Int J Clin Exp Pathol 2016;9(10):10682-10687
www.ijcep.com /ISSN:1936-2625/IJCEP0037188

Original Article

Endotoxin and bone turnover markers in postmenopausal women with and without osteoporosis

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Received August 3, 2016; Accepted August 21, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Bacterial lipopolysaccharide (LPS) also known as endotoxin, which represents the outer cell wall membrane of gram-negative bacteria has been implicated as the major bacterial bone-resorbing factor. Recently, the most prevalent form of clinically significant osteopenia and osteoporosis involves periodontitis and otitis media by gram-negative bacteria. The aim of the study is to evaluate circulating endotoxin levels and to study the association amongst endotoxin and bone turnover markers in a cohort of Saudi postmenopausal women with or without osteoporosis. We determined the levels of endotoxin, bone turnover markers, 25-OH vitamin D total and corrected calcium in 100 Saudi postmenopausal women with osteoporosis and 100 women without osteoporosis were taken under the supervision of qualified physicians in the primary care centers in Riyadh. Serum endotoxin, NTx, osteocalcin, PTH, 25-OH vitamin D total and calcium were analyzed. Serum NTX and PTH levels in patients with osteoporosis were significant higher than controls. Serum endotoxin was significantly and positively associated with calcium in all subject and controls. Endotoxin was positively associated with NTX in both groups but not with osteocalcin, PTH or 25-OH vitamin D. Findings of the present study implicate a role for endotoxin-mediated inflammation in patients with osteoporosis.

Keywords: Endotoxin, osteoporosis, postmenopausal, 25-OH vitamin D, bone turnover marker

Introduction

Osteoporosis is an important metabolic bone disease characterized by low bone mineral density (BMD) due to calcium and bone protein depletion [1]. The World Health Organization (WHO) [2] operationally defines osteoporosis as having a BMD score >2.5 standard deviations (SD) below the mean for a young, healthy adult woman. Osteoporosis affects 200 million people worldwide [3]. In Saudi Arabia, the prevalence of osteoporosis (≥50 years) is as high as 44.5% in Saudi women and 33.2% in Saudi men [4]. Moreover, the incidence of fragility fractures jumped from 2.9/1000 in 1999 [5] to 6/1000 in 2007 at an annual cost of SR 4.27 billion ($1=SR3.75) [6].

Currently, osteoporosis is viewed as a heterogeneous condition which can occur at any age, but certain populations such as postmenopausal women have a higher risk due to factors such as changes in estrogen and low vitamin D [7]. Studies have reported significant associations between inflammation and osteoporosis. These studies have identified that osteoporosis is more frequent in patients with rheumatoid arthritis [8] and systemic lupus erythematosus [9]. Furthermore, the most prevalent form of clinically significant osteopenia and osteoporosis involves chronic inflammation of bone in disorders such as periodontal disease [10] and otitis media [11].

The physiological activities of lipopolysaccharides (LPS) are mediated mainly by the lipid A component. Lipid A delivered by LPS-binding protein (LBP) to (CD14) on the surface of osteoblasts/stromal cells associates with Toll-like receptor-4 (TLR4) [12]. This complex can stimu-

late receptor activator of nuclear factor kappa-B ligand (RANKL) expression through calcium/protein kinase C signals [13, 14]. RANKL can interact with its receptor RANK on osteoclast precursors and provide essential signals for osteoclast differentiation and maturation [15, 16].

During bone resorption, bone degradation products are produced. These include N-telopeptides of type I collagen (NTx) and carboxy-terminal telopeptides of collagen type I (CTx) into the circulation [17]. On the other hand, during the bone formation process, a number of molecules are released into the circulation such as bone specific alkaline phosphatase, procollagen 1 carboxyterminal propeptide and osteocalcin. These bone turnover markers (BTM) are not used for the diagnosis of osteoporosis because there is a great overlap between values of osteoporotic and non-osteoporotic patients [18]. However, prospective studies have demonstrated greater and faster reductions in BMD in those with higher bone turnover [19]. Low BMD in the presence of high BTM is more predictive of fracture then either risk factor alone [20]. The present study aims to evaluate circulating endotoxin levels and determine whether endotoxin correlates with BTM in Saudi post-menopausal women with and without osteoporosis.

Materials and methods

Study population

This study included a total of 200 Saudi post-menopausal women aged ≥50 years old [N=100 with osteoporosis and N=100 without osteoporosis] recruited from the Primary Care Centers (PCCs), King Salman Hospital and King Fahd Medical City, Riyadh, kingdom of Saudi Arabia (KSA). All measurements were performed systematically from August, 2013 to September, 2014. Participant's history was recorded from a generalized questionnaire including age, age of menarche, age of menopause, family history of osteoporosis, disease status, etc. Ethics approval was granted by the Ethics Committee of the College of Science, King Saud University, Riyadh, KSA.

