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Transmission and scanning electron microscopy confirm that bone microstructure is similar in osteopenic and osteoporotic patients

Transmisyon ve tarama elektron mikroskopisi kemik mikromimarisinin osteopenik ve osteoporotik hastalarda benzer olduğunu doğrulamaktadır

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Objectives: The objective was to **confirm the finding of "Bone microstructure is similar in osteopenic and osteoporotic patients with femoral neck fracture." obtained in previous "light microscopy study", which was new and important data.**

Patients and methods: Fourteen patients (5 males, 9 females) who were admitted with proximal femoral fracture following low energy trauma (patients who participated in the **light microscopy study**) were included. The patients were divided into two groups based on the bone mineral density (BMD) measurement, including osteopenic group (n=7, mean age 69 years; range 63 to 74 years) and osteoporotic group (n=7, mean age 74.1 years; range 67 to 78 years). **Cortical and trabecular bone samples were taken** from the patients who underwent endoprosthesis during partial hip arthroplasty **and these samples were analyzed using transmission electron microscopy and scanning electron microscopy evaluations which are more sophisticated higher resolution techniques.**

Results: The mean cortical bone thickness was 3622.14 µm in osteopenic group and 2323.14 µm in osteoporotic group (p<0.005). Transmission electron microscopy and scanning electron microscopy evaluations revealed similar findings for both groups.

Conclusion: Although a significant difference in cortical thickness was found between the groups, **transmission and scanning electron microscopy confirmed that bone microstructure shared similar characteristics in osteopenic and osteoporotic patients with low-energy femoral neck fracture, as it was in previous light microscopy study.**

Key words: Bone microstructure; osteopenia; scanning electron microscopy; osteoporosis; transmission electron microscopy.

Amaç: Daha önce "ışık mikroskopi çalışması" ile elde edilen "Femur boyun kırığı olan osteopenik ve osteoporotik hastalarda kemik mikromimarisi benzerdir" bulgusunu ki, önemli ve yenidir, teyit etmek amaçlandı.

Hastalar ve yöntemler: Düşük enerjili travma sonrası femur proksimal uç kırığı nedeniyle başvuran 14 hasta (5 erkek, 9 kadın) çalışmaya alındı **(ışık mikroskopi çalışmasına katılan hastalar)**. Hastalar kemik mineral yoğunluğu (KMY) ölçümlerine göre osteopenik (n=7, ort. yaş 69 yıl; dağılım 63-74 yıl) ve osteoporotik (n=7, ort. yaş 74.1 yıl; dağılım 67-78 yıl) olarak iki gruba ayrıldı. Parsiyel kalça artroplastisi sırasında endoprotez uygulanan hastalardan **kortikal ve trabeküler kemik örnekleri alındı ve alınan bu örnekler, daha gelişmiş ve yüksek çözünürlüklü transmisyon ve taramalı elektron mikroskopisiyle incelendi.**

Bulgular: Ortalama kortikal kemik kalınlığı osteopenik grupta 3622.14 µm; osteoporotik grupta ise 2323.14 µm idi (p<0.005). Transmisyon elektron mikroskopisi ve taramalı elektron mikroskopisi değerlendirmesinde her iki grupta da benzer bulgular saptandı.

Sonuç: Gruplar arasında kortikal kalınlık açısından anlamlı farklılık bulunmuş olsa da, **transmisyon ve taramalı elektron mikroskopisi, daha önceki ışık mikroskopi çalışmasında olduğu gibi, kemik mikromimarisi düşük enerjili femur boyun kırığı olan osteopenik ve osteoporotik hastalarda benzer özellikler taşıdığını teyit etmiştir.**

Anahtar sözcükler: Kemik mikromimarisi; osteopeni; taramalı elektron mikroskopisi; osteoporoz; transmisyon elektron mikroskopisi.

