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The role of NADPH Oxidase 4 in Iraqi Osteoporosis Patients

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ABSTRACT: Background: Osteoporosis is a common condition that occurs in bone leading to the loss of bone mass. It is caused by increased osteoclast activity which is differentiated by low bone density measured by dual-energy X-ray absorptiometry (DEXA).**Objective**: This study aimed to estimate the levels of NADPH oxidase (NOX4), Alkaline phosphatase, and vitamin D in Iraqi osteoporosis patients diagnosed by DEXA. **Methods**: A total of 88 volunteers were split into two groups for this investigation. There were 58 patients in group 1 and 30 controls in group 2 , the samples were taken from both sexes of range age (20-60) years, the levels of Alkaline phosphatase (ALP), Vitamin D, and NOX 4 were evaluated. NADPH oxidase 4 levels were measured by enzyme-linked immunosorbent assay (ELISA), Alkaline phosphates levels were measured using a spectrophotometer, and the concentration of vitamin D was measured by electrochemiluminescence binding assay using Cobas e 411. Whereas the T-score was measured using DEXA.**Results**: The results of NADPH oxidase 4, AlP, and vitamin D did not show any significant change (P>0.05) when compared osteoporosis group to the control group. Whereas T score showed a significant variance when contrasting between the studied groups (P<0.05). **Conclusion**: according to the presented results NADPH oxidase 4 is not affected by osteoporosis.

Keywords: ALP , Dual- energy X-ray absorptiometry, Osteoporosis, NADPH Oxidase 4 , NOX 4

1. INTRODUCTION

Bones have a role as a scaffold and protection for muscles and organs, they are a tissue that is constantly remoldeing to a high degree . Bones are active metabolic tissues that undergo metabolism , which is continuously replayed by opposing processes called bone resorption and bone formation. Under normal conditions, these processes rely on the activity of osteoblasts (responsible for formation), osteoclasts (responsible for resorption), and osteocytes (responsible for maintenance) . The bone formation and resorption processes are interwoven, with the amount of bone created equal to the amount of reabsorbed bone [1]. Mesenchymal stem cells give rise to osteoblasts , which create osteoid tissue by synthesizing specific collagen and proteins such as osteocalcin and osteopontin . Large multi-nucleated cells called osteoclasts originate from the hematopoietic progenitor of the myeloid lineage and are responsible for resorbing bone and eliminating deteriorated or old bone matrix [2]. Osteoporosis is a skeletal disease differentiated by decreasing bone density leading to weakened bone strength and increased risk of fractures [3], The World Health Organization (WHO) defines osteoporosis in practical terms as low bone mineral density (BMD), which is measured by dual-energy X-ray absorptiometry (DEXA), the industry standard for bone mineral density measurement. It provides data on changes in the composition of bone minerals and is useful for tracking changes in a patient's bone mass and studying those changes [4]. Primary osteoporosis, or postmenopausal osteoporosis, is linked to a decrease in estrogen, exacerbating bone loss as people age [5].The hormonal variables that impact osteoporosis explain adrenal androgen hypersecretion, which is linked to increased bone mass, and glucocorticoids promote

osteoclastogenesis by down-regulating the expression of osteoprotegerin (OPG) and upregulating the expression of CSF-1 (Colony Stimulating Factor 1) and RANKL (Receptor Activator of nuclear factor kappa-B ligand). Insulin-like growth factor I (IGF-1) expression is further suppressed by glucocorticoids, which reduces the function of the remaining osteoblasts. Also, osteoblast differentiation was enhanced by melatonin, while RANKL production was also observed to be inhibited by melatonin. Recent research shows that bone remodeling processes epigenetically regulate histone post-translational modifications and DNA methylation. Genes linked to osteoblast and osteoclast differentiation, such as RANK/RANKL/OPG, RUNX2 (Runt-related transcription factor 2), OSX (osterix), OCN (osteocalcin), ALP (Alkaline phosphatase), and Wnt (wingless and int) pathways, are modulated by DNA methylation status [6].