Bone mineral density BMD (g/cm^2) in the femoral neck was measured using dual-energy X-ray absorptiometry DEXA (Hologic QDR 2000 Inc., Woltham, MA, USA) for all participants. The diagnostic criteria of osteoporosis was based on the T-score for BMD established by WHO (T-score value of -2.5 SD or below the mean for a young healthy adult woman indicate osteoporosis). T-scores value between -1.0 and -2.5 SD indicate osteopenia and T-score value of -1.0 SD or more as normal.

Patients with acute medical conditions that require immediate medical attention and with other associated diseases and inflammatory condition (illnesses fall beyond routine cold or flu infections) and no history of any other bone disease were excluded.

Anthropometry and blood collection

Anthropometry included height (m) and weight (kg), determined using standardized conventional methods. Waist (cm) and hip circumferences (cm) were obtained using a standardized non-stretchable fiber measuring tape, Waist-to-hip ratio (WHR) was calculated as the ratio of waist and hip circumferences. Blood pressure (systolic and diastolic in mmHg) was also measured. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2).

Five (5) ml fasting venous blood samples were collected in tubes without anticoagulant (serum separator tubes). Samples were then left to clot at room temperature for 30 minutes, and then centrifuged at 5000 rpm for 10 minutes. After that the collected sera was transferred to pre-labeled plain tubes and stored in ice before being transported to\Biomarkers Research Program (BRP) in King Saud University, Riyadh, KSA, for immediate storage at -80°C until analysis.

Sample analysis

Serum endotoxin was analyzed using a commercially available QCL-1000 LAL End Point Assay. Kits were purchased from Lonza, U.S. License No. 1775. Catalog Number: 50-647U. The intra- and inter-assay coefficient of variation (CV) were (3.9 60 0.46)% and (9.6 60 0.75)% respectively.

Serum NTx was measured using a competitive-inhibition enzyme-linked immunosorbent assay (ELISA) kits purchased from Alere Scarborough, Inc. 10 Southgate Road Scarborough, ME

Table 1. Differences in clinical, anthropometric characteristics, and biochemical parameters of controls versus cases

Parameter	Control	Osteoporosis	*P*-value	*Adjusted for Age, BMI, Menarche and Age of Menopause*
N	100	100		
Age (years)	55.2 ± 8.8	56.2 ± 7.2	0.374	
BMI (kg/m^2)	34.4 ± 5.7	31.1 ± 5.9	<0.001	
Menopause (years)#	5.6 ± 4.9	10.6 ± 7.2	<0.001	
Menarche age (years)	13.1 ± 1.5	13.6 ± 1.8	<0.030	
Waist circumference (cm)	103.8 ± 15.0	98.2 ± 15.9	<0.008	
Hip circumference (cm)	114.9 ± 13.1	108.1 ± 14.8	<0.001	
Waist-Hip ratio	0.9 ± 0.1	0.9 ± 0.1	0.321	
Systolic Blood Pressure (mmHg)	123.8 ± 21.2	128.4 ± 19.6	0.110	
Diastolic Blood Pressure (mmHg)	77.1 ± 14.9	76.3 ± 12.6	0.662	
Parathyroid Hormone (pg/ml)#	41.9 ± 87.3	7.06 ± 106.9	<0.038	
BMD lumbar volume (g/cm^2)	-0.2 ± 0.6	-3.1 ± 0.5	<0.001	0.000
Endotoxin (IU/ml)	1.9 ± 0.9	2.1 ± 1.1	0.329	0.206
NTX (nMBCE)#	61.0 ± 44.6	76.8 ± 56.2	<0.035	0.287
Osteocalcin (ng/ml)	11.3 ± 6.3	11.1 ± 6.9	0.885	0.193
25(OH) Vitamin D (nmol/l)	59.2 ± 30.8	63.2 ± 35.4	0.418	0.828
Calcium (mmol/l)	2.3 ± 0.2	2.3 ± 0.2	0.109	0.233

Note: Data presented as mean ± SD; significant at P<0.05.

04074 USA. Reference 9021. Intra- and inter-assay CV were 4.6% and 6.9% respectively. Osteocalin and 25-OH vitamin D were determined by electrochemiluminescence immunoassay, kit purchased from (Roche Diagnostics, Mannheim, Germany). Intra- and inter-assay CV were 4% and 6.5% respectively. BRP is an accredited laboratory by the 25-OH vitamin D External Quality Assessment Scheme (DEQAS). Serum PTH was determined using Luminex Multiplex Assay System (Luminex Inc.). (Intra- and inter-assay CV were 4% and 9% respectively). Serum calcium was carried out using a chemical analyzer (Konelab, Espoo, Finland) kit purchased from Thermo Fisher Scientific Oy, ref 981367. Intra- and inter-assay CV were 0.2% and 0.4% respectively.

