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Osteoprotegerin (OPG) levels, total soluble receptor activator of nuclear factor-Kappa B ligand (total sRANKL), and RANKL/OPG ratio in patients with rheumatoid arteritis

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Rheumatoid arthritis (RA) is one of most important collagen vascular diseases. It has an unknown origin. The aim of this study was to evaluate circulating levels of osteoprotegerin (OPG), total soluble receptor activator of nuclear factor-Kappa B ligand (total sRANKL), and RANKL/OPG ratio in patients with RA. Forty-five females with RA, who fulfilled the American college of rheumatology (ACR) criteria for RA were included in this cross-sectional study. The overall disease activity was evaluated by the disease activity score based on 28 joint counts (DAS-28). The OPG and sRANKL were measured by the enzyme-linked immunosorbent assays (ELISA). The levels of C-reactive protein (CRP) were measured by ELISA.We used Pearson's correlation for our comparisons. There was no statistically significant difference between the levels of CRP, OPG, sRANKL and RANKL/OPG ratio in terms of DAS-28 grades in our patients. No significant correlation was found between the serum levels of OPG and DAS-28 (P= 0.525), duration of the disease (P= 0.884), Z-score of the femur (P= 0.546) and Z-score of the spine (P= 0.492), T-score of the femur (P= 0.137) and T-score of the spine (P= 0.821) in the patient group. No significant correlations were found between sRANKL levels with DAS-28 (0.919), Z-score of the femur (P= 0.971), Z-score of the spine (P= 0.832) and T-score of the femur (P = 0170) in the studied groups. Our study showed that there was no significant correlation between CRP, OPG, sRANKL and RANKL/OPG ratio in DAS-28 grading of our patients. For this reason they will not be used for evaluating disease activity. However, there was a significant difference between case and control groups except for sRANKL (pg/mL).

Keywords: ACR criteria, OPG, rheumatoid arthritis, sRANKL.

Introduction

Rheumatoid arthritis is a chronic disease, which involves several systems in the body. Even though it has an unknown cause, interventions of immunological factors have been considered as effective factors in RA. Different studies have indicated that the activity of the disease and the process of inflammation in RA have a close correlation with joint destruction, osteoporosis and finally disability [1, 2]. Patients with RA have higher cardiovascular mortality and morbidity compared with the healthy population [3, 4]. Endothelial Dysfunction (ED) is a major risk factor for the development of atherosclerosis and subsequent cardiovascular events [5,6]. Inflammation also has an important role in the formation of atherosclerosis via inducing vascular calcification (VC). Vascular calcification, in turn, mediates cardiovascular disease (CVD) in these patients [7, 8]. Proinflammatory cytokines and plasma acute phase reactant proteins levels are increased in RA patients. The disease is associated with cartilage breakdown, juxtaarticular and generalized bone loss and also reduced bone mass. Mineral metabolism abnormalities cause osteodystrophy at the beginning and induce CVD in RA patients. Bone resorption causes calcium efflux from bone to plasma and results in precipitation of calcium through intima and media of vessel walls leading to vascular calcifications. Bone regeneration is complex and is mediated by systemic and local factors that affect osteoclasts and osteoblasts activation [9, 10]. The process of coordinated resorption

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and formation of bone may be up or down-regulated by the receptor activator of nuclear factor-ƙB ligand (RANKL), osteoprotegerin (OPG), prostaglandin E2 (PGE2), systemic hormones (PTH, calcitriol) or local factors (interleukins [IL-1, IL-6], growth factors [TNF-a], and insulin-like growth factor-1) [11-13]. Osteoprotegerin belongs to the TNF receptor super family and regulates bone resorption and absorption processes mainly by inhibiting osteoclastic bone resorption. Furthermore, RANKL is a homotrimeric trans-membrane protein member of the TNF receptor super family located on osteoclast and dendritic cells [14, 15]. The RANK mediates differentiation of osteoclast and their functional activation [16, 17]. It is the main stimulatory factor for osteoclastogenesis providing an essential signal to osteoclast progenitors signal transduction [18]. It has been shown that imbalance of RANKL/OPG ratio could be related to the pathogenesis of bone metastases and secondary hypercalcemia [19, 20].

