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Research Article

Evaluation of Human β-defensin-3 Diagnostic Role in a Group of Iraqi Patients with Osteoporosis

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Abstract

Background: Osteoporosis (OP) is a prevalent age-related condition that increases the risk of fracture and bone fragility, as a result of loss of bone mass as well as micro-architectural degradation of the bone, thereby reducing the mass and strength of bone. Human β-defensin (HBD-3) is an anti-inflammatory peptide and a crucial part of the human innate immune system. Giving early therapeutic intervention for OP requires an early diagnosis.

Objectives: To evaluate the serum HBD-3 accuracy of diagnosis in patients with osteoporosis.

Methods: The study was conducted in the National Joint Center at Yarmouk Teaching Hospital in Baghdad during September - October 2023.

Eighty participants were recruited, all of whom had clinical examinations and had their bone status measured by dual-energy X-ray absorptiometry (DXA). Levels of serum HBD-3 and vitamin D3 (Vit D3) were determined by the ELISA technique. Calcium level (Ca^{+2}) was measured using a spectrophotometer. A comparative study was conducted between forty patients with OP and 40 control. The study included females and males with an age range between (40-60) years.

Results: The serum level of HBD-3 in the OP group was significantly higher ($p < 0.001$) than that of the healthy controls. The area under the curve (AUC) was found to be (1.000) in the ROC curve analysis for serum HBD-3 level.

Conclusion: Serum HBD-3 can be a valuable indicator of OP in middle-aged individuals, and may be a helpful biological marker for OP diagnosis.

Keywords: Calcium; Human β-defensin-3; Osteoporosis; T-score; Vitamin D3.

Introduction:

Osteoporosis is a disease of the skeleton caused by the interaction of intricate and composite pathways in molecules that lead to the loss of bone mass as well as micro-architectural degradation of bone, thereby reducing the mass and strength of bone. Reduced bone mineral density (BMD) is a significant effect linked to weak, fragile, and broken bones (1-4) Both sexes are susceptible to OP and its worst effects (5-7) include fractures and chronic pain. However, women are more susceptible than men, due to accelerated bone mass loss caused by decreased estrogen levels, with the prevalence being higher in postmenopausal women (8-9). Increased bone resorption results in a phase of faster bone depletion and Ca^{+2} exhaustion from the skeleton into the extracellular fluid. These changes exacerbate bone loss by creating an imbalance of Ca^{+2} throughout the body (10-12). Vit D3 has a major impact on calcium-phosphate homeostasis and ideal bone growth. It is worth mentioning that an inadequate amount of Vit D3 raises the risk of OP fractures. Physiologically active Vit D3 enhances Ca^{+2} intestinal absorption while promoting osteoclastic maturation and bone growth (13-14) by modulating Ca^{+2} transport proteins in the small intestine. Adequate serum Ca^{+2} concentrations are necessary for the correct mineralization of bones (15-16). Vitamin D3, obtained from food or cutaneous synthesis, is first converted to the physiologically active version 25-hydroxyvitamin D [25(OH)D] in the liver and undergoes a hydroxylation process. The kidneys then use this form to create 1,25-dihydroxyvitamin D [1,25(OH)2D] which is named calcitriol. The latter is linked to calcium homeostasis and phosphate absorption in the intestine maintaining adequate levels of calcium and phosphate in the bloodstream (15,17). One important element of hydroxyapatite is calcium, the mineral compound that makes up bone tissue. For maintaining the strength and mineralization of bones, this process is essential (18). Vit D3 also binds to its receptor, known as the vitamin D receptor (VDR), which is found on the surface of bone cells. This binding stimulates the production of proteins involved in bone formation, such as osteocalcin and collagen. Ultimately, this leads to increased bone matrix formation and mineralization, which promotes bone growth and density and reduces osteoporosis (19) Defensins are a subclass of antimicrobial peptides (AMPs) which are 3.5–4.5 kDa tiny cationic proteins rich in cysteines that are present in immune system cells with a range between 33 - 47 amino acid residues in length have varying order (20-21), and composition that serve as

