Comparison Bone Mineral Density, Receptor Activator Nuclear Factor Kappa B Ligand, Osteoprotegerin and Some of Bone Metabolism Parameters Between Chronic Hepatitis B Patients and Healthy Controls

Kronik Hepatit B Hastalarında Kemik Mineral Yoğunluğu, Reseptör Aktivatör Nükleer Faktör Kappa B Ligand, Osteoprotegerin ve Bazı Kemik Metabolizma Parametrelerinin Sağlıklı Kontrollerle Karşılaştırılması

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ABSTRACT Objective: Osteoprotegerin (OPG), receptor activator nuclear factor kappa B (RANK), receptor activator nuclear factor kappa B ligand (RANKL) are important for osteoclast production, fusion, activation, and apopitosis. The effect of OPG is to supress the activity of mature osteoclasts. RANKL differentiates and activates osteoclasts. We intended to investigate the relation between bone mineral density (BMD), bone turnover parameters, OPG and RANKL serum levels in non cirrhotic chronic hepatitis B patients and them to compare with healthy controls. **Material and Methods:** 16 patients admitted to the gastroenterology and infectious disease policlinics with the diagnosis of chronic hepatit B infection without any previous hepatitis B virus infection treatment and 31 healthy controls were included to study. OPG and RANKL serum levels and other bone metabolism markers were measured in all patients. BMDs of all subjects were measured by Dual-X-ray Bone Absorbtiometer. **Results:** There were not statistically significant difference between chronic hepatitis B and control group's mean lomber and femur neck bone mineral density values. Serum OPG levels were higher in the chronic hepatitis B group compared to control (p= 0.029). On the other side, RANKL serum level was found lower in chronic hepatitis B group (p= 0.004). **Conclusion:** BMD values between groups were not statistically different. There were not any correlation between bone metabolism parameters, BMD, OPG and RANKL serum levels. Increased level of OPG and reduced RANKL level in chronic hepatitis B patients may probably results from inflamation in the liver instead of change in bone metabolism.

Key Words: Hepatitis B; osteoprotegerin; RANK ligand

ÖZET Amaç: Osteoprotegerin (OPG), reseptör aktivatör nükleer faktör kappa B (RANK), reseptör aktivatör nükleer faktör kappa B ligand (RANKL) osteoklast üretimi, füzyonu, aktivasyonu ve apopitozu için önemlidir. OPG'nin etkisi erişkin osteoklastların aktivitesini baskılamaktır. RANKL osteoklastların aktifleşmesini ve farklılaşmasını sağlar. Biz kemik mineral yoğunluğunun (KMY), kemik döngü parametreleri, OPG, RANKL serum düzeyleri ve diğer kemik metabolizma belirteçleri ile ilişkisini siroz olmayan kronik hepatit B tanılı hastalarda araştırmayı ve bunları sağlıklı kontroller ile karşılaştırmayı planladık. **Gereç ve Yöntemler:** Gastroenteroloji ve enfeksiyon hastalıkları kliniklerine başvuran tedavi almamış 16 kronik hepatit B hastası ile 31 sağlıklı kontrol hastası çalışmaya alındı. OPG ve RANKL serum düzeyleri ve kemik metabolizma belirteçleri ölçüldü. Dual X-ray Kemik Absorbtiometre ile deneklerin kemik mineral yoğunluk ölçümü yapıldı. **Bulgular:** Kronik hepatit B hastaları ile kontrol grubu arasında femur boyun ve ortalama lomber vertebra kemik mineral yoğunluğu ölçümleri arasında istatistiksel olarak anlamlı bir fark izlenmemiştir. Kronik hepatit B grubunda OPG serum düzeyleri istatistiksel olarak anlamlı yüksek bulunmuştur (p= 0.029). Diğer taraftan, RANKL serum düzeyi kronik hepatit B grubunda istatistiksel olarak anlamlı düşük saptanmıştır (p= 0.004). **Sonuç:** Gruplar arasında KMY değerleri arasında anlamlı bir fark tespit edilmedi. Kemik metabolizma parametreleri, KMY, OPG ve RANKL serum seviyeleri arasında korelasyon saptanmadı. Kronik hepatit B hastalarında artmış OPG ve azalmış RANKL serum düzeyleri kemik metabolizmasındaki değişiklikten ziyade karaciğerdeki inflamasyondan kaynaklanıyor olabilir.