We studied atherosclerosis and vascular calcification factors including the levels of OPG, RANKL and their relationship with Bone Mineral Density (BMD) indexes in female RA patients.

Methods and Materials

Patients

This study was performed in clinics related to our rheumatology department of Tabriz University of Medical Sciences (TBZMED) and enrolled patients were referred to these clinics. This was a type of cross-sectional study. The ethical committee of TBZMED approved the study according to the declaration of Helsinki. All candidates filled a written consent and they were allowed to leave the study at any point (Code: TBZMED.REC.1387.34).

The patients were recruited from April 2011 to November 2012. Regarding previous studies, forty-five females with definite RA according to the American college of rheumatology criteria were enrolled in the study and five patients were excluded [21]. Sample size calculations were performed using power and sample size calculation (PS) version 3.1.2. Based on information obtained from a pilot study by focusing on serum OPG levels, it was estimated that 45 patients are required to achieve 95% confidence interval and a power of 80%. Given the dropout rate of 10%, the sample size increased to 50 in the study group. Finally, 45 patients were enrolled. The exclusion criteria were: Body Mass Index (BMI) > 30 kg/m^2, history of smoking or alcoholism, cancer, coronary heart diseases, uncontrolled hypertension (systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg), diabetes mellitus, nephrotic syndrome, hepatic, renal, cardiac or genetic diseases, cushing syndrome, thyroid disorders, or other metabolic diseases and treatment with micronutrients and anti-oxidants supplements, lipid-lowering drugs and hormone replacement therapy. The patients with changed medication schedule in the previous two months and during the study period were also excluded. We used the simple sampling method. Medication schedule was kept constant throughout the study period. The patients were not under standard protocol of RA treatment because their disease was in the early phase and were not receiving any drug treatment (except for Prednisone and non-steroid inflammatory drugs). Blood samples were obtained after overnight fasting and the separated serums were collected and stored at -70°C until laboratory tests were done.

Clinical and para clinical experiments

Clinical examination was carried out by a rheumatologist and disease activity score of 28 (DAS-28) was calculated using the numbers of swollen and tender joints, (hs-CRP) and patient general health using the visual analog scale (VAS) [21, 22]. The patients were divided to the case groups according to their DAS, under 3.2 (low grade), between 3.2 and 5.1 (medium grade) and more than 5.1 (high grade), and the control group consisted of healthy individuals without RA that were matched with the case group in terms of demographic features following history report and physical examination; they were chosen with consideration of the inclusion and exclusion criteria. Measurement of bone density was done by the means of USA set of Hologic QDR 4500 elite and methods of energy X-Ray absorptiometry dual. In this method, the rate of T-score (density of bone in comparison with young people), Z-score (density of bone in comparison with their peers), bone mineral density (BMD) and bone mineral content (BMC) were studied. A T-score of more than +1 was considered as “very high T-score”, between +1 and -1, was considered as “normal T-score”, between -1 and -2.5 as “osteopenia T-score” and more than -2.5, as “Osteoporosis T-score” [23]. The levels of C-reactive protein (CRP) were measured by the ELISA method using high sensitive monobind kits (Pars Azmoon Co.). Serum receptor activator of nuclear factor-ƙB ligand was measured by immunoassay (Biomedia Mediziprodukte GmbH, Wien, Austria). The method was designed to detect soluble free human RANKL

directly in the serum. In brief, human sRANKL binds to the precoated recombinant OPG and forms a sandwich with the detection antibody. Following a wash, streptavidin-horse radish peroxidase (HRP) conjugate was added to the wells and after addition of substrate, the sRANKL was quantitated by an enzyme catalyzed color change. The analytical limit detection of the assay was 0.02 pmol/L, with inter-assay coefficient of variation (CV) of 3% and intra-assay CV of 9%. The OPG was determined by sandwich ELISA (Boster biological technology, Wauhan, China) method with sensitivity of < 5 pg/mL, which detects both monomer and dimer forms in human.