This article confirms, using transmission electron microscopy and scanning electron microscopy evaluations which are more sophisticated higher resolution techniques, the finding of "Bone microstructure is similar in osteopenic and osteoporotic patients with femoral neck fracture." Obtained in previous "light microscopy study", which was new and important data, and published before as *"Gul O, Atik OS, Erdoğan D, Goktas G. Is bone microstructure different between osteopenic and osteoporotic patients with femoral neck fracture?: Light microscopy for evaluation of bone. [Article in Turkish] Eklem Hastalik Cerrahisi 2012;23:15-9."*

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In our previous "light microscopy study" in which the same cohort of patients was used, we found that "bone microstructure is similar in osteopenic and osteoporotic patients with femoral neck fracture."[1] This finding was new and important.

We also found **no histological study in our literature search** comparing a patient group with bone mineral density (BMD) in the osteopenic range to one with BMD in the osteoporotic range.

For these reasons, we aimed to **confirm our data using the same cohort of patients used in the previous "light microscopy study," with transmission electron microscopy and scanning electron microscopy evaluations which are more sophisticated higher resolution techniques.**

PATIENTS AND METHODS

Fourteen patients (9 females and 5 males) presenting to our clinic with a proximal femur fracture after low-energy trauma **(same cohort of patients used in the previous "light microscopy study")** were included in this study. Only patients with normal serum electrolytes and alkaline phosphatase were included, while those with thyrotoxicosis, male hypogonadism, malabsorption syndromes, malignancy, chronic liver disease and other causes of secondary osteoporosis and those receiving treatment for osteoporosis before trauma were excluded.

Patients were assigned into two groups as follows: seven patients with a BMD value ≤2.5, and seven patients with a BMD value from -1 to -2.49 at the proximal femur.[2] To support the reliability of the results, care was given to form groups with similar age ranges. The overall mean age of 14 patients was 71.5 (range 63-78) years. The mean age was 69 (range 63-74) years in the osteopenia group, and 74.1 (range 67-78) years in the osteoporosis group.

A Hologic QDR® 4500 X-ray bone densitometer device (Hologic, Inc., Waltham, MA, USA) was used to obtain BMD measurements, after providing stabilization and appropriate conditions for the patients.

Urine N-terminal telopeptide (NTx) levels were determined by using Osteomark NTx test (Ostex International, Inc., Seattle, WA, USA). Urine samples were obtained during second morning voiding in both groups, and stored at -20 °C until testing. Test procedures were performed in accordance to manufacturer's instructions. Normal values were considered as 5-65 nM BE/mM creatinine for women, and 3-63 nM BE/mM creatinine for men.[3]

All patients underwent partial hip arthroplasty with endoprosthesis after preoperative evaluations. During operation, cortical bone samples (8x4 mm) were taken from the inferior-anterior region of the femoral neck, while trabecular bone samples (8x8x8 mm) were taken from 2 cm below the subcapital region of the femoral head. Samples were placed in glutaraldehyde solution and delivered to the histology department for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) evaluations.

For TEM evaluations, tissue samples were decalcified by placing into ethylenediaminetetraacetic acid (EDTA) solution prepared by glutaraldehyde. Then, tissue samples were placed in 1% osmium tetraoxide for one hour, followed by fixation and staining. Then, samples were dehydrated with alcohol series and tissues were placed in propylene oxide for 30 minutes, followed by a 30 minute waiting period in embedding material, enabling tissue passage into embedding material. After this step, tissues taken into embedding material were placed into a rotator at room temperature for two hours, then placed into a oven at 40 °C for another two hours. Finally, tissues were embedded into horizontal embedding blocks within the same mixture.[4] Then, contrast staining was achieved by using uranyl acetate and lead citrate and they were evaluated and captured using Carl Zeiss EVO LS10 electron microscope (Carl Zeiss Microscopy Ltd., Cambridge, UK).[5]

For SEM evaluations, specimens were placed into 2.5% buffered glutaraldehyde solution. Tissues were dehydrated by using acetone series with increasing degree. Then, complete dehydration was achieved by drying with a Leica EM CPD030 critical point drying device (Bal-Tec AG, Balzers, Liechtenstein). After the drying process, they were mounted on aluminum reservoirs by liquid silver and coated with gold/palladium in Denton Vacuum, LLC Desk V sputter/etch unit coating device (Denton vacuum LLC, Moorestown, NJ).[6] The tissue loaded was evaluated and captured using a Carl Zeiss EVO LS10 microscope.[5]

On electron microscopy, the thickness of the cortex and trabecular wall as well as the diameter of the haversian canal was measured in the tissue samples obtained from both groups.