Anahtar Kelimeler: Hepatit B; osteoprotegerin; RANK ligandı

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Osteoporosis can be defined as a skeletal disease characterized by reduced bone strength and increased fracture risk.[1] Osteoporosis is a worldwide common pathology with high morbidity.[2]

The structure and function of bone depends on the ongoing bone turnover. Remodeling of the bone are carried out by two different cell groups namely osteoblasts and osteoclasts. Under physiological conditions, bone resorbtion and mineralization is a balanced continuous process. This balance was impaired under pathologic conditions. If bone loss overcomes construction osteoporosis appears.

Cytokines, growth factors, and hormones all seems to affect this homeostasis. The key molecules that are responsible for osteoclast differentiation are osteoprotegerin (OPG) and receptor activator nuclear factor kappa B ligand (RANKL). RANKL is a tumor necrosis factor (TNF) family member. In vitro, without macrophage-colony stimulating factor (M-CSF), RANKL stimulate mature osteoclasts to induce bone resorption. All these RANKL effects could be blocked by OPG.[3] RANKL shows its effect on osteoclast differentiation and activation with binding to the high affinity receptor on osteoclasts.[3] RANKL could be induced by almost all factors that bring about bone resorbtion and hypercalcaemia. This effect was antagonized by OPG that binds and blocks RANKL.[4] OPG has been accepted as a decoy receptor because it has no transmembrane component. That means, it has no effect in the absence of RANKL. TNF-α, IL-18, IL-1, α steroid hormones, estrogen, 1,25 dihydroxy vitamin D3, tumor growth factor (TGF)-β, bone morphogenic protein (BMP) are molecules that known to increase OPG mRNA synthesis. On the other hand glucocorticoids, parathyroid hormone, cyclosporine A, prostaglandin E2, basic fibroblast growth factor are molecules that suppress OPG mRNA synthesis. Activation, differentiation and survival of osteoclasts are regulated by a balance between RANKL and OPG.

Chronic liver disease because of increased mortality and severe complications is a catastrophic disease. Osteoporosis is also a complication of liver cirrhosis.[5-7] Because of improving conditions and health care; cirrhotic patients live longer and experience cirrhotic bone disorders. Increased osteoclastic activity was shown in chronic liver disease patients in addition to reduced osteoblastic activity.[8] OPG/RANKL/RANK pathway is one of the most important pathways in the pathogenesis of osteoporosis in chronic liver disease. Cirrhotic patients show reduced osteoid thickness, osteoblastic surface, and bone formation rate.

On the other side; there are few studies which investigate the effect of chronic hepatitis B virus infection on bone turnover. Reduced bone mineral density in non-cirrhotic chronic hepatitis B and C patients was shown.[9] Although, there is a lot of study which evaluates cytokines, bone metabolism, and osteoporosis in cirrhotic patients, there is not much in non-cirrhotic hepatitis C and B patients. The etiology of osteoporosis detected in chronic hepatitis B patients was not exactly known. Because of that we evaluated the relation between bone mineral density, bone turnover parameters, serum RANKL and OPG levels in non-cirrhotic hepatitis B patients.

MATERIAL AND METHODS

We evaluated 16 new diagnosed chronic hepatitis B and 31 healthy control patients admitted to Gastroenterology and Infectious Diseases departments. According to conversation and physical examination; body mass index, cigarette smoking, physical activity, menstruation, gestation, coffee consumption of patients were noted. Patients who have consumed corticosteroid, calcium, vitamin D, and the other drugs effecting bone metabolism were excluded from the study. Patients taking hormone replacement treatment were excluded from the study. Positive serologic signs for hepatitis B infection have been shown with positive polymerase chain reaction in chronic hepatitis B patients for at least 6 months. Exclusion criteria's are the presence of antibody against hepatitis D and C virus, HIV positivity, 1/160 or higher titer of nuclear antibody (ANA) positivity or history of autoimmune hepatitis, decompensated liver disease, metabolic liver

disease, poor clinical condition, diabetes mellitus, malnutrition, cardiomyopathy, neurologic, metabolic, autoimmune, neoplastic disease. Postmenopausal women have also been excluded from the study. Liver biopsy was performed to all hepatitis B patients. Knodell Method was used for histopathological grading.

BONE DENSITOMETRY

DXA, bone density evaluation was performed with Hologic QDR 4500W Elite series. Dual energy used is at 100/140 Kv, 40 mA level. Daily calibration of machine was made with phantom (lumbar vertebrate QC phantom) including calcium hydroxyapetide crystals which reflects the mean of four lumbar vertebrates bone mineral content. Bone mineral density evaluation was performed at AP (anterior-posterior) L1, L2, L3, L4, L1–4 lumbar vertebrate position and femur neck, great trokanter, intertrocanteric area, ward's triangle. Every metallic goods were excluded from the study field.