Statistical analysis

Statistical analysis was performed using the SPSS software version 18. Values were expressed as the mean±standard deviation (SD), frequency and percentage as appropriated. Differences among groups were assessed by Mann–Whitney U test, independent samples T test and one way analysis of variance (ANOVA) as appropriate. Spearman's coefficient was calculated to determine the correlation between biochemical parameters. P values of <0.05 were considered statistically significant.

Results

Demographic characteristics of the RA and control subjects are shown in Table 1. The control group consisted of healthy individuals without RA that were matched with the case group in terms of demographic features following history report and physical examination; they were chosen with consideration of the inclusion and exclusion criteria. There was no significant difference in the mean age and BMI between the two groups. Laboratory findings of the patients with RA and control groups are seen in Table 2. As it is clear in the content of Table 2, only sRANKL (pg/mL) was not statistically significant between the control and case groups (P= 0.49).

Laboratory findings in the patients with RA according to the DAS grades are shown in Table 3. There were no statistically significant differences between the HsCRP, OPG, sRANKL and RANKL/OPG ratio in terms of DAS grades in the patient group.

Table 4 summarizes the findings in the patients with RA according to the T-score grades.

The reverse correlations of sRANKL levels with disease duration (r= -0.34, P= 0.024) was seen and direct correlation was detected between sRANKL levels with T-score in the patient group (r= +0.41, P= 0.005) (Figs. 1 and 2). No significant correlation was found between serum OPG levels and DAS-28 (P= 0.525), duration of the disease (P= 0.884), Z-score of the femur

Table 1. Descriptive statistics for general characteristics of the study subjects with rheumatoid arthritis and control groups[a]

Characteristics	Categories	Patient Group	Control Group	P Value
Age (years)		40.7±10.7	42.8±12.5	0.4
BMI (kg/m^2) (1)		25.3±3.8	24.7±3.9	0.6
Disease duration (month)		20.51±20	-	
Das-28		3.93±0.48	-	
Tender joint count	< 5	(68%)	-	
	5-10	(28%)	-	
	> 10	(2.2%)	-	
Disease Activity	Low Activity, DAS<3.2	4 (8.9%)	-	
	Medium Activity, DAS:3.2-5.1	41 (91.1%)	-	
	High Activity, DAS>5.1	0 (0%)	-	
Swollen joint count	< 5	(82.2%)	-	
	5-10	(17.8%)	-	

[a] Mean±SD for continues variables and No. (%) for the categorical variables.

Table 2. Results of laboratory findings of patients with rheumatoid arthritis and control groups

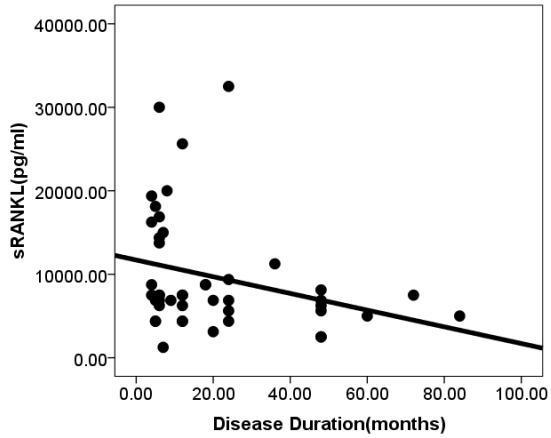
Characteristics	Patients group	Control group	Correlation	P Value
CRP (mg/dl)	1.27±2.95	2.95±1.27	0.863	0.001
OPG (pg/ml)	18.73±103.7	176.1±28	0.741	0.001
sRANKL (pg/ml)	108.2±9652.7	631.8±8986.1	0.134	0.49
sRANKL/OPG ratio	161.2±141.5	79.6±87.2	0.782	0.002

