones cells. After osteoclast migration the osteoblastic cells come at that place which formed new bone matrix through osteocytes cells, further calcification of bones takes place and finally new bone formed. In the third type of bone cell activity osteocytes fixed into the bone surface membrane and then undergo differentiation for the new bone formation (Back *et al.,* 2010; Abdelmagid *et al.,*2015). Osteocytes interconnect with other bone surface area which originates into cytoplasmic series of other nuclei through canaliculi of bones in the matrix sites. Osteocytes act as mechanical sensors of cytoskeleton that detect and responding to many changes in the bones fluid that run in the canaliculi of bones surface (Pacureanu *et al.,*2012). Bone remodeling has controlled by various hormones, such as bone parathyroid hormone, 1, 25 dihydroxy-vitamin D3, sex hormones, calcitonin and some other certain local factors such as NO, prostaglandins, growth factor and inflammatory markers were also involved in bone remodeling. The main influencing local expression of receptor activator of nuclear factor k-B ligand (RANKL), receptor activator of nuclear factor (RANK) and osteoprotegrin (OPG), that has function in the regulation and

differentiation of many osteoclastic cells activity to maintain the bone structure (Ahmed and Elmantaser, 2009; Boyce and Ting, 2008). Oxidative stress defines as the uncontrollable condition of reactive oxygen species oxidized several biomolecules that are present in biological system. Natural antioxidants levels were reduced due to scavenger activity against the formation of free radicals. Through oxidation of macromolecules, free radicals directly cooperate with the bone resorption which activates the Osteoclastic differentiation so it can say that oxidative stress causes the higher bone resorption (Valacchi *et al.,* 2012). Likewise concentration of antioxidants decreases by the disturbance creates by free radicals. Furthermore, these radicals cause DNA damage and also secret the specific oxidative stress biomarkers including 8-OHdG, Isoprostanes and MDA (Boyce and Ting, 2008; Valacchi *et al.,* 2012).

In the response of oxidative stress, the levels of antioxidants also decrease because it will be try to compete with free radicals' action and given the protection against cellular damaging process. There are several types of antioxidants either enzymatic or non-enzymatic including CAT, GSH, SOD and vitamins etc. Intake of antioxidant vitamins show beneficial effect on bone quality and decrease the risk of mineral deficiency (Shweta and Khoshhal, 2007; Cervellati *et al.,* 2013). The molecular mechanism of bone structure in healthy and skeletal pathologies are not been found yet. But according to previous studies at cellular levels, the regulated mechanism of bone structure and bone turnover will be different (Basu *et al.,* 2001). There has been associated with bone resorption and bone formation cells that regulate the activity of bone cells by increasing the expression of tumor necrosis factor ligand superfamily 11 like RANKL and tumor necrosis factor ligand superfamily 11b likeOPG. RANKL is mostly present at the surface of bone forming stromal cells and stimulate the activation of its particular receptor. The RANKL present at osteoblastic cells surface and promote the bone cells formation through inhibition of the osteoclastic cell death. OPG produced by the influence of bone forming cells and stromal cells causes binding with RANKL and play role as a competitive inhibitor. There has been necessary to maintain the balance RANKL and OPG which determines osteoclastic function and bone resorption (Altindag *et al.,* 2008; Hofbauer *et al.,* 2000). Osteoprotegrin acts as natural antagonist factor for the RANKL activity that can be seen in early stages of menopause. During

the initial phase of menopause, the levels of estrogen were decreased that trigger to up-regulation of osteoclastic activity and down-regulate the activity of osteoprotegrin, that results in the process of bone loss will be increased and causes the osteoporosis. Furthermore, menopause is a medical condition in which sex hormone may induced, for instance, in men a disease which is common prostate cancer, and in women breast cancer related receptor positive that has linked with an activated RANKL pathway which elevated the more resorption of bones (Hofbauer and Schoppet, 2004; Bergmann, 2009).

AIM AND OBJECTIVE:

The aim of present study was to find out the relationship of circulating biochemical markers of oxidative stress, inflammatory biomarker and vitamin D in osteoporotic females.

MATERIAL AND METHOD

For the current research work fifty females with osteoporosis and thirty age-sex matched controls were screened at the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore. All the patients were provided signed consent form before including them in study. All protocols were according to the Ethical committee of University of Lahore. 5ml blood was taken in the vial and serum was separated and stored at -80^{o}C for the respective protocol.

INCLUSION CRITERIA

Females with osteoporosis age 20-70 were added in the current study

EXCLUSION CRITERIA

Females on any drug, alcohol and smoking. Patients with any congenital disease such as HCV, HIV, and Diabetes were excluded out.

