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Boron intake, *osteocalcin* polymorphism and serum level in postmenopausal osteoporosis

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ABSTRACT

The relationship between daily boron intake and osteocalcin-mediated osteoporosis was studied in boron-exposed postmenopausal women. It is known that boron and osteocalcin are important in bone metabolism, however the effect of boron in bone metabolism has not been fully discovered. The study was performed on 53 postmenopausal women aged 55–60 living in parts of Balikesir, Turkey, where the subjects are naturally exposed to high ($\geq$1 mg/L) or low ($<$ 1 mg/L) boron concentration in drinking water. 24-h urine samples were collected from all participants and creatinine clearance was detected. Boron intake levels of the subjects whose clearance levels were between 80–124 mL/min were measured by inductively coupled plasma-optical emission spectrometry (ICP-OES) in urine samples. Serum osteocalcin levels of the subjects were measured by osteocalcin enzyme-linked immunosorbent assay (ELISA) kit. *Osteocalcin* polymorphism rs1800247 was detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Serum osteocalcin levels in boron-exposed postmenopausal women were significantly higher than that of control group ($P \leq 0.05$) and the correlation between the serum osteocalcin level and rs1800247 polymorphism was not significant in both groups ($P > 0.05$). The differences in the distribution of *osteocalcin* genotypes and alleles in postmenopausal women were not significant between the boron exposed and the control groups ($P > 0.05$). Serum osteocalcin level in the CC genotype was significantly higher compared to the TC genotype in boron-exposed group ($P \leq 0.05$). Our study suggests that daily boron intake of 1 mg/L may affect bone metabolism in postmenopausal women positively.

1. Introduction

Osteoporosis is classified in the group of multifactorial diseases whose development partly depend on genetic and environmental factors [1,2]. Despite the limited number of studies, daily boron intake draws attention among the environmental factors affecting the Bone Mineral Density (BMD). In nature, boron is not found in elemental form but in the forms of borax, boric acid, colemanite, ulexite, and borate. In the light of the limited data, the normal amounts of daily boron intake accepted by the WHO are 0.44 µg/day from air, 0.2–0.6 mg/day in drinking water, and 1.2 mg/day in diet [3].

In Turkey, the daily boron intake was found to be 6.77 mg/day according to a recent study conducted in boron-rich regions [4]. Boron associated with magnesium, vitamin D, and calcium also plays an important role in bone metabolism. Especially in postmenopausal women, total plasma density decreased, excretion of calcium and magnesium minerals decreased, and 17-beta estradiol and testosterone levels increased in boron-supplemented diet. Although the evidence suggests that elemental boron plays a regulatory role in macromineral metabolism and affects cell metabolism at the membrane level, the role of boron in bone metabolism has not been fully elucidated [5–8].

Several twin and family studies showed that the effect of genetic factors, i.e. gene polymorphisms, on bone formation is around 60% with the associated bone parameters [9,10]. Polymorphism in the *osteocalcin* gene is an effective and important genetic factor. Osteocalcin synthesis is induced by 1,25 vitamin D_3 and contains three glutamic acid residues [10]. It is gamma-carboxylated through vitamin K and gains the ability to bind calcium. It has high affinity to hydroxyapatite and is believed to be associated with the crystals of hydroxyapatite in bone. In various metabolic diseases, osteocalcin is a sensitive and specific indicator of osteoblast function. Osteocalcin level is elevated in hyperparathyroidism, hyperthyroidism, acromegaly, and Paget's disease, where bone turnover is rapid [9,10]. Genetic polymorphisms located in *osteocalcin* gene were reported to be linked with the serum

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osteocalcin levels and bone fracture incidents [11]. Among these polymorphisms rs1800247 is the most well-known and researched single nucleotide polymorphism (SNP) [12]. The rs1800247 polymorphism has been reported to affect BMD levels in adults [13–16], adolescent females [17], and young children [18]. While a study conducted in Taiwan [19] linked rs1800247 with the BMD and osteoporosis in Chinese postmenopausal women, while some studies were not able to draw relationships between the BMD and rs1800247 in Chinese population [20–22] indicating the potential complexity. As rs1800247 is located in the promoter region, it has potential functionality and could alter gene expression and subsequently serum osteocalcin concentrations. It has been examined for its association with serum osteocalcin levels in a few studies [11,14,23]. From this point of view, the current study was planned on the idea that there may be a multifactorial relationship between the daily intake of boron mineral responsible for bone metabolism and the *osteocalcin* gene and its serum level.

2. Material and methods

2.1. Location of study

Study locations were İskele, Osmanca, and Beğendikler regions within the borders of Bigadiç district of Balıkesir city with boron concentrations of 1 mg/L or more in the drinking water, and were defined as boron-rich regions based on previous studies [3,4,24,25]. Control locations were Çağış, Çömlekçi, and Esenli regions that were also within the borders of Bigadiç, Balıkesir with boron concentration below 0.3 mg/L in drinking water [24].