Table 3. Results of laboratory findings of patients with rheumatoid arthritis according to the DAS grades

Characteristics	Low activity, DAS < 3.2	Medium activity, DAS: 3.2-5.1	P Value
CRP (mg/dl)	7.6±4.6	13.5±10.8	0.25
OPG (pg/ml)	95.1 ±26.9	107.5±11.0	0.92
sRANKL (pg/ml)	10000.0±9652.7	9618.9±673.3	0.48
RANKL/OPG ratio	190.1± 26.6	158.4±11.8	0.6

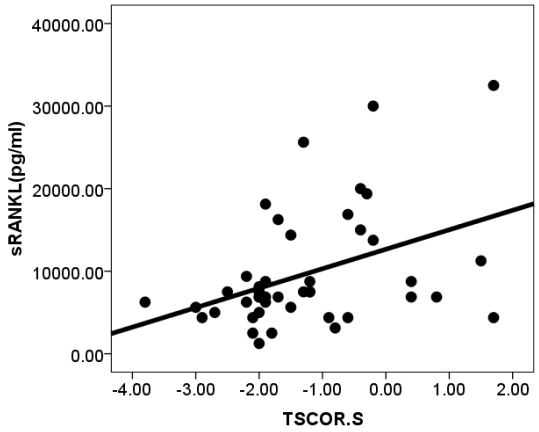
Table 4. Results of laboratory findings of patients with rheumatoid arthritis according to the T-score grades

Characteristics	Normal T-score, -1> T-score> +1	Osteopenia, -1> T-score> -2.5	Osteoprosis, -2.5> T-score	P Value
CRP (mg/dl)	14.0±10.9	13.3±11.0	8.7±5.4	0.51
OPG (pg/ml)	79.3±55.3	119.2 ±156.1	83.8±45.9	0.63
sRANKL (pg/ml)	12644.2±821.4	8865.7±667.9	6125.0±703.2	0.17
sRANKL/OPG ratio	210.4±178.3	151.1±128.8	87.8±41.7	0.19



Spearman's coefficient was calculated to determine the correlation between biochemical parameters (r= -0.34, P= 0.024).

Fig. 1. The correlations between sRANKL levels and disease duration in the patients group



Spearman's coefficient was calculated to determine the correlation between biochemical parameters (r= +0.41, P= 0.005).

Fig. 2. The correlations between sRANKL levels and T-score in the patient group

(P= 0.546) and Z-score of the spine (P= 0.492), T-score of the femur (P= 0.137) and T-score of the spine (P= 0.821) in the patient group. On the other hand, there was no significant correlation between sRANKL level with DAS-28 (P= 0.919), Z-score of the femur (P= 0.971) and Z-score of the spine (P= 0.832), and T-score of the femur (P= 0170) in the patient group. There were significant associations between RANKL/OPG ratio with CRP (P= 0.013), disease duration (P= 0.028), femur T score (P= 0.030) and spine T-score (P= 0.042).

Discussion

The roles of OPG/RANK/RANKL system in pathogenesis of osteodystrophy have been shown in many studies [24, 25]. It has been demonstrated that the OPG/RANK/RANKL system has an important role in vascular calcification and bone disorders by different mechanisms and mainly by cytokine misbalancing [22, 26]. Conflicting results have been described regarding prevention or the induction role of OPG for arterial calcification [24, 27, 28]. Although OPG could primarily prevent arterial calcification, its secretion secondary to inflammatory processes could mediate an arterial calcification [29-31]. It seems the latter effect is due to expression and up regulation of endothelial OPG, which belongs to the TNF-a super-family. Evidence also suggests that OPG may act as a proinflammatory molecule and inducer of vascular calcification and atherosclerosis [32, 33]. The OPG can inhibit the production and differentiation of osteoclasts by binding the RANKL and acting as a decoy receptor to inhibit RANKL interaction with its RANK, because of which bone resorption is inhibited. In the present study, RA patients showed increased CRP and OPG as inflammatory markers, suggesting persistent inflammatory vascular disease and atherosclerosis, which is implicated by the OPG/RANK/RANKL system.