BIOCHEMICAL ASSAY

Lipid peroxidation was determined by Ohkawa method. About 200µl of the sample was obtained in the test tube and subsequently 200µl of 8.1% SDS, afterwards acetic acid (20%) and TBA (0.8%) having volume 1.5ml were finally added in the tube and were allowed to heat for 60min. It was then cooled down, and about 4ml of n-butanol was then added and was allowed to centrifuge for 10mins at 3000rpm. At last, supernatant was separated in a cuvette and absorbance was measured with the help of spectrophotometer at 532nm against the blank. The levels of NO and antioxidants such as SOD, CAT and GSH were also estimated by spectrophotometry methods. Isoprostanes were estimated by the help of commercially available ELIZA kits (Caymen Chemicals) (Morrow *et al.,*1990).

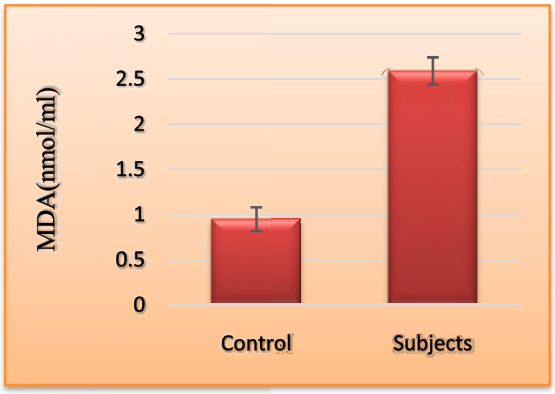
Determination of 8-hydroxy-2-deoxyguanosine with the help of commercially available ELIZA kits (Enzo-USA) (Bruskov *et al.,* 1996). Vitamin D was estimated by the help of ELISA kit (by Caymen Chemicals) (Wallace *et al.,* 2010).

STATISTICAL ANALYSIS

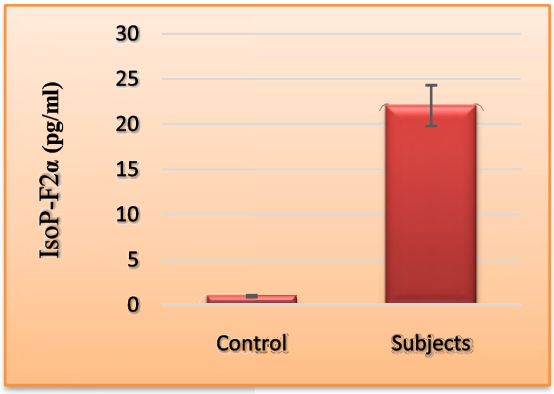
Statistical analysis done using SPSS (v.16 or more) applied test was Independent T-test. Results were expressed (Mean ±S.D) taking ($p \leq 0.05$) for significance.

RESULTS

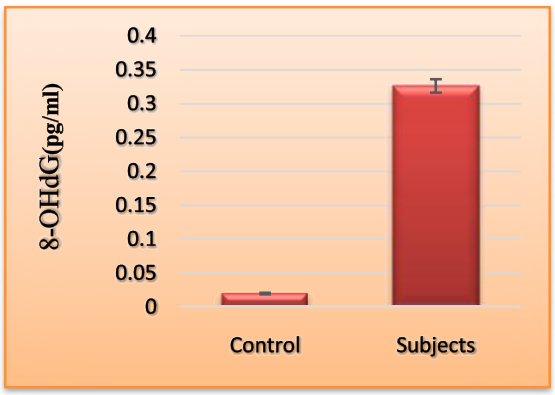
According to the results of present study the levels of MDA and NO were increased in osteoporotic females as compare to healthy individuals. As show in figure 01 the levels of oxidative stress biomarkers like MDA and NO (2.59±0.15 nmoles/ml,57.91±3.93 µmol/L) were significantly high in patients as compare to control group (0.95±0.13 nmoles/ml, p=0.014) (19.46±1.38 µmol/L, p=0.025) respectively as show in figure 01 (A, H). Another oxidative stress biomarker that also represent to specific side of damaging in which including 8-OHdG and Isoprostanes (0.326±0.01 pg/ml, 22.06±2.26 pg/ml) were significantly increased in osteoporotic females'parallel to control group (0.02±0.001 pg/ml, p=0.011), (0.99±0.13 pg/ml, p=0.005) respectively, figure 01 (C, B).As show in present study the levels of antioxidants were also decreased such as SOD, GSH and CAT(0.09±0.008 U/ml, 4.23±0.53 µmol/L, 2.21±0.18 U/L) in subjects as compare to healthy groups (0.50±0.013 U/ml, p=0.023), (9.80±0.81 µmol/L, p=0.032) and (3.91±0.25 U/L, p=0.027) respectively as show in figure 01 (E, G, F). Deficiency of vitamin D and calcium also the major risk factor to cause the osteoporosis because the levels of calcium and vitamin D (4.16±0.61 mg/dL, 14.04±1.26 ng/ml) were also decreased in osteoporotic females Vs to control group (9.67±0.33 mg/dL, p=0.023), (26.56±1.58 ng/ml, p=0.018) respectively figure 01 (I, D). The levels of inflammatory biomarker such as IL-7 also disturbed in subjects because it has the significantly role in the regulation of bone remodeling through RANKL activation. So, the levels of IL-7 were significantly increased in patients (6.74±0.21 pg/ml) as compare to control group (5.65±0.18 pg/ml, p=0.016), show in figure 01 (J).