NADPH oxidase (NOX) (EC1.6.3.1) is an essential enzyme in charge of generating superoxide (O_2 •) radicals in mammal cells where seven genes encode NOX isozymes in human cells. It has a role in many processes like cellular signal transduction, host apoptosis, defense, oxygen sensing, and angiogenesis. All NOX family members are thought to be transmembrane proteins that move electrons from reductio (O_2) to superoxide (O_2•) across cellular membranes [7]. NADPH oxidase isoforms participate in controlling and directing the formation of ROS in both pathophysiology and physiology.They contribute to some bone processes such as the formation, survival, and activity of osteoblasts and osteoclast. The members of the NADPH oxidase family vary in the way they are activated according to the function of this enzyme [8].

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Other studies indicate that in both human and murine osteoblasts, the increase of iron accumulation leads to an increase in NOX4 enzyme accumulation and mitochondrial dysfunction. NOX4 transcription is triggered by iron, which facilitates the IRP1(Iron regulatory proteins 1) protein's separation from IRE (Inositol-requiring enzyme)-like sequences at the NOX4 promoter. Increased ROS generation and intracellular lipid peroxide buildup caused by elevated NOX4 enzyme levels cause osteoblasts to undergo ferroptosis [12]. Further experiments showed that it could inactivate ROS/JNK (c-Jun N-terminal kinase) signalling pathway by suppressing NOX4 expression in osteoblasts [13].

Alkaline phosphatase (ALP) is a metalloenzyme, Each isoenzyme is a glycoprotein linked to the membrane encoded by particular loci genes [14] Because of skeletal growth, bone isoforms predominate during childhood and adolescence. Osteoblasts produce bone-specific ALP during the process of bone formation. Even though its precise function is still unknown, it is known that ALP expression positively correlates with bone formation and that ALP must be present in the cell membrane of osteoblasts for bone mineralization to occur [15]. Developing strong bones and preserving proper calcium levels are significantly dependent on vitamin D (calciferol), a fat-soluble vitamin. The two most important forms of vitamin D are vitamin D2 and D3. In addition, it plays an important role in maintaining proper calcium and phosphate concentrations, vitamin D is essential for the immune system, as well as for the proliferation and differentiation of cells [16].

The current study focuses on identifying the correlation of NADPH oxidase 4 with Iraqi osteoporosis patients, also finding the relationship between osteoporosis and other parameters such as BMI, T-score percentage, ALP, and Vitamin D for early diagnosis as novel biomarkers for the disease.

2. MATERIAL AND METHODS

2.1 Study design and setting

At Baghdad Teaching Hospital - Medical City, Baghdad - Iraq, serum blood samples were obtained from 30 healthy individuals of both sexes and ages ranging from 30 to 60 years old, as well as 58 patients with osteoporosis, the patients were diagnosed using Dual-energy X-ray absorptiometry (DEXA) . The enzyme-linked immune sorbent assay (ELISA) was used to determine NOX 4 (NADPH oxidase 4 assay following the manufacturer's instructions (Human NADPH oxidase 4, catalog Number. CSB-EL015961HU). A spectrophotometer (APEL PD 303) was used to determine the absorbance of ALP, an electrochemiluminescence binding assay using Cobas e 411(Roche Company)

was used to detect the concentration of vitamin D, DEXA was used to calculate the T score %, which is equivalent to bone mineral density.

2.2 Exclusion Criteria

Patients suffering from diabetes mellitus, rheumatoid arthritis, hypothyroidism, or hyperthyroidism, pregnant women, patients who are treated with steroid drugs, and smokers.

3. Statistical Analysis

The results have been analyzed using SPSS 25, a statistical analysis program. The general descriptive statistic described the primary results, and the significant differences were found using the Mann-Whitney Test . ROC curves have also been Applied.

4. RESULTS

The information about the donors' demographics is summarized in Table 1 about the demographic character of individuals with osteoporosis and those in the control group.