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the body natural defense system. Pathogenic microorganisms are killed by defensins by permeabilizing their cytoplasmic membranes (22-24). Mostly expressed in certain epithelial tissues, human β-defensin (HBD) is a crucial portion of the human innate immune system and it plays a vital function in tumor formation, metastasis, injury repair, and inflammatory diseases (25-27). HBD-3 is an antibacterial and immunomodulatory protein secreted by skin, salivary glands, and bone marrow cells (27). It is an essential component of the innate immune system, which serves as the first line of defense against microorganisms on mucosal surfaces like the skin, lungs, eyes, and airways (28,29). HBD-3 is typically expressed at a low concentration as part of the oral mucosa's natural immune barrier (27,30,31). Moreover, rheumatoid arthritis (RA) and other autoimmune diseases have also been connected to HBD-3. HBD-3 has a positive charge (+11) (32). HBD-3 is installed from three anti-parallel β-strands and an N-terminal α-helix that make up the 45 residues of HBD-3, which is stabilized via three disulfide bonds inside the molecule made up of six cysteine residues: C11-C40, C18-C33, and C23-C4 (28). According to certain findings HBD-3 can be utilized as a marker for treatment follow-up for patients with RA due to its association with proinflammatory cytokines (24,31). Correspondingly, HBD-3 is a physiological component that rises during term labor and is found in amniotic fluid, demonstrating that this defensin engages in host defense processes in the amniotic cavity to ward off pathogens or warning signs (33). A previous study recommended using HBD-3 as a therapeutic target to treat cutaneous conditions marked by impaired autophagy and skin barriers, such as atopic dermatitis (AD) (34). Furthermore, HBD-3 was able to support bone repair *in vivo* while also reducing the inflammatory destruction caused by periodontitis (35). In a different research, scientists examined how human-defensin-3-C15, a component of HBD-3, inhibits osteoclast activity to stop bone resorption. HBD-3 has prevented the rise in tartrate-resistant acid phosphate (TRAP+) multinucleated cell formation that was brought about by RANKL. Moreover, the establishment of the RANKL-induced podosome belt is prevented by HBD-3, a feature of osteoclasts that are mature and capable of resorbing bone (36-38). This research aims to measure the levels of HBD-3 in the serum of patients with OP and of healthy individuals to see if there is any relationship between them and disease features.

Patients and Methods:

Osteoporosis Patients:

It is a cross section study which was conducted on 80 participants between (40 -60) years of age, who were not suffering from any significant diseases and were recruited from the National Joint Center at Yarmouk Teaching Hospital in Baghdad for the period between September and October, 2023. The participants were divided into two groups: 40 participants suffering from OP (study group), and 40 healthy individuals (control group). All participants underwent an examination of OP activity by a joint physician using the dual energy X-ray absorptiometry (DXA scan) at the lumbar and femoral neck spine regions vertebrae (L1–L4). Patients were categorized according to the following: The T-score was used to identify OP (< -2.5 standard deviations) and healthy individuals (BMD within 1 SD) of a young normal adult (T-score ≥ -1). A blood sample was tested for (Vit D3, Ca^{+2}) as a routine biochemical blood procedure for all subjects, in addition to measuring the levels of serum HBD-3 in the blood samples for all patients. The weight (Kg) and height (m) of each participant was measured to calculate the body mass index (BMI) Kg/m^2. The waist and hip circumferences were also measured to calculate the waist/hip ratio (WHR). People suffering from diabetes mellitus, heart disease, rheumatoid arthritis, kidney diseases, cancer, hysterectomized women, Addison's disease and other diseases were excluded from this research.

This study was approved by the Iraqi Ministry of Health / Center for Education and Human Development, Yarmouk Iraqi Teaching Hospital Committee of Ethics, and the University of Baghdad Ethical Committee. The ethical standards of the Helsinki Declaration were adhered to in the procedures.

Blood Sample Collection and Laboratory Analysis:

Five milliliters of blood were collected from the participants without using a tourniquet, leaving them to coagulate in a clot activator tube for 15 minutes at room temperature. The serum was separated using a centrifuge for 5 minutes, was stored in 2ml Eppendorf containers and kept at -4◦C. Serum HBD-3 (Cloud-Clone Corp., USA, SEE132Hu), and Vit D3 (Cloud-Clone Corp., USA, CEA920Ge) concentrations were assessed using an ELISA plate reader from Germany's Human. Serum Ca^{+2} (Linear Chemicals, Spain) concentration was evaluated using A spectrophotometer.

Statistical Analysis:

Statistical analysis was conducted using version 26 of SPSS. The median, 25^{th} and 75^{th} percentiles were used. The Mann-Whitney test was used to identify numerical elements that weren't normally distributed. The ROC curve method was utilized to assess the serum HBD-3 level cut-off value. Additionally, calculations were made for the specificity, sensitivity, negative predictive value, and positive predictive value. *P*-values that are less than 0.05 were regarded as significant.

Results:

Table 1 shows the median (25th and 75th percentiles) values for age, BMI and WHR for the OP cases and their healthy controls. The differences between the means of the two groups were not statistically significant.