MDA(nmol/ml)
3
2.5
2
1.5
1
0.5
0
Control
Subjects

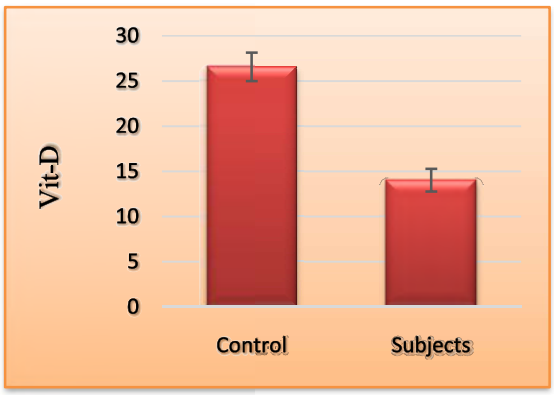
A


IsoP-F2α (pg/ml)
30
25
20
15
10
5
0
Control
Subjects

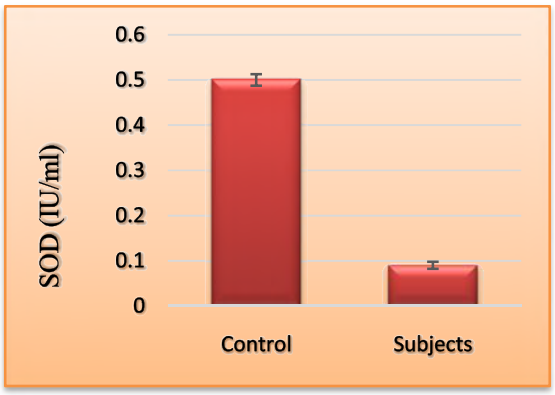
B


8-OHdG(pg/ml)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
Subjects

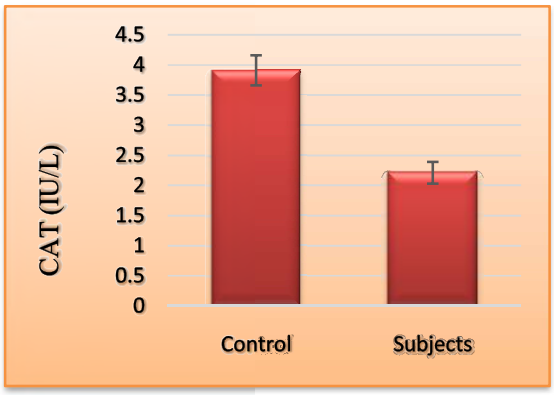
C


Vit-D
30
25
20
15
10
5
0
Control
Subjects

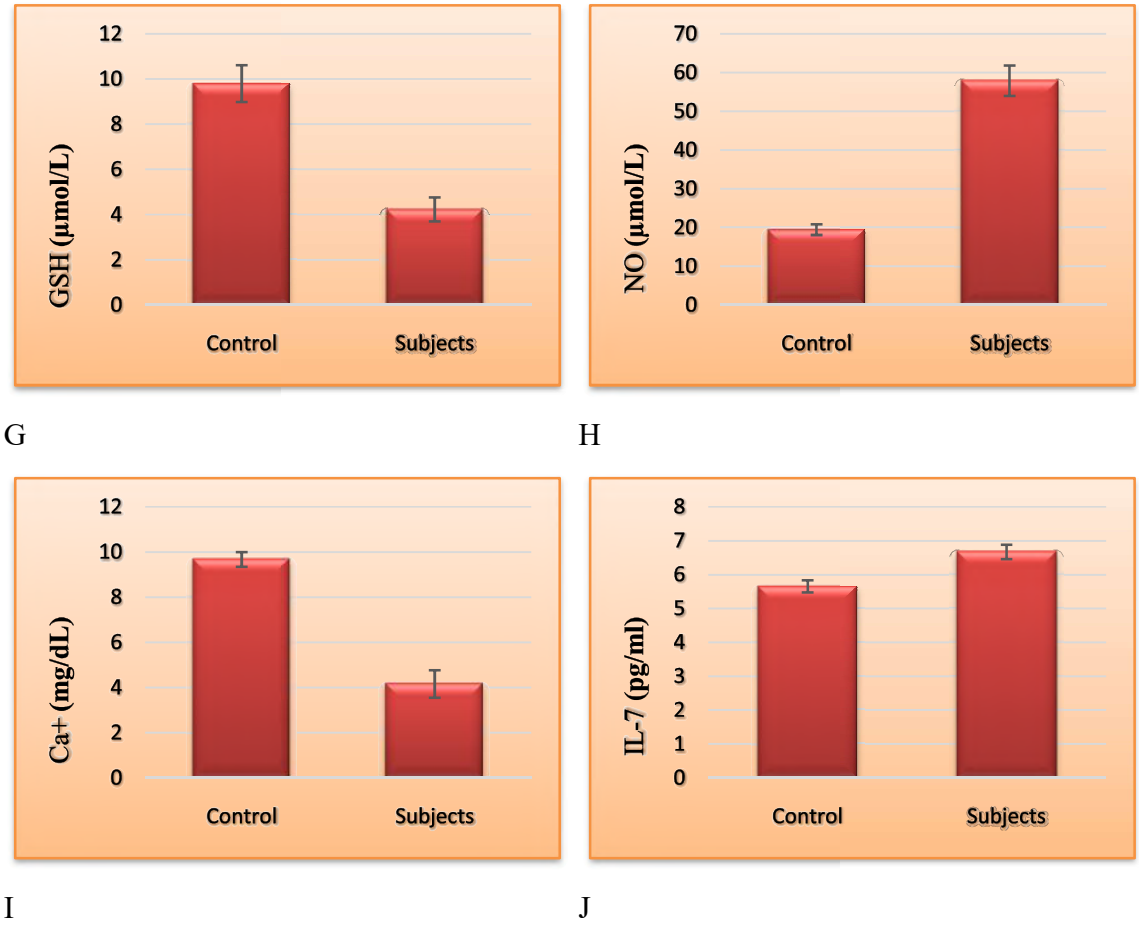
D


SOD (IU/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Subjects

E


CAT (IU/L)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Subjects

F



G H

I J

Figure 01: Circulation Biochemical Profile and of Different Variables In Osteoporotic Females

DISCUSSION

Osteoporosis may occur at age under 45 years in women who have undergone hysterectomy and oophorectomy. Osteoporosis is a common disorder of old age, creating a worldwide health problem, as the aged tend to suffer from bone fractures from mild injury or even without injury. These fractures increase the morbidity and mortality rates, and health funding, and reduce the quality of life of the patients (Lindsay *et al.,* 2005; Lespessailles *et al.,* 2009). According to the prior works vitamin D have the significant role in several complications including osteoporosis because vitamin D have the countless potency to reabsorb the Ca^{+} from intestine for bone structure. It was well established that the normally vitamin D synthesized in body through two-timehydroxylation in different location including liver and kidney, due to abnormality in both position leads to cause Vitamin-D deficiency. Finally, the low levels of Vitamin-D results in osteoporosis

(Compston, 2017). Reactive oxygen and nitrogen species also have the significantly role in osteoporosis either directly or through cascade of reactions. The oxidative stress might be occurring due to the overproduction of free radicals and antioxidant defense mechanism because the antioxidant such as SOD, CAT, and GSH have the significant role to prevent the osteoporosis by neutralize the toxic effects of free radicals. SOD have the greater affinity in the conversion of oxygen free radicals into hydrogen peroxides but it is also toxic then it further converted into neutralize form like water and oxygen by the action of CAT as well as glutathione peroxidase have the greater affinity to prevent the osteoporosis through the inhibition of IL-7 secretion. It is the major inflammatory markers that involved in the up-regulation of RANKL (Koleshikova *et al.,*2015).