The increased CRP serum level that represent immune-inflammatory activity, with the presence of activated immune cells as source of RANKL, which could

interact with its RANK receptor in endothelial cells and osteoclasts, certainly played an important role in the atherosclerotic and osteogenic process in our studied RA patients.

The results of the present study showed that OPG serum level was markedly high in patients with RA. In addition, we also found that sRANKL was correlated to T-score and inversely correlated to disease duration, which was consistent with the study of Ziolkowska et al., in which serum level of OPG was significantly higher in patients with RA [34] and the study of Isioro, who could not find a significant association between RANKL serum levels with CRP and duration of disease in RA patients [35].

The reason for having higher circulating OPG level in RA patients may represent the rapid bone loss period and defense mechanism for resistance to rapid bone loss. Studies on the general population have shown that increased serum OPG is related to increased risks for osteoporosis and vertebral fracture in women. These results are consistent with that of the RA patients in our study [35, 36].

Although the elevation of OPG may be due secondary to vascular damage and active inflammatory processes, its meaningless association with DAS grade, disease duration and T-score indicate that many confounding factors such as malnutrition, inflammation and the type of drugs may have important roles on the level of serum OPG and RANKL levels in RA patients. However, in this study, similar to the study of Ueland et al., no association between serum OPG level and densitometry results (Z-score and T-score) were found [37].

Although in the present study we could not find any significant differences between CRP, OPG and sRANKL in different DAS grades and T-scores, a significant negative correlation was observed between sRANKL and disease duration and positive correlations with T-score in RA patients. The RANKL has been demonstrated in the extracellular matrix surrounding the calcium mineral deposit of plaques [38]. Moreover, RANKL transcripts were detected in the calcified arteries of OPG-deficient mice [31]. These findings suggest that RANKL may be involved in the activation of osteoclasts and the consequent promotion of bone resorption, which decreases T-score in the RA patients. Increased RANKL can interact with RANK receptor in endothelial cells and osteoclasts that may play an important role in the osteogenic process and atherosclerotic process in the patients. These results may indicate the persistence of other mechanisms in addition to the immune inflammatory process, which accompanied with activated immune cells as a source of RANKL and induce many cytokines that increase the expression of RANKL in these patients, and suggests an association between bone reduction and vascular disease in RA patients. These findings indicate a pathophysiological link between calcification and osteoporosis related diseases [39].

The results of the present study indicated that although the estimation of OPG levels in the detection of significant marker of bone mineral deficit in RA patients may be clinically useful, there was no significant association between the serum level of OPG, RANKL and the ratio of RANKL/OPG, and the activity of RA. However, it seems that OPG level for evaluating the disease for a long period is not an appropriate marker and does not have adequate adaptation conformity with densitometry results.

All of our patients and controls were women (the majority of patients had RA), thus we cannot generalize the results. On the other hand, it will be better to do this study with a bigger sample sizes. Some other weaknesses of our study were the lack of the measurement of the Intima-Media Thickness (IMT) and radiological scores. Also, the possible effects of consumed drugs in RA patients on OPG as well as hs-CRP levels are major limitations of our study.

Conclusion

Our study showed that there was no significant correlation between hsCRP, OPG, sRANKL and RANKL/OPG ratio in DAS-28 grading of our patients. For this reason they will not be used for evaluating disease activity. However, there was a significant difference between case and control groups except for sRANKL (pg/mL).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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