BIOCHEMICAL PARAMETERS

Blood samples were collected following overnight fast. 24 hour urine was collected for calcium and phosphorus measurements. Deoxypridinoline was measured from spot urine sample. Aspartate and alanine aminotransferase, direct and indirect bilirubin, gamma glutamyl transpeptidase, alkaline phosphatase, urea, creatinine, calcium, phosphorus, urine calcium and phosphorus measurement was performed by Beckman Coulter LX20 machine. TSH, LH, FSH, estradiol, cortizol, prolactin measurements were performed by Beckman Coulter UniCel DxI 800 machine. Osteocalcine measurement was performed by B.R.A.H.M.S Cryptor machine with chemiluminescence method. Deoxypridinoline measurement was performed by Chrosystems 1100 machine using HPLC method. RANKL and OPG measurement was performed with sandwich ELISA method. Hepatitis B surface antigen (HBsAg), antibody against hepatitis surface antigen (anti-HBs), antibody against hepatitis B core antigen (anti-HBc), antibody against hepatitis B early antigen (anti-HBe) (BIOKIT, s.a. Barcelona-Spain), hepatitis B early antigen (HBeAg) (Dia. Pro Diagnostic Bioprobes, Milano-Italy) and antibody against human immune deficiency virus (Anti-HIV) (Biomerieux, Marcy I'Etoile-France) were evalutated with enzyme immune assay method. Anti-nuclear antibody (ANA) (Euroimmun, Luebeck-Germany) was evaluated with immunofluoresence test.

STATISTICAL ANALYSIS

Data's were given as mean and standard deviation for continuous variables. For non continuous variables frequency interval was shown. Homogeneity of data distribution between groups was analyzed according to One-Sample Kolmogorov-Smirnov test. Difference between groups was evaluated with independent sample-t test for parametric variables and Mann Whitney U test for nonparametric variables. Comparing osteoporosis risk factors between groups was performed with Chi-square test. $P< 0.05$ was accepted as statistically significant. Correlation between groups was done with Spearman correlation test.

RESULTS

The mean age of 16 chronic hepatitis B patients and 31 healthy controls were 37.56 ± 10.26 and 36.87 ± 9.89 years respectively. Gender distribution of patients were 13 male and 3 female chronic hepatitis B patients, 18 male and 13 female healthy controls. Demographics and data's related disease of both groups were shown in Table 1. There was not statistically significant difference between both groups according to gender (NS) and age (NS). Also, there was not statistically significant difference between both groups according to cigarette smoking (NS), physical activity (NS), coffee consumption (NS), presence of osteoporosis in the family (NS), and body mass index (NS). Mean and standard deviation values of HBV DNA in chronic hepatitis B patients were shown in Table 2 (3128636.69 ± 4410069.23). Liver biopsy grading scores and percentile of chronic hepatitis B patients were shown in Table 3 (Knodell grade 1 n= 3 (18%), grade 2 n= 8 (50%), grade 3 n= 3 (18%), grade 4 n= 1 (6.3%), grade 5 n= 1 (6.3%)). Osteo-

TABLE 1: Comparing clinical findings and osteoporosis risk factors between hepatitis B and control groups.

	Hepatitis B group (n= 16)	Control group (n= 31)	P
Gender (female/male)	3/13	13/18	NS
Age (year)	37.56 ± 10.26	36.87 ± 9.89	NS
Body Mass Index	27.19 ± 4.66	26.60 ± 5.30	NS
Systolic blood pressure	117.50 ± 13.41	114.19 ± 13.85	NS
Diastolic blood pressure	73.12 ± 10.62	74.51 ± 12.40	NS
Cigarette smoking (consumer/nonconsumer)	6/10	13/18	NS
Physicial activity (minimal/medium/good)	3/10/3	6/17/8	NS
Coffee consumption (consumer/nonconsumer)	16/0	26/5	NS
Osteoporosis in family (present/not present)	0/16	6/25	NS

NS= nonsignificant.

TABLE 2: Minimum, maximum and standart deviation of HBV DNA values of chronic hepatitis B patients.