Imbalance between the production of reactive oxygen or nitrogen species and defense activity of antioxidants cause the oxidative stress (OS). In this response the free radicals were bind with membrane bilayer and initiate the process of lipid peroxidation. The end product of lipid peroxidation is malondialdehyde (MDA). This universal stress biomarker assessed to check the severity of oxidative damaging process. Furthermore, the MDA directly binds with DNA and generates DNA adducts that were isolated in the form of 8-OHdG that is the specific biomarker to check the severity of DNA damage (Baron and Hesse, 2012; Zhou, 2016).During the OS condition the excess production of reactive nitrogen species including NO also influenced on the activity of gonadotrophic releasing hormone (GnRH) from the hypothalamus and this abnormality further migrate into anterior pituitary glands for the secretion of FSH and LH hormone. The LH has the major role in the transportation of cholesterol for the production of estrogen in ovaries (Mansell and Jason, 2008). But the overproduction of NO directly influenced to the activity of 17 beta hydroxysteriod dehydrogenase (17β-HSD) because this enzyme has the significant role in the conversion of estrone to estradiol. Due to the inhibition of 17β-HSD enzyme through NO the levels of estradiol decrease but the levels of estraone were high. Due to the deficiency of estradiol the RANKL is activated and it binds with RANK those results in the increased activity of osteoclastic cells that leads to accelerate the osteoporosis (Sanchez-Rodriguez *et al.,* 2007; Nichols and Scott, 2012). Estrogen has the positive correlation with calcium levels because the desire levels of estrogen

facilitate to the absorption of calcium from the gastrointestine side and reabsorption from the renal tubular sides. But according to the results of present study low levels of estrogen also lead to cause calcium deficiency due to improper function and concentration of vitamin D that might be lead to cause osteoporosis (Horowitz, 1993; Martin-Millan, 2010).

The abnormal secretion of parathyroid hormone (PTH) is another major risk factor of osteoporosis because it regulates the secretion of calcium and phosphate to manage the levels of calcium from the bone because the levels of PTH were inversely proportional to the levels of vitamin D. Low levels of calcium triggers the secretion of PTH that maintain the concentration of calcium through bones and it also trigger to osteoclastic activity to regulate bones remodeling and facilitate vitamin D activation through kidney that also collaborate to manage the desire levels of calcium by the process of reabsorption from intestine. Because low levels of calcium trigger the secretion of PTH then PTH further stimulate to vitamin D activation by the stimulation of specific kidney enzyme that is called 1-hydroxylase and it has the potential role for the activation of vitamin D and maintains the levels of calcium that results in the decreased prevalence of osteoporosis (Gallagher and Sai, 2010; Pacheco-Costa, 2016). According to some prior studies the osteoclastic activity might be up regulated through the activation of RANKL signaling in bone cells. Furthermore, intracellular oxidative stress markers increase and thus might be increase the chance of bone fragility. RANKL is belong to the family of TNF and it have the significantly role in the remolding of bones regulated by activity of osteoblastic cells. The secretion of RANKL was inversely proportional to the activity of osteoprotegrin that also have the important role to maintain the balance between osteoclastic and osteoblastic activity. It was well-established that the higher levels of nitrogen species like NO activate the inflammatory markers including IL-7 that is the major risk factor for the activation and secretion of RANKL to cause the osteoporosis by cascade of reactions (Kassem, 2015). The RANKL bind with RANK, in this response the activity of osteoclastic cells increased as well as osteoprotegrin also another receptor that activated by the osteoblastic cells to inhibit the activity of osteoclastic cells but in osteoporosis condition the binding affinity of RANKL with RANK was increased as compare to osteoprotegrin,that results in

decreased activation of osteoprotegrin that is the another major risk factor for osteoporosis. According to the results of prior study the desire levels of estrogen might be regulate the secretion of RANKL and osteoprotegrin activity and make the balance between osteoclastic and osteoblastic cells activity but in present study the levels of estrogen also reduce that is another risk factor to increase the activity of RANKL and inhibits the activity of osteoprotegrin. So, all aforesaid risk factors might be significant causing agents to induce osteoporosis (Unnanuntana *et al.,* 2010; Kohli and Kohli, 2011).

CONCLUSION

It was concluded that the calcium played a significant role in osteoporosis that might be regulated by vitamin D as well as oxidative stress is the major risk factor in osteoporosis that occur in the result of cellular damaging through free radicles production and secretes inflammatory cytokines including IL-7 that is the major factor in up-regulation of RANKL and down-regulation of osteoprotegrin through deficiency of estrogen in osteoporotic females.

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CONFLICT OF INTEREST

Authors declared no conflict of interest.

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