Data were compared by using Mann Whitney U test, Statistical Package for Social Sciences for Windows version 10.0 (SPSS Inc., Chicago, Illinois, USA), and $p<0.05$ was accepted as significant.

RESULTS

When gender distribution was assessed by chi-square test, no significant difference was found between groups (p>0.05).

In the osteopenia group, the measurement of BMD T-scores revealed the following mean values: -1.72 (-0.80, -2.40) at the femur neck, -1.52 (-0.33, -2.16) at the trochanter and 1.60 (0.29, -2.80) at L_1-L_4. In the osteoporosis group, corresponding values were as follows: -3.08 (-2.59, -3.53), -2.96 (-2.51, 3.39), and -2.27 (-2.01, -3.12), respectively.

The thickness of the trabecular bone wall was higher in the osteopenia group ($\overline{X}$=113.13 μm) than in the osteoporosis group ($\overline{X}$=86.41 μm) according to measurements in the spongious bone. However, no significant difference was found in the thickness of the trabecular bone wall between osteopenia and osteoporosis groups (p>0.05).

The cortex thickness was significantly higher in the osteopenia group ($\overline{X}$=3622.14 μm) than in the osteoporosis group ($\overline{X}$=2323.14 μm) according to measurements in the cortical bone. This difference was found to be statistically significant (p<0.05).

N-terminal telopeptide values were found to be higher in the osteoporosis group ($\overline{X}$= 84.71 nM BCE/mM creatinine) than in the osteopenia group ($\overline{X}$=83.00 nM BCE/mM creatinine). However, no significant difference was found between groups regarding NTx measurements (p>0.05).

The diameter of the haversian canal was found to be higher in the osteopenic group ($\overline{X}$=49.02 μm) than in the osteoporotic group ($\overline{X}$=44.65 μm). No significant difference was found between groups regarding haversian canal measurement (p>0.05).

Compact bone

Transmission electron microscopy evaluations revealed that lamellae were sporadically effaced with formation of homogenous areas between them in the osteoporosis group (Figure 1a). Despite the presence of osteocytes, it was noted that they were sporadically degenerated (Figure 1b). In the osteopenia group,