Table 1. - Demographic characteristics of individuals

Characteristics		Patient's	Control
Numbers of samples		58	30
Age Range		20-60	20-60
Gender	Female	53	23
	Male	5	7

Table 2. - Statistical distribution of parameters level in the serum of osteoporosis patients and controls

No	Parameters	Group	Mean ±Std. Error[1]	Minimum	Maximum	Mean Rank	P value [2]
1	Age	Osteoporosis	44.7931± 1.49039	20.00	60.00	51.25	<0.05
		control	35.9667±1.82290	20.00	56.00	31.45	
2	BMI	Osteoporosis	30.3040± .75299	19.82	45.44	45.79	>0.05
		control	30.6070±1.55100	18.67	55.86	42.00	
3	T_Score_per	Osteoporosis	-.22483± .018853	-.450	.300	31.82	<0.05
		Control	-.03933± 0.018238	-.160	.140	69.02	
4	NOX 4	Osteoporosis	6.01390 ± 3.436356	.006	172.342	41.50	>0.05
		control	10.74840 ± 6.279718	.000	186.915	38.83	
5	ALP	Osteoporosis	93.43621±4.845528	23.300	190.000	44.37	>0.05
		Control	89.96667±4.628369	49.000	153.000	44.75	
6	Vitamin D	Osteoporosis	16.95621±1.533895	5.700	63.000	36.92	>0.05
		control	21.82316±4.609926	2.800	98.000	45.34	

The data obtained were evaluated by Mean ± standard Error 1.

Mann Whitney test was performed, Significant level at P value <0.05 [2] .

The (mean ± SE) of age between the patients and control groups is displayed in Table 2, where were [(44.7931±1.49039)(35.9667±1.82290)] with the results indicating a significant difference (P<0.05),the (mean± SE) of BMI showed a non - significant various where were [(30.3040± .75299)(30.6070±1.55100)] (P>0.05) .

T-score-percentage analysis indicated a significant difference (P<0.05) in the mean ± SE between the patients and the control group, which is [(-.22483±.018853)(-.03933± 0.018238)] as shown in Figure 1.

The results of NOX 4 showed non-significant difference (P>0.05) where mean ± SE between patients and control are [(6.01390 ± 3.436356)(10.74840 ± 6.279718)] as shown in Figure 2.

Table 2 also shows the mean ± SE of ALP and vitamin D for patients and control where were [(93.43621±4.845528)] [(89.96667±4.628369)] [(16.95621 ±1.533895) (21.82316±4.609926)] respectively. The results indicated a non-significant difference (P<0.05) between the two groups as shown in Figure 3,4.