Table (1): The median and (25th and 75th percentiles) of age, BMI and WHR for the OP cases and their healthy controls

Variable	OP case(n = 40)	Control (n = 40)	*P* value
Age (year)	57.0 (52 - 59)	55.0 (45 - 58.75)	0.648
BMI (kg/m^2)	28.2 (24.5 - 33.2)	30.5 (26.7 - 34.7)	0.273
WHR	0.93 (0.91- 0.95)	0.92 (0.90 - 0.93)	0.407

-The median (25th and 75th percentiles) of the obtained data were evaluated using the Mann-Whitney test at the 0.05 threshold; The two independent group differed significantly from one another.

As it turns out, there was a clear significant difference between the concentration levels of each (Vit D3, Ca) for the OP patients [6.44 (4.05-9.44) ng/dl, 7.99 (7.81-8.15) mg/dl] with [67.0 (38.30-79.10) ng/dl, 9.3 (8.80-9.80) mg/dl] healthy individuals, p-value (<0.001), table 2. Serum HBD-3 levels were noticeably higher in OP patients (2.84 (2.52-3.36) ng/ml) than in the healthy individuals (0.990 (0.890-1.10) ng/ml) with p-value (<0.001). The statistical analysis demonstrated that the OP group and the healthy individuals differ significantly as displayed in (Table 2).

Table (2): The median (25th and 75th percentiles) of lab and radiological investigations for the OP cases and their controls

Variable	OP case (n = 40)	Control (n = 40)	*P*-value
Vit D3 (ng/dl)	6.44 (4.05-9.44)	67.00 (38.30-79.10)	<0.001
Ca (mg/dl)	7.99 (7.81-8.15)	9.30 (8.80-9.80)	<0.001
T/score%	-2.95 (-3.27- -2.60)	0.05 (-0.80-0.38)	<0.001
HBD-3 (ng/ml)	2.84 (2.52-3.36)	0.99 (0.89-1.10)	<0.001

The median (25th and 75th percentiles) of the obtained data were evaluated using the Mann-Whitney test at the 0.05 threshold; The two independent group differed significantly from one another.

Table 3 shows the specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) for studied biomarkers. For each biomarker in the study, the ideal cut-off value was obtained from the ROC curve using the Youden index to determine how well the serum HBD-3 concentration can distinguish between OP cases and healthy individuals using ROC curve analysis, (Figure 1) with enhanced validity high sensitivity and specificity (100.0, 100.0), respectively. The ROC curve, which has an AUC of 1.000 (P-Value <0.001) reached the ideal degree of accurate diagnosis of OP.

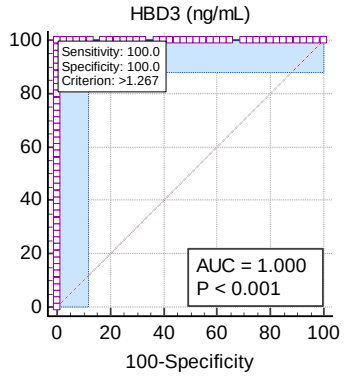

Figure (1): The ROC curve for the predictive value of HBD-3 serum concentration in OP patients (n = 40) compared to controls (n = 40) AUC is 1.000 (95%), *P*-Value 0.001.

Table (3): Validity criteria of test variables to distinguish between groups of people with and without OP

Variable	AUC	P-Value	cut off value	Sensitivity	Specificity	Accuracy	PPV	NPV
HBD-3	1.000	0.001	>1.267	100.0	100.0	1.000	100.0	100.0

*AUC and the ability to discriminate between osteoporosis sufferers and healthy individuals.

Discussion:

The study results showed no significant differences in (age, BMI and WHR) between the OP cases and healthy controls, indicating that age is not related to the severity of OP, and that it may be related to factors like race, lifestyle, diet, prescription drugs and concomitant diseases. This result is in line with the study of Ahmed *et al.* (22), while it disagrees with the study of Alfadhul (10) which showed that younger age groups were more knowledgeable about OP and therefore less susceptible to the disease. When comparing the OP patients to the healthy controls, there was significantly lower concentrations of vitamin D3, Ca+2 among those with OP. This is in line with the results of Jafer, et al. (39) and Farhan, *et al.* (40). The findings of the current study emphasized the role of vitamin D in preventing OP, as all the OP cases had low levels of vitamin D, which may be one of the main causes of the disease. The current study showed a highly significant difference in HBD-3 levels among OP cases compared to healthy controls. The T-score was significantly higher in OP cases. Park, et al. (36) pointed to the role of the human-defensin-3-C15, a component of HBD-3 in inhibiting osteoclast activity to stop bone resorption. The prohibited HBD-3 prevents the rise in tartrate-resistant acid phosphate (TRAP+) multinucleated cell formation that is brought about by RANKL, and inhibits the formation of the RANKL-induced podosome belt, a feature of osteoclasts that are mature and capable of resorbing bone. So HBD-3 was evaluated as an anti-bone resorption agent (36). This