	Total number	Minimum	Maximum	mean
*HBV DNA	16	707	10000000	3128636.69 ± 4410069.23

*HBV DNA: hepatit B virüs DNA.

porosis risk factors were not different between groups. Statistically non significant difference were observed between groups for indirect bilirubin, globulin, international normalization ratio (INR), glucose, urea, creatinine, calcium, phosphorus, osteocalcin, TSH, cortizol, LH, FSH, eostradiol, 24 hours urine calcium excretion, 24 hours urine phosphorus excretion, and urine deoxypridinoline. On the other side, statistically significant difference was observed between alanine aminotransferase, aspartat aminotransferase, albumin, direct bilirubine values between groups. Mean values, standard deviation and p values of these parameters were presented in Table 4. There were not any statistically significant difference between hepatitis B group and healthy controls according to mean lumbar and femur neck BMD values. Bone densitometry data's were presented in Table 5. OPG and RANKL serum values were found to be statistically significantly different between hepatitis and healthy control group. Higher OPG and (p= 0.029), lower RANKL serum levels (p= 0.004) were found in hepatitis B compared to control group. There were not any correlations between groups according to lumbar and femur neck

TABLE 3: Liver biopsy grading score and percent values of chronic hepatitis B patients.

Histopathological grade of chronic hepatitis	Frequency	Percent
1.00	3	18.8
2.00	8	50.0
3.00	3	18.8
4.00	1	6.3
5.00	1	6.3
Total	16	100.0

BMD values, urine deoxypridinoline, serum osteocalcin values, RANKL and OPG serum levels with Spearman's correlation test. Correlation p and r values were presented in Table 6.

DISCUSSION

In this study, we did not find statistically significant difference in serum calcium, phosphorus, urine calcium and phosphorus levels between hepatitis B and control groups. They show us that; there is not any difference in calcium, phosphorus intake and absorption between hepatitis B patients and healthy controls. Again, statistically significant different serum osteocalcin and de-

TABLE 4: Comparing the OPG, RANKL, bone mineral metabolism and liver function parameters between hepatitis B and control groups and p values.

	Hepatitis B group (n=16)	Control group (n=31)	P
Alanine transaminase	87.81±74.92	22.87±9.71	0.03
Aspartat transaminase	67.12±60.28	21.76±5.59	0.009
Direct bilirubine	0.20±0.08	0.13±0.05	0.008
Indirect bilirubine	1.07±0.58	0.81±0.34	NS
Alkaline phosfatase	73.56±24.26	60.03±14.84	NS
Gamma glutamyl transpeptidase	32.18±13.47	21.70±8.77	NS
Albumine	3.96±0.43	4.26±0.27	0.02
Globuline	3.04±0.47	2.99±0.38	NS
INR*	1.01±0.07	1.00±0.08	NS
Calcium	9.46±0.47	9.52±0.43	NS
Phosporus	3.61±0.74	3.58±0.57	NS
Osteocalcin	22.72±7.17	23.04±9.87	NS
Deoxypridinoline	8.67±3.67	10.43±3.82	NS
Urine calcium	140.15±125.30	127.92±116.62	NS
Urine phosphorus	673.12±345.31	618.17±223.66	NS
RANKL	0.16±0.00	0.17±0.01	0.004
OPG	569.24±173.40	441.13±189.08	0.029

Note= Values are given mean and standard deviation, INR= International normalisation ratio, NS= nonsignificant.

TABLE 5: Comparing bone densitometric parameters of hepatitis group with control group.

Densitometry parameters	Hepatitis B group	Control group	P values
Lumbar (L1-4)			
BMD	0.99 ± 0.16	1.00 ± 0.15	0.91
t-score	-0.78 ± 1.51	-0.61 ± 1.38	0.70
z-score	-0.63 ± 1.46	-0.45 ± 1.37	0.64
Femur Neck			
BMD	0.94 ± 0.11	0.89 ± 0.12	0.24
t-score	-0.18 ± 1.05	-0.47 ± 1.18	0.41
z-score	0.44 ± 0.91	0.14 ± 1.22	0.32
Femur Trochanter			
BMD	0.78 ± 0.12	0.73 ± 0.08	0.10
t-score	0.03 ± 1.15	-0.30 ± 0.91	0.27
z-score	0.29 ± 1.07	-0.06 ± 0.92	0.23

Values are given as mean±standart deviation.

oxypridinoline levels between both groups were not detected. That also means that bone turnover isn't different between both groups. High serum OPG levels and low RANKL levels were found in hepatitis B groupcompared to control. Szalay et al. have also found increased OPG and reduced RANKL serum levels in primary billier cirrhosis patients. It has been pointed out that increase in serum OPG level is not a specific marker of primary billier cirrhosis instead secondary to inflammatory reaction in chronic liver disease. OPG is synthesized mainly by osteoblasts. Although Szalay et al. have detected high serum OPG levels; suppressed osteoblastic function in primary billier cirrhosis should result in reduced serum OPG level.[10] It was proposed that; increased synthesis

TABLE 6: Correlation results of RANKL and OPG serum levels with osteocalcin, deoxypridinoline, lumbar BMD (bone mineral density), femur neck BMD, lumbar t-score, femur neck t-score, lumbar z-score, femur neck z-score values.