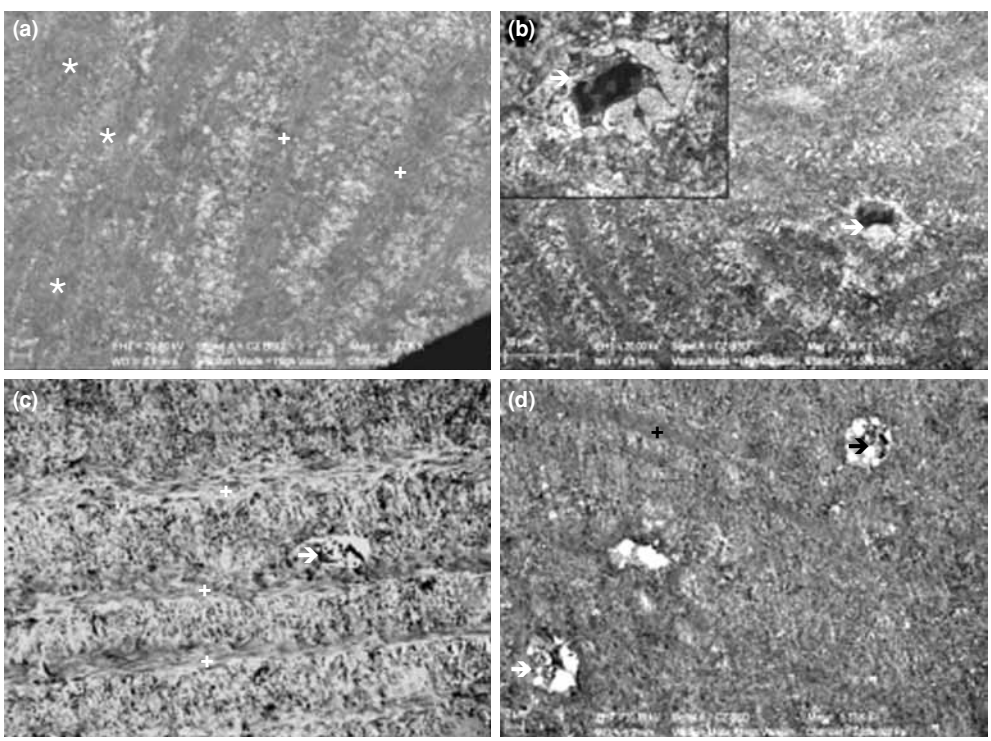

Figure 1. On transmission electron microscopy evaluation of compact bone from the osteoporosis group: Sporadic effacement of lamellae (+) and formation of homogenous area (★) **(a)** Presence of osteocyte degeneration (➔) is seen (**b** and inset). On transmission electron microscopy evaluation of compact bone from the osteopenia group: It is seen that lamellar structure (+) is impaired in some areas, while it is preserved in other areas similar to the osteoporosis group. Degeneration of osteocytes (➔) can be recognized. **(c)** Osteocytes (➔) are obvious. Lamellar structure (+) is impaired in some areas, while it is preserved in other areas **(d)** (Uranyl acetate-Lead citrate A x 6.37, B x 4.36, inset x 9.75, C x 4.97, D x 5.33).

it was seen that the lamellar configuration was defective in some areas, while it was preserved in others, similar to the osteoporotic group (Figure 1c). It was recognized that osteocytes were scarce and degenerated (Figure 1d).

Scanning electron microscopy evaluations demonstrated that lamellae in the haversian canal system were degenerated in some areas; although they were preserved in other areas in the osteoporotic group. Sporadic decomposition of lamellae and prevalence of homogenous material deposition between them were noteworthy (Figure 2a, b). In the osteopenia group, it was seen that lamellae were normal in some areas, although they were impaired in other areas (Figure 2c). Moreover, it was found that the haversian canal system had a defective distribution and there was homogenous material deposition as seen in the osteoporosis group (Figure 2d).

Spongious bone

On TEM evaluation, degeneration in trabecular structure was noteworthy in the osteoporosis group. In this group, it was detected that collagen disappeared due to lysis and homogenized areas were formed in the lytic areas (Figure 3a). In the osteopenia group, it was detected that although trabecular structure was preserved better, there were homogenized areas in this group similar to the osteoporosis group (Figure 3b).

On SEM evaluation, it was found that the structure of whole trabecula were degenerated and thinned in the osteoporosis group (Figure 4a, b). It was also detected that trabecular structure had a similar appearance in the osteopenia group (Figure 4c, d).

DISCUSSION

In recent years, it has been understood that a significant proportion of osteoporotic fractures occur in patient groups with BMD in the osteopenic range. This suggests that BMD measurements are not the only criteria in the diagnosis of osteoporosis but several factors such as bone turnover, bone micro-architecture and genetic predisposition are also important.[7-9]

No histological study was found in our literature search, comparing a patient group with BMD in the osteopenic range, and one with BMD in the osteoporotic range. There were studies regarding histological comparisons of normal versus