2.2. Subjects

The study materials were composed of blood and urine samples taken from 25 women in the postmenopausal period between 50 and 60 years old living in the boron-rich regions. Materials belonging to the control group were taken from 28 postmenopausal women of the same age group living in the control regions. All study related samples from a total of 53 subjects were collected in November 2014. All of the subjects were free from spontaneous menses for at least a year at the time of study.

2.3. Ethics

This study was conducted in accordance with the good clinical practice and applicable regulatory requirements by the Declaration of Helsinki. The study was approved by the Celal Bayar University Institutional Review Board (IRB) in March 2014. IRB-approved informed consent forms were obtained from each subject prior to any study-related procedure.

2.4. Boron measurement and determination of individual daily boron intake levels

Boron levels in drinking water in the study region, individual daily boron intake levels of study subjects, boron levels in urine samples from the study subjects, and the creatinine clearance tests were performed based on the methods described by Orenay-Boyacioglu et al. [4].

2.5. Serum osteocalcin level determination

Blood samples were allowed to clot for 30 min at 4 °C and total serum was isolated by centrifugation at 3000 rpm for 10 min at 4 °C and stored at −20 °C until use. Serum osteocalcin levels of the study and control groups were measured by the sandwich ELISA method using a commercial kit (ChemoKine Osteocalcin, Millipore, Billerica, MA, USA) following the manufacturer's guidelines.

2.6. Isolation, quantity and purity of DNA

Genomic DNA was isolated from peripheral blood according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA concentration and purity were evaluated by absorbance methodology using a NanoDrop 1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Wilmington, DE, USA).

2.7. PCR amplification

Osteocalcin polymorphism rs1800247 −298C/T was detected by PCR-based RFLP method as described by Specjalski et al. [19]. The PCR reaction was performed with 20 µL of DNA, 2 µL of 10X PCR buffer, 1.5 mM of $MgCl_2$, 2.5 U of Taq DNA polymerase, 200 µM of dNTPs, and 200 nM of forward and reverse primers. The primer pair used for the amplification of *osteocalcin* rs1800247 polymorphism is 5′-CCGCAGCT CCCAACCACAATAAGCT-3′ (forward) and 5′-CAATAGGGCGAGGA GTG-3′ (reverse). PCR protocol employed started with an initial denaturation step at 95 °C for 5 min; continued with 30 cycles of denaturation step at 94 °C for 30 s, annealing step at 58 °C for 30 s, and extension step at 72 °C for 60 s; and ended with a final extension step at 72 °C for 7 min.

2.8. Genotyping

Amplified PCR products were incubated with restriction enzyme *Hin*dIII (New England Biolabs, Ipswich, MA, USA) at 37 °C for 1 h. The restriction-cut PCR products were evaluated on a 3% agarose gel. The rs1800247 polymorphism yielded a 253 bp product for homozygous CC genotype, 253 bp and 232 bp products for heterozygous CT genotype, and a 232 bp product for homozygous TT genotype.

2.9. Statistical analysis

Statistical analyses were performed using SPSS v.11.5.1 program. The results were evaluated by analysis of variance (ANOVA) and Chi-squared tests with 5% level of significance ($P \leq 0.05$).

3. Results

Of the two subject groups in the study, boron exposed group was composed of 25 subjects and control group had 28 subjects (Table 1). All subjects in the boron exposed group had lived in the boron-rich area for an average of 57.5 ± 12.0 years. None of the control subjects had lived in the boron-rich areas at any time during their life for an average of 59.8 ± 9.0. Majority of the subjects in both groups had an education level of elementary school.

The concentrations of boron in drinking water were detected as 1.59 ± 0.04 mg/L in boron rich study area and 0.012 ± 0.05 mg/L in the area where the control subjects lived. On the other hand, creatinine clearance was found to be in the range of 80–124 mL/min in the daily urine samples from 25 subjects in the boron exposed group and 28 subjects in the control group. Average creatinine clearance levels were determined to be 115.00 ± 41.00 and 125.50 ± 15.34 mL/min for boron exposed and control groups, respectively. The difference between the groups was not statistically significant ($P = 0.768$). Boron exposed subjects were calculated to have a daily average of 6.99 ± 2.90 mg/day boron intakes while control subjects had 1.20 ± 0.12 mg/day boron intake. Boron exposed individuals showed 5.83 times more boron exposure in average than that of the control subjects. Average level of boron excreted in urine was found to be 4.42 ± 0.07 mg/L in the boron exposed group and 0.99 ± 0.10 mg/L in the control group.

Average serum osteocalcin level was 27.55 ± 3.20 ng/mL in the boron exposed group and 23.47 ± 12.55 ng/mL in the control group. Also, analyses showed that serum osteocalcin level in postmenopausal women subjects in the boron exposed regions were significantly higher