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# The Effects of Some Phosphodiesterase 5 Inhibitors on Oxidative Stress, VEGF, BMP 2 and 9 in the Liver Tissue of Ovariectomized Rats

## *Bazı Fosfodiesteraz 5 İnhibitörlerinin Ovariektomize Sıçanların Karaciğer Dokusunda Oksidatif Stres, VEGF, BMP 2 ve 9 Üzerine Etkileri*

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### Abstract

**Introduction:** Osteoporosis is an important health problem and there is no effective treatment yet. phosphodiesterase 5 inhibitors are promising agents for the treatment of osteoporosis. In this study, we aimed to determine the effects of phosphodiesterase 5 inhibitors (vardenafil, tadalafil, and udenafil) on bone morphogenic protein-2 and 9 (BMP-2 and 9), vascular endothelial growth factor (VEGF), and oxidative stress markers (malondialdehyde and CoQ10) in liver tissue of rats with ovariectomy-induced osteoporosis.

**Materials and Methods:** 50 Albino wistar female rats were randomly divided into 5 groups of 10 rats per group. Groups were the sham-operated, ovariectomise (OVEX), OVEX + Tadalafil, OVEX + udenafil and OVEX + vardenafil, respectively. VEGF, BMP-2 and 9 levels were measured by ELISA kits. To detect levels of MDA and CoQ10, we used high pressure liquid chromatography method.

**Results:** The levels of VEGF, BMP-2 and 9 levels in the groups that applied PDE-5 inhibitors were significantly higher than in the sham and OVEX groups. There was no significant difference between the OVEX+vardenafil and OVEX+udenafil groups in terms of VEGF, BMP-2 and 9 levels. The levels of MDA and CoQ10 were significantly lower in the groups that applied PDE5 inhibitors than in the OVEX group. When the histological and immunohistochemical results were examined, it was seen that angiogenesis was significantly higher in PDE-5 inhibitor groups.

**Conclusion:** In conclusion, we can say that these inhibitors may have positive effects on bone mineralization and remodelling by inducing the expression of VEGF, BMP-2 and 9 in liver tissue.

**Keywords:** Osteoporosis; phosphodiesterase 5 inhibitors; vascular endothelial growth factor A; bone morphogenic proteins.

### Özet

**Amaç:** Osteoporoz önemli bir sağlık sorunudur ve henüz etkili bir tedavisi yoktur. Fosfodiesteraz 5 inhibitörleri osteoporoz tedavisi için umut verici ajanlardır. Bu çalışmada, fosfodiesteraz 5 inhibitörlerinin (vardenafil, tadalafil ve udenafil) ovariektomi ile osteoporoz oluşturulan sıçanların karaciğer dokusunda kemik morfojenik protein-2 ve 9 (BMP-2 ve 9), vasküler endotelyal büyüme faktörü (VEGF) ve oksidatif stres belirteçleri (malondialdehit ve CoQ10) üzerindeki etkilerini belirlemeyi amaçladık.

**Gereç ve Yöntem:** 50 Albino wistar dişi sıçan her grupta 10 sıçan olacak şekilde rastgele 5 gruba ayrıldı. Gruplar sırasıyla sham-operated, ovariectomise (OVEX), OVEX + Tadalafil, OVEX + udenafil ve OVEX + vardenafil idi. VEGF, BMP-2 ve 9 seviyeleri ELISA kitleri ile ölçülmüştür. MDA ve CoQ10 seviyelerini tespit etmek için yüksek basınçlı sıvı kromatografi yöntemi kullanılmıştır.

**Bulgular:** PDE-5 inhibitörleri uygulanan gruplarda VEGF, BMP-2 ve 9 seviyeleri sham ve OVEX gruplarına göre anlamlı derecede yüksekti. OVEX+vardenafil ve OVEX+udenafil grupları arasında VEGF, BMP-2 ve 9 düzeyleri açısından anlamlı bir fark bulunmamıştır. PDE5 inhibitörü uygulanan gruplarda MDA ve CoQ10 düzeyleri OVEX grubuna göre anlamlı derecede düşüktü. Histolojik ve immünohistokimyasal sonuçlar incelendiğinde, PDE-5 inhibitörü gruplarında anjiyogenezin anlamlı derecede yüksek olduğu görüldü.

**Sonuç:** Sonuç olarak, bu inhibitörlerin karaciğer dokusunda VEGF, BMP-2 ve 9 ekspresyonunu indükleyerek kemik mineralizasyonu ve yeniden şekillenmesi üzerinde olumlu etkileri olabileceğini söyleyebiliriz.

**Anahtar Kelimeler:** Osteoporoz ;posfodiesteraz 5 inhibitörleri; vasküler endotel büyüme faktörü A; kemik morfojenik proteinleri.