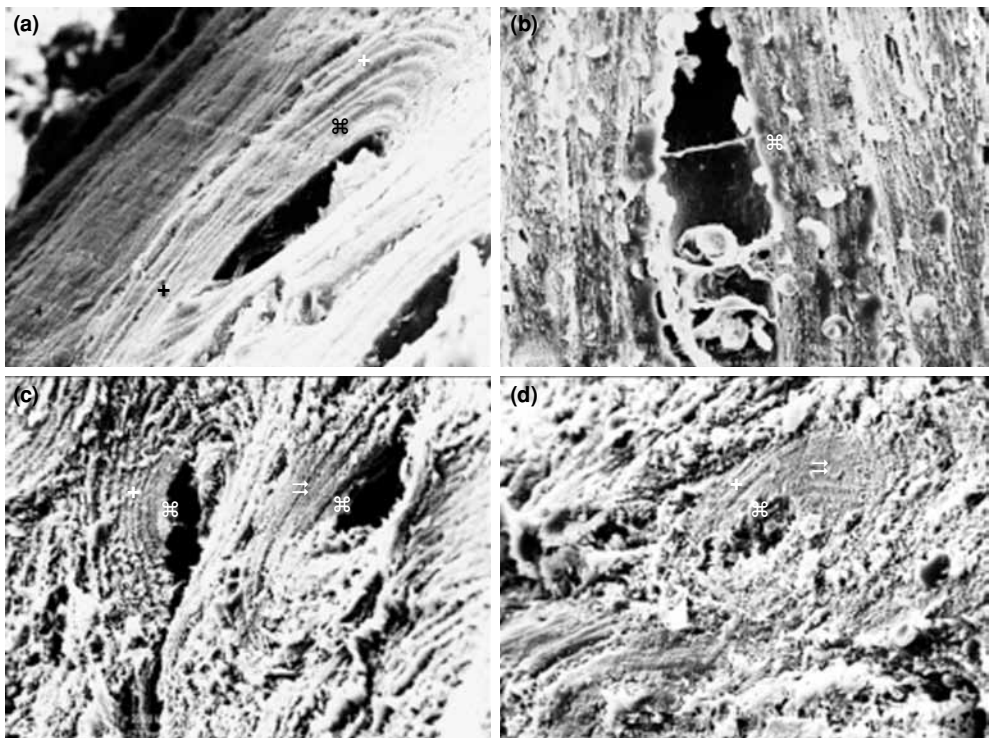

Figure 2. On scanning electron microscopy evaluation of compact bone from the osteoporosis group: **(a, b)** Lamellae (+) are seen in the Haversian canal (⌘) system. On scanning electron microscopy evaluation of compact bone from the osteopenia group: **(c)** It is seen that lamellar structure is normal (+) in some areas, while it is impaired (⇉) in other areas. **(d)** The presence of homogeneous material deposition (⇉) is clear between lamellae (A x 5.33, B x 1.20, C x 1.21, D x 1.20).

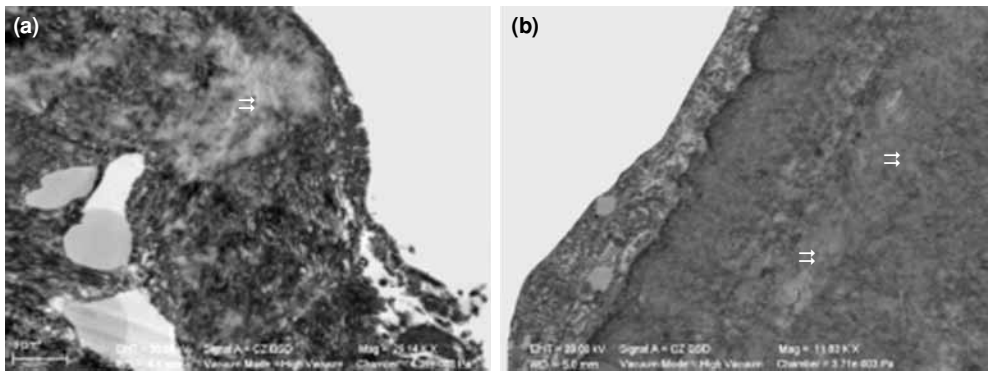

Figure 3. On transmission electron microscopy evaluation of spongious bone from the osteoporosis group: Homogenized areas (⇉) replacing collagen that disappeared due to lysis are seen. **(a)** On transmission electron microscopy evaluation of spongious bone from the osteopenia group: It is seen that trabecular structure is relatively preserved when compared to the osteoporosis group; however, homogenized areas (⇉) can be recognized as similar to the osteoporosis group. **(b)** (Uranyl acetate-Lead citrate A x 29.14, B x 11.83).

osteoporotic bones[10,11] and osteoarthritic versus osteoporotic bones.[12,13] Thus, we aimed to make a histological comparison between a patient group with hip fracture that had osteopenic BMD and those with hip fracture that had osteoporotic BMD.