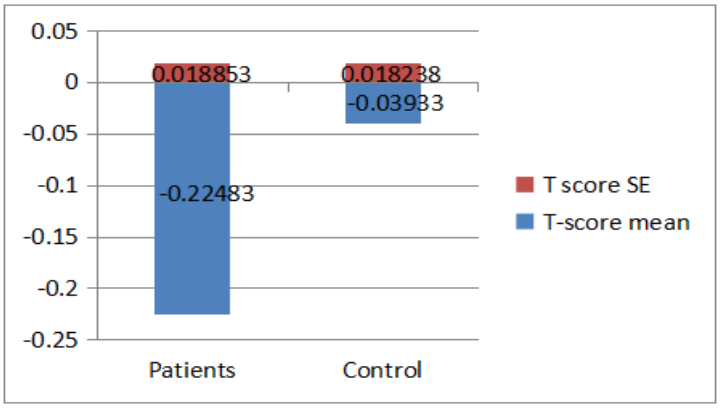

FIGURE 1.- The mean ± SE of T- score values between Patients and control

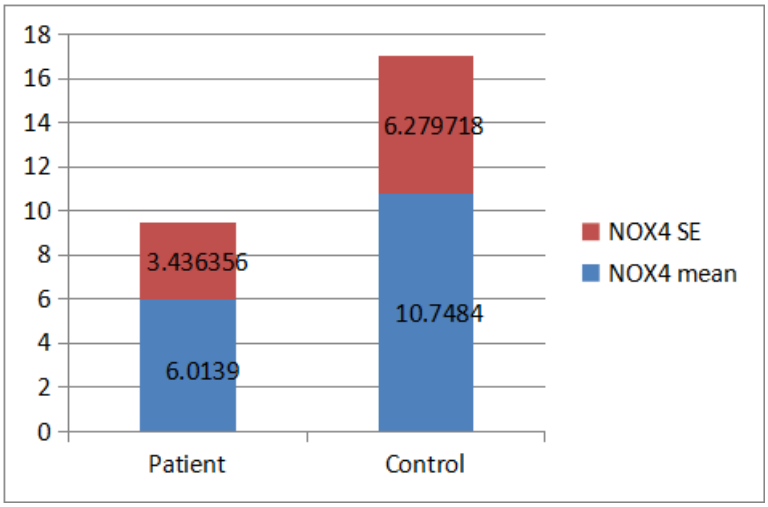

FIGURE 2. - Mean ± SE of NOX4 in studied groups

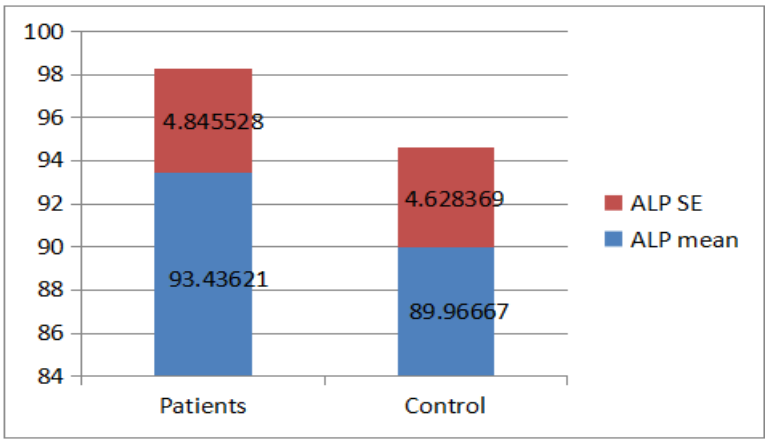

FIGURE 3.- The mean± SE of ALP values between two groups

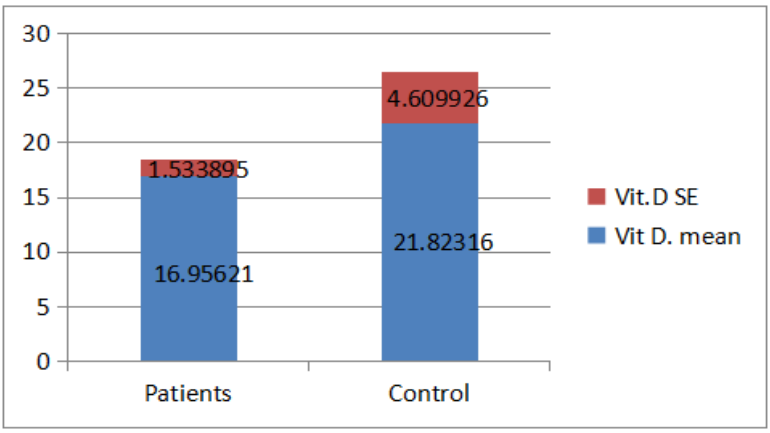

FIGURE 4. - The mean± SEof Vitamin D values between two groups

4.1 The receiver operating characteristics curve (ROC)

ROC is a statistical study that uses a plot of the connection between sensitivity and 1-specificity to determine the right level of specificity and sensitivity for the diagnostic test [17]. The NOX4 ROC cutoff point was determined. Figure 5 indicates that NOX 4 did not exhibit a distinct cut-off point, and the area under the curve was (0.530).