		OPG	RANKL
Osteocalcin	Correlation coefficent	-0.019	-0.356
	p-values	0.947	0.193
	N	15	15
Deoxypridinoline	Correlation coefficent	-0.092	-0.156
	p-values	0.735	0.564
	N	16	16
t-lumbar	Correlation coefficent	-0.258	0.088
	p-values	0.335	0.745
	N	16	16
z-lumbar	Correlation coefficent	-0.253	0.167
	p-values	0.343	0.537
	N	16	16
BMDlumbar	Correlation coefficent	-0.238	0.070
	p-values	0.374	0.797
	N	16	16
t-neck	Correlation coefficent	-0.366	-0.313
	p-values	0.163	0.237
	N	16	16
z-neck	Correlation coefficient	-0.279	-0.216
	p-values	0.296	0.421
	N	16	16
BMDneck	Correlation coefficent	-0.312	-0.347
	p-values	0.240	0.188
	N	16	16
t-trochanter	Correlation coefficent	-0.393	0.048
	p-values	0.132	0.860
	N	16	16
z-trochanter	Correlation coefficient	-0.370	0.100
	p-values	0.158	0.713
	N	16	16
BMD trochanter	Correlation coefficent	-0.341	0.016
	p-values	0.196	0.953
	N	16	16

p< 0.05 was accepted expressive.

of OPG from cells other than osteoblasts namely T cells, B cells, fibroblasts and dysfunctional osteoblasts may contribute to high OPG serum levels.[11] That may explain our results.

Marek et al. showed increased TGF β1 serum levels in chronic hepatitis B patients.[12] Hegedus et al. reported high serum OPG levels in Wilson's disease patients. High serum OPG levels in patients with Wilson's disease were explained as increased secretion from fibroblasts and immunocompetant cells due to inflammatory process.[13]

Invariant NK T cells (natural killer T cells) presented by human cluster of differentiation-d (CD1d) are a group of auto reactive cells that was thought to recognize endogen lipid ligands and regulates host response to tissue damage and cell stress

with rapid cytokine production. Invariant NKT cells increase markedly in chronic hepatitis B infected liver. These cells produce cytokines like type 2 profibrotic IL-4 and IL-13.[14] IL-13 has been known as cytokine that reduce serum RANKL level, increase serum OPG level, and suppress osteoclastogenesis.[15] It has been shown that IL-13 suppress osteoclast differentiation and bone resorbtion by activating receptors via signal transducers and activators of transcription 6 (STAD6) pathways located on osteoclasts and osteoblasts which show its effects via RANKL/RANK/OPG system.[16] This supports our hypothesis that changes in the level of OPG and RANKL occurs as a result of inflammation in chronic viral hepatitis.

Udagawa reported increased OPG and reduced RANKL serum levels in new onset Crohn's disease patients. In Crohn's disease, peripheral CD4+ Th1 cells exposed to intestinal antigens responds with interferon gamma (IFN-γ) secretion. It was known that IFN-γ increase serum OPG and suppresses serum RANKL level. Although, reduced osteoblastic activity explains the reduced RANKL secretion from osteoblasts, but it does not explain the increase in OPG serum level. It might be explained by OPG secretion from cells other than osteoblasts like colonic cells. Udagawa et al pointed out that; IFN-γ is a key cytokine in the inhibition of bone construction at the same time suppression of bone resorbtion. Although T cells secrete RANKL, the secretion of another cytokine IFN- γ predominates with a net decrease of serum RANKL level.[17]

We have not detected statistically significant difference in terms of bone mineral density between chronic hepatitis B and control groups. Schiefke et al was found increased osteopenia and osteoporosis incidence in non cirrhotic viral hepatitis B and C patients. In this study we excluded postmenopausal women. It was known that high frequency of osteoporosis occurs in postmenopausal women. Also Schiefke et al. did not compare their data's with healthy control group.[9]

One limitation of our study is low number of subjects. Second limitation of our study is cross sectional nature. In addition to these; we did not evaluate TGF β1 and IL-13 serum levels in this study. Because of that; we could not able to prove our hypothesis related with liver inflammation and serum OPG and RANKL levels.

As a conclusion, we did not find any correlation between OPG, RANKL serum levels, BMD values, and bone remodeling parameters. Inflammation in the liver rather than change in bone metabolism might probably be the reason of change in OPG and RANKL serum levels. The other probably reason of not to see osteoporosis in chronic viral hepatitis B patients might be insufficient time for osteoporosis development or insufficient serum levels of cytokines secondary to inflammation.

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