Blain et al.[12] compared samples obtained from 21 patients with osteoarthritis and 20 patients with osteoporosis using a light microscope. They found that there was cortical effacement and reduction in the density and integrity of trabecular bone in patients with osteoporosis. In that study, thickness measured in the cortical bone samples obtained from the inferior of the neck was between 2500 and 3000 μm. In another study, Bell et al.[14] reported that cortical

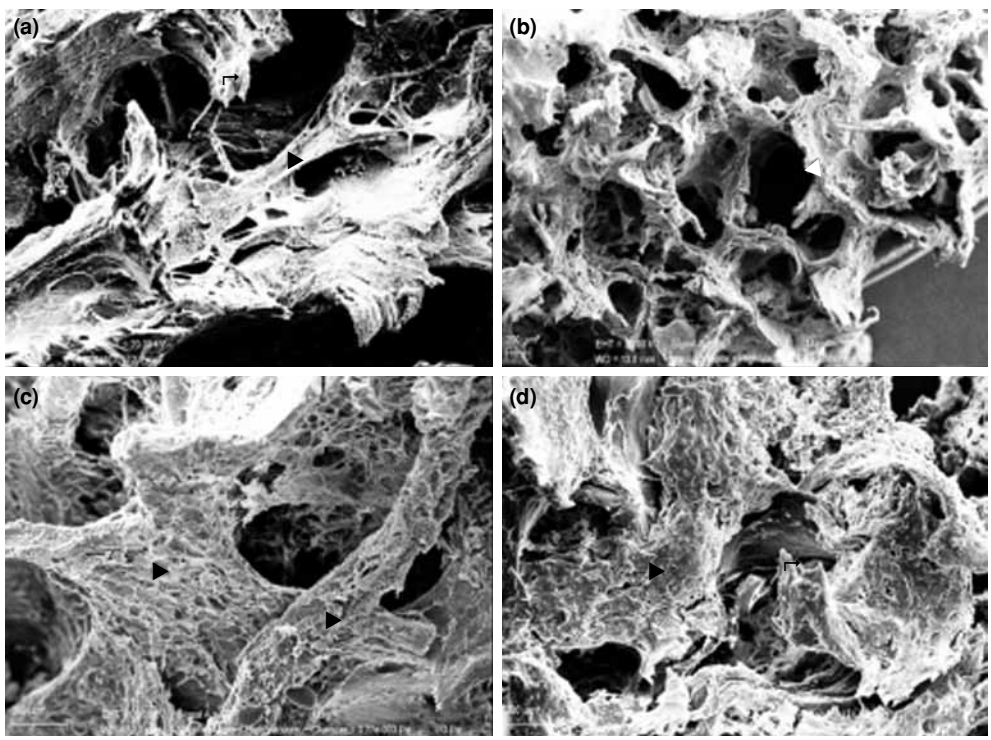

Figure 4. On scanning electron microscopy evaluation of spongious bone from the osteoporosis group: **(a)** Thinned trabeculae (▶) and fracture site (↱) are seen. **(b)** The structure of whole trabecula (▶) is impaired and thinned. **(c)** On scanning electron microscopy evaluation of spongious bone from the osteopenia group: It is seen that trabeculae (▶) have similar appearance to those in the osteoporosis group. **(d)** Trabecular wall (▶) and fracture site (↱) are seen (A x 174, B x 75, C x 173, D x 174).

bone measurements were between 2000-2500 μm in the inferoanterior region of the femur neck in patients with osteoporosis. We found that lamellae were destroyed and cortical bone was thinned in the semi-thin sections of the majority of patients in the osteoporosis group. Mean cortical thickness was 2323.14 μm in patients with values in the osteoporotic range. These findings were similar to those obtained in other studies. In our study, a significant difference was found in the comparison of osteopenia and osteoporosis groups in terms of cortical thickness (osteoporosis group: 2323.14 μm; osteopenia group: 3622.14 μm; $p<0.05$).