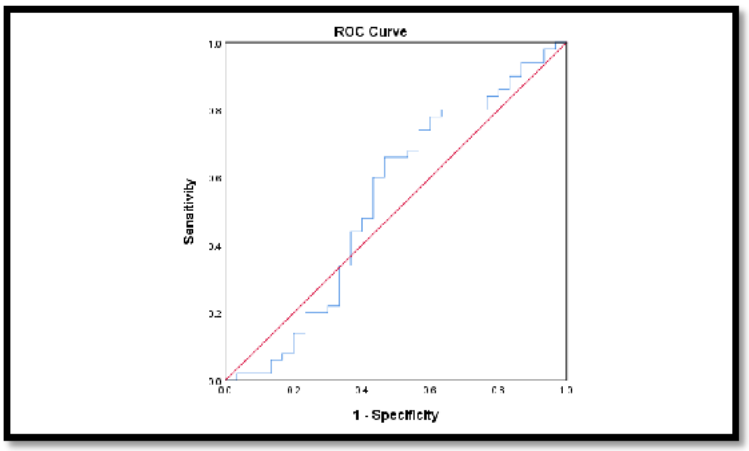

FIGURE 5. - NOX 4 ROC curve

5. DISSCUTION

One of the most common bone illnesses is osteoporosis, which is one of the most prevalent conditions affecting the bones, resulting in brittle and easily fractured osteoporotic bones. The DEXA device is the most effective diagnostic tool for predicting osteoporosis. When determining the mineral density, fat mass, and lean mass, it calculates a T-score value where the normal value is less than -2 [18]. According to the study, age is a risk factor for osteoporosis patients because it affects the ability of stem cells to differentiate and generate new bone marrow and osteoblasts, which is related to age changes [19]. Also, this study shows the level of NOX4 in osteoporosis patients and healthy people where NOX4 shows a non-significant effect and this study is the first which measure this enzyme in the serum of osteoporosis patients where most previous studies were about NOX 4 effects in mice or cell lines. A study in 2020 shows that NOX 4 in knockout mice has higher bone density and less osteoclast activity where expression of NOX4 and free radicals H2O2 production is stimulated by monocyte with M-CSF (macrophage-colony stimulating factor) and RANKL [20], but Schröder, K. 2015 shows the effect of NADPH oxidase in bone remodeling and bone homeostasis by the different effects of NADPH isoforms where NOX type 2 leads to form osteoblast whilst NOX type 4 leads to form osteoclast, which means it has a role in osteoporosis occurring [7] and this contradicts our results. According to Renaudin F. 2023's data, removing NOX4 in mice could slow the advancement of osteoporosis. However, it is unclear if these statistics are relevant in a therapeutic situation. The effects of NOX4 on human joints and bone disorders have not been thoroughly studied in prior research [21].

By calculating the bone mineral density (BMD) of the central and axial skeleton, double -energy X-ray absorptiometry (DEXA) scanners were used to evaluate T-score [22]. This test was frequently used to diagnose osteoporosis.

ALP shows a negative correlation with bone mineral density and this agrees with a previous study by (Zhou, P.) which explains the effect of ALP with bone mineral density [23], while a high level of ALP was found with hip fracture [24]. When osteoporosis patients have low ALP, it may indicate hypophosphatasia, a disease that can be misdiagnosed as osteoporosis [25].

These findings are consistent with the earlier research on vitamin D, which found no variation in osteoporosis based on bone mineral density [23]. Vitamin D levels do not correlate with declining bone mineral density, according to a different 2017 study by Alkhenizan [26]. There were twenty studies total, 16 of which were cohort studies and 4 case-cohort studies. The meta-analysis comprised 41,738 participants from 20 studies, of which 5916 had fractures, 3237 of which were hip fractures. Lower levels of serum 25 (OH)D have been proposed as a potential risk factor for fractures, especially hip and total fractures [27]. As a result, they cannot be used as a marker for osteoporosis diagnosis, however, they may be a risk factor.

6. CONCLUSION

This study investigated the causes of osteoporosis, but no significant results emerged that could be considered one of the causes of osteoporosis, it has a role in bone remodeling and mineralization where NADPH oxidase 4 in serum showed no alteration in its relation to osteoporosis a therefor it needs more studies about the mechanisms of this enzyme and its role in osteoporosis by study more patients number from different regions where many factors affect like age, environment, genes, osteoporosis 'time occurring.

The T-score percentage was demonstrated as an indicator of osteoporosis. Also, the results of other biomarkers mentioned that the ALP and vitamin D levels did not impact osteoporosis, and this needs more future research to explain their mechanisms for causing osteoporosis. However, these biomarkers are still considered risk factors for bone health.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest

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