is consistent with the results of the current research, which found high concentrations of anti-inflammatory protein HBD-3 in OP patients compared to controls, indicating its defensive activity against the causes of OP. Mohammed, *et al.* (32), studied rheumatoid arthritis patients and was in agreement with the results of the current study, as they found that higher HBD-3 levels were observed in rheumatoid arthritis cases, an indication that it can be used as a marker to monitor the treatment of the disease, as that disease leads to bone loss and OP over time (32). A high concentration of HBD-3 in OP cases in the current study compared to controls, indicating its defensive activity against the causes of osteoporosis. This confirms its diagnostic role for the disease, and probably suggests future research to explore the potential therapeutic possibilities of HBD-3 for OP patients.

Conclusions:

Serum HBD-3 can be a valuable indicator of OP in middle-aged individuals. The activity of the disease is reflected in the levels of this marker, with OP patients having higher levels of HBD-3 than healthy individuals. Serum HBD-3 levels may be a helpful biological marker for OP diagnosis.

Authors' Declaration:

We hereby confirm that all the Figures and Tables in the manuscript are ours. The Department of Chemistry, College of Science for Women, University of Baghdad approved the project according to the code number (4168 /22 on 26/7/2023).

Conflicts of interest: None.

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Authors' Contributions:

Study conception & design: (Layla Othman Farhan & Zahraa Salim Hassan). Literature search: (Zahraa Salim Hassan). Data acquisition: (Layla Othman Farhan & Zahraa Salim Hassan). Data analysis & interpretation: (Layla Othman Farhan & Zahraa Salim Hassan).Manuscript preparation: (Zahraa Salim Hassan). Manuscript editing & review: (Zahraa Salim Hassan).

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دراسة بيتا-ديفينسين-3 البشري في المرضى العراقيين المصابين بهشاشة العظام

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الخلاصة:
خلفية البحث: هشاشة العظام هي حالة منتشرة مرتبطة بالعمر وتزيد من خطر الإصابة بالكسور وهشاشة العظام، نتيجة فقدان كتلة العظام وكذلك التدهور المعماري الدقيق للعظام، وبالتالي تقليل كتلة العظام وقوتها. بيتا-ديفينسين-3 البشري هو الببتيد المضاد للالتهابات وجزء مهم من جهاز المناعة الفطري البشري.
الأهداف: تقييم دقة تشخيص بيتا-ديفينسين-3 البشري في مصل الدم لدى المرضى الذين يعانون من هشاشة العظام.
الحالات والمنهجية: تمت دراسة ثمانين مشاركا، خضعوا جميعا لفحوصات صحية وتم قياس حالة عظامهم بواسطة قياس امتصاص الأشعة السينية المزدوج الطاقة (DXA) وتحديد مستوى بيتا-ديفينسين-3 البشري في مصل الدم وفيتامين D3 (Vit D3) بواسطة تقنية ELISA. تم قياس الكالسيوم (Ca) باستخدام مقياس الطيف الضوئي. أجريت دراسة مقارنة بين أربعين مريضا يعانون من هشاشة العظام و 40 من الاصحاء. وشملت الدراسة الإناث والذكور الذين تتراوح أعمارهم بين (40-60) سنة. أجريت الدراسة في المركز الوطني للمفاصل في مستشفى اليرموك التعليمي في بغداد خلال الفترة من أيلول إلى تشرين الأول 2023.
النتائج: بالمقارنة مع مجموعات الأفراد الأصحاء، كان مستوى بيتا-ديفينسين-3 البشري في مصل مجموعة هشاشة العظام أعلى بكثير ($P < 0.001$). تم العثور على المنطقة تحت المنحنى (AUC) لتكون (1.000) في تحليل منحنى ROC لمستوى بيتا-ديفينسين-3 البشري في الدم.
الاستنتاجات: يمكن أن يكون مستوى بيتا-ديفينسين-3 البشري في مصل الدم مؤشرا قيما لهشاشة العظام لدى الأفراد في منتصف العمر، وقد يكون علامة بيولوجية مفيدة لتشخيص هشاشة العظام.
مفتاح الكلمات: الكالسيوم، بيتا-ديفينسين-3 البشري، هشاشة العظام، T-score، فيتامين د.