In a study of three patients with osteoporosis, Rubin et al.[11] found that there was atypical lamellar configuration, elevation in inorganic sites and degeneration in osteocytes in the osteoporotic spongious bones using TEM. Shen et al.[13] conducted a study of eight patients with osteoporosis and seven patients with osteoarthritis using TEM, in which they reported no osteocytes were found in the osteoporosis group. They found irregularity and reduction in collagen fibrils. In our study, degeneration was detected in trabeculae of the spongious bone of patients with osteoporosis on TEM evaluation. It was also found that collagen became irregular and underwent lysis and disappeared. Homogenized areas were formed in these lysis areas. Findings in the present study were similar to those obtained in previous studies in terms of above-mentioned features. In the osteopenia group, TEM evaluation in spongious bone revealed that, although trabecular structure was preserved better, there were also homogenized areas in this group similar to the osteoporosis group. No study was found in the literature in patients with osteopenia.

Suvorova et al.[10] compared trabecular structures of normal and osteoporotic bone using SEM and TEM. On SEM evaluation, it was reported that trabeculae of osteoporotic bones were more irregular and thinner, despite more organized structure of normal bones. The same authors noted that the inorganic structure was replaced by collagen, resulting in weaker bone structure. We found that in the osteoporosis group all trabecular structure was degenerated and thinned in the evaluation of spongious bone at the SEM level. This finding suggested that changes in internal structure of trabecula might be caused by degenerated collagen structure. It was also noted that trabecular structure had similar appearance in the osteopenia group.

Blain et al.[12] found a mean thickness of 80.9 μm in the measurement of trabecular bone of patients with osteoporosis. In our study, mean thickness was 86.41 μm in patients with osteoporosis. When compared to patients with osteopenia, no significant difference was observed ($p>0.05$).

Gabet and Bab[15] demonstrated that the number of pores was increased and haversian canals were dilated in cortical bone; and bone became thinner by advancing age. We found that lamellae were sporadically effaced with formation of homogenous areas between them in the osteoporosis group on TEM evaluation. Despite the presence of osteocytes, it was noted that they were sporadically degenerated. In the osteopenia group, it was found that similar to osteoporosis group, some lamellae were effaced, while others were preserved. It was also recognized that the number of osteocytes was lower and they were degenerated.

On SEM evaluation in the osteoporosis group, we found that lamellae were preserved in some regions, while they were extremely degenerated in other regions of the haversian canal system. Sporadic decomposition of lamellae and prevalence of homogenous material deposition between them were noteworthy. In the osteopenia group, lamellae were normal in some areas, although they were impaired in other areas. Moreover, it was found that haversian canal systems had a defective distribution and there was homogenous material deposition as seen in the osteoporosis group. No significant difference was found between groups, when measurements of the haversian canal diameter were compared ($p>0.05$).

When we assessed the findings obtained, we observed that there was no significant difference between the patients with osteoporotic BMD values and those with osteopenic BMD values in terms of trabecular, organic and cellular structures and micro-architecture in spongious bone. In qualitative evaluations using TEM and SEM, it was also seen that there was no significant difference in cortical bone between the two groups. However, it was found that the cortex of the cortical bone was thicker in the osteopenic group when compared to the osteoporotic group, suggesting an explanation for the difference in BMD values.

In addition, no significant difference was found in urine NTx measurements between groups ($p>0.05$). There is probably a destructive process in both groups, and irregularities of collagen configuration are associated with this process.

Kazakia et al.[16] studied the variations in morphological and biomechanical indices at the distal radius in subjects with identical BMD by using HR-QCT. They found that substantial variations in microstructural indices associated with biomechanical

competence and fracture risk exist among subjects with identical BMDs. Gül et al.[1] investigated whether bone microstructure is different between osteopenic and osteoporotic patients with low energy femoral neck fractures, and using a light microscope they demonstrated that there are similar characteristics for both groups, although a significant difference was found between the groups in terms of cortical thickness.

Osteoporosis definition based on T-score on BMD measurements is no longer sufficient.[1,7-9,16] Importantly, although a majority of osteoporotic fractures occurs in osteopenic patients, antiosteoporotic drugs are used only for patients with osteoporotic BMD values. Lifestyle changes and weight bearing exercises are recommended for osteopenic patients in addition to calcium and vitamin D supplementation in some cases. But these measures may not be enough. Therefore, the following question should be asked: Which subgroups of osteopenic patients will undergo anti-osteoporotic drug therapy? The FRAX™ tool which was recently developed by WHO may be a good answer[17] but it still has serious limitations for some countries.[18]

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