Statistical analysis

Data was analyzed using SPSS version 16.5 (Chicago, IL, USA). Continuous data were represented as mean ± SD. Categorical data was presented as frequencies and percentages (%). Continuous variables were checked for normality (Kolmogorov-Smirnov test). Differences between groups were done using Student T-test. For non-Gaussian variables, Mann-Whitney U test was done to compare groups. Associations between variables were determined using Spearman's correlation. Univariate and multivariate linear regression analysis were performed to identify independent factors affecting endotoxin. A *P* value <0.05 was considered statistically significant.

Results

Differences in the clinical, anthropometric and biochemical characteristics of subjects are summarized in **Table 1**. The mean age of subjects with and without osteoporosis was not statistically different from one another. There was a significant difference with respect to BMI, menopause, menarche age, waist and hip circumference (P<0.001), (P<0.001), (P<0.03), (P<0.008), (P<0.001) respectively. The BMD lumbar volume was significantly lower in the osteoporosis group than controls even after adjusting for age, BMI, menarche and age of menopause (P<0.001). There was a significantly higher NTx and PTH levels in the osteoporosis group than controls (P<0.035), (P<0.038) respectively. These significant differences were lost after adjusting for confounders. No other significant differences were elicited.

The bivariate associations of endotoxin with anthropometric and biochemical metabolic markers of all subjects, controls and cases are

Table 2. Bivariate associations of endotoxin with anthropometric and biochemical metabolic markers of all, controls and cases cohort

Parameter	All	Control	Osteoporosis
N	200	100	100
Waist circumference (cm)	-0.04	-0.02	-0.04
Hip circumference (cm)	0.04	0.02	0.14
Menarche Age (years)	0.07	0.08	0.001
Menopause (years)#	0.01	-0.08	0.03
First pregnancy age (years)	0.12	0.07	0.21
Systolic blood pressure (mmHg)	0.02	-0.06	-0.09
Diastolic blood pressure (mmHg)	-0.03	0.03	0.000
BMI (kg/m^2)	-0.03	-0.15	-0.22
BMD femoral neck volume (g/cm^2)	0.11	0.17	-0.16
NTX (nM BCE)#	0.09	0.15	0.02
Osteocalcin (ng/ml)	-0.0001	-0.04	-0.02
25(OH) Vitamin D (nmol/l)	-0.11	-0.1	-0.09
Parathyroid Hormone (pg/ml)#	0.05	-0.008	-0.08
Calcium (mmol/L)	0.24**	0.48**	0.10

Note: **denotes significance at 0.01 level.

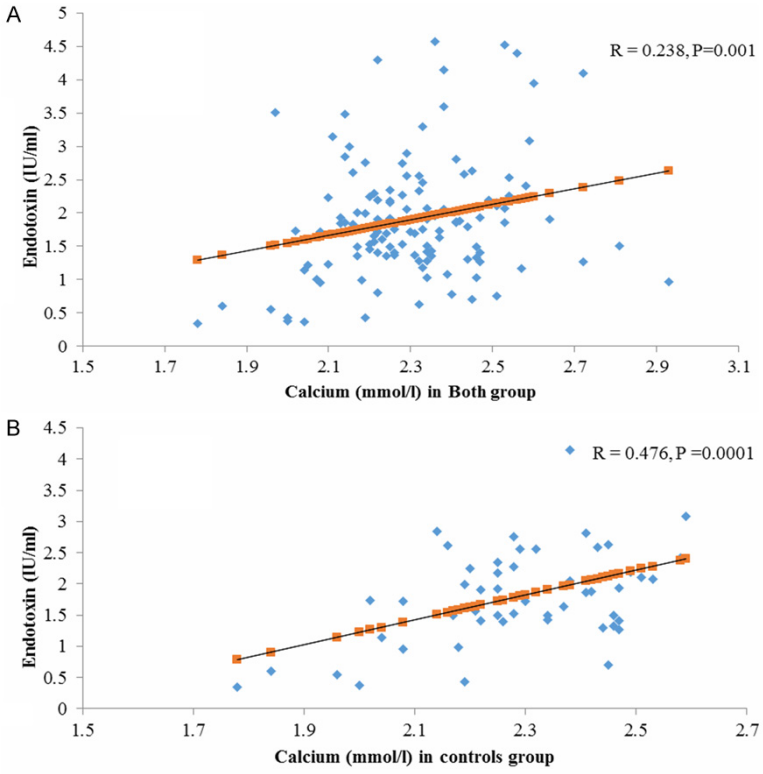

Figure 1. A. Correlation between endotoxin and calcium concentrations in all subjects; B. In control group.

summarized in **Table 2**. Serum endotoxin showed a significant positive association with calcium in all subjects and controls (r=0.238, P<0.001; r=0.476, P<0.001, respectively). A significant positive correlation was found between endotoxin and calcium in all subjects (**Figure 1A**) and controls (**Figure 1B**). Results also showed positive but insignificant association between NTX in all subjects. No significant associations were found between endotoxin with waist, hips, menopause, menarche, age of 1st pregnancy, blood pressure, BMI, BMD, osteocalcin, 25(OH) vitamin D and PTH.

Discussion

In the present study, we determined the associations between endotoxin with BTMs in postmenopausal Saudi women with and without osteoporosis. Data obtained showed significantly lower BMI in postmenopausal women patients with osteoporosis than controls, which is in agreement with the previous observations of Reid [21]. Furthermore, a significant positive association between circulating endotoxin and calcium was elicited. LPS have been recently observed to upregulate calcium concentrations in animal models through T-type calcium channels activated by the NFκB/ET-1 signaling pathway [22]. In humans, LPS also induce intra- and extracellular calcium increase in human lung epithelial and microvascular endothelial cells, highlighting its role in calcium mobilization [23]. The increase in extracellular calcium is parallel to higher endotoxin levels and may represent higher bone resorption observed in patients with osteoporosis.

Endotoxin was previously described to partially cause osteoclast formation. Abu-Amer et al. [24] have shown that endotoxin stimulated osteoclast formation in vivo and in vitro. On the other hand, several studies demonstrated that LPS was a potent stimulator of various pro-inflammatory cytokines and mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and prostaglandin E2 (PGE2), produced chiefly by macrophages and T-lymphocytes. These cytokines play an important role in the stimulation of RANKL and maturation and differentiation of osteoclasts and bone loss [25]. Surprisingly, the mechanism of endotoxin action is not fully understood. Sismey-Durrant and Hopps reported that bacterial endotoxin failed to activate osteoclasts using purified Porphyromonas gingivalis [26].

The relationship between BTM and osteoporosis has been previously analyzed. In an observational study among osteoporosis patient, significantly higher NTX levels were observed in osteoporosis [27]. Bone degradation process involves breakdown of type I collagen as CTx and NTx are used to determine the rate of bone resorption [18, 28]. These observations are in agreement with our finding since NTx was higher in women with osteoporosis than controls. These results also support a high rate of bone resorption in women with osteoporosis. Moreover as expected, the BMD values were significantly lower in patients with osteoporosis than controls. These results suggest that low BMD in the presence of high bone markers is more predictive of fracture than either risk factor alone [20]. Furthermore, though insignificant, results showed positive associations between endotoxin and NTX in all subjects. Therefore, we hypothesize that endotoxin, as a marker of inflammation, could be significant positively correlated to NTX and negatively correlated with osteocalcin.

A significantly higher PTH levels in the women with osteoporosis than controls was observed similar to our previous findings [27]. PTH indirectly stimulates bone resorption though its receptors on osteoblasts. Binding PTH to its receptor, this occurs via activation of heterotrimeric Gs protein to increase PKA activity and thereby cAMP-mediated transcriptional activity. IGF-1 is essential for this activity. PTH1R also stimulates osteoblasts to increase expression of RANKL via activation of cAMP/PKA pathway and subsequently MAPK, result in an increase in RANKL and CSF, and a decrease in the decoy receptor OPG, which enhance the recruitment of osteoclast precursors to form osteoclasts in a controlled manner, lead to stimulate osteoclastogenesis In the kidney, PTH suppresses reabsorption of phosphate, while stimulating tubular reabsorption of calcium [28].

The authors acknowledge several limitations. The cross-sectional study design cannot suggest any causal and temporal correlations. Further investigations are needed to improve our understanding of endotoxin implication in inflammation in large scale to estimate association endotoxin with BTM, also to establish on cytokine pattern such as TNF-α, IL-1 and PGE2 and bone metabolism.

In conclusion, serum endotoxin was positively associated with NTx, PTH, and calcium. Prospective studies are needed to determine whether elevated endotoxin levels constitute osteoporosis risk.

Acknowledgements

This project was funded by the national plan for science for science, Technology and Innovation (MAARIFAH), King Abdulaziz City for science and Technology, kindom of Saudi Arabia, Award number (11-MED-2113-02).

Disclosure of conflict of interest

None.

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