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## Introduction

Osteoporosis is a medical condition that involves a reduction in bone mass and the degradation of bone tissue microarchitecture (1). In postmenopausal women, the ovarian production of oestrogen is reduced, and this is the main cause of rapid bone loss, resulting in postmenopausal osteoporosis. Fractures not only affect patient comfort and lifestyle but can also result in more serious conditions, such as permanent disability and death (2). For this reason, many studies on osteoporosis treatment and management have been carried out. The transforming growth factor-beta (TGF-β) superfamily includes a substantial subfamily known as bone morphogenetic proteins (BMPs). BMPs are important growth and differentiation factors that provide morphogenic signals for bone development during embryogenesis (3). There is a suggestion in the scientific literature that BMPs have a role to play not only in bone formation, but also in various physiological processes (4, 5). BMPs with bone-inducing properties are subdivided according to the homology of amino acid sequences (such as BMP2/BMP4 and BMP9/BMP10); there is no proven information on the osteogenic properties of BMPs outside these subgroups (6). BMP2 stands out as an important growth factor, inducing both osteoblast and osteoclast activity. However, long-term use of BMP2 has been reported to cause bone resorption (7). BMP9 has been reported to play a role in bone remodelling (5, 8). Although the positive effects of BMP2 and BMP9 on bone formation and remodelling are known, the effects of oestrogen deficiency on BMPs, especially BMP2 and BMP9, are not clear. The phosphodiesterase (PDE) enzyme family is an important superfamily that regulates intracellular concentrations of cyclic nucleotides by hydrolysing phosphodiester bonds. PDE5, a member of the PDEs, is specific for the hydrolysis of guanosine 5'-monophosphate (cGMP). Selective inhibitors of PDE5 induce relaxation of the vascular smooth muscle cells as a result of the nitric oxide (NO)-dependent accumulation of cGMP (9). In addition to the effects of PDE5 inhibitors on endothelial dysfunction, a previous study conducted by our group showed that vardenafil, tadalafil and udenafil had a positive effect on trabecular bone thickness and bone mineralisation, as well as increased angiogenesis via the NO/cGMP/Protein kinase G signal pathway in the bone tissue of rats with osteoporosis (10). In later studies, the positive effects of PDE5 inhibitors on bone were reported (11, 12). Although positive effects of PDE5 inhibitors on bone have been reported, the effects of these inhibitors on BMP2 and 9, which have important effects on bone remodelling, are not clear. The effects of PDE5 inhibitors on erectile dysfunction and bone tissue have been particularly focused on, but their effects on liver tissue have also been examined. Studies have reported that PDE5 inhibition decreased liver damage and reduced oxidative damage in septic rats (13). PDE5 inhibition has also been shown to have positive effects on liver function (14, 15). Liver function is an essential factor in the regulation of BMPs and their activity, and any changes in liver function can affect bone metabolism and contribute to osteoporosis in postmenopausal women. In particular, BMP9 is mainly produced in the liver. Therefore, it is important to maintain healthy liver function to promote bone health in this population. This study aims to measure the levels of vascular endothelial growth factor (VEGF), BMP 2, BMP 9, malondialdehyde (MDA), coenzyme Q10 (CoQ10) as markers of oxidative damage in liver tissue of rats with osteoporosis following ovariectomy and treatment with vardenafil, udenafil, and tadalafil as PDE5 inhibitors. The objective is to ascertain whether the beneficial effects of these inhibitors on bone tissue are mediated through BMP 2 and 9, and to evaluate their impact on VEGF.

## Materials and Methods

**Chemicals:** All agents and chemicals were obtained from commercial suppliers. The study utilized three different tablets: vardenafil (Levitra 10 mg), tadalafil (Longis 10 mg), and udenafil (Zydena100 mg), all of which were sourced from different manufacturers in Istanbul, Turkey. The vardenafil was obtained from Bayer, the tadalafil from Santa Pharma, and the udenafil from Abdi Ibrahim. Thiobarbituric acid and 1,1,3,3-tetraethoxypropane used for MDA measurement were also obtained from analytical grade CoQ10 sigma used as a standard for CoQ10 analysis (5500, T9889, and C9538 respectively, Sigma Aldrich, USA).

**Experimental design:** The experimental cohort of this study comprised 50 female Albino Wistar rats, which were never used for breeding purposes. The rats weighed between 200-250 g and were of the same age, eight months old, and born in the same week. To conduct the experiment, the rats were divided into five different groups, each with an equal number of

rats (n=10). The groups were designated as sham, ovariectomized (OVEX), OVEX+vardenafil, OVEX+tadalafil, and OVEX+udenafil. A stable living environment was provided for the rats during the experiment, with consistent conditions such as a 12-hour light-dark cycle and controlled temperature and humidity levels. All rats were fed a standard pellet feed purchased from Bayramoğlu Yem in Erzurum, Turkey.

**Surgical procedure and PDE5 inhibitors application:** During all surgical procedures, 20 mg/kg ketamine was administered intraperitoneally to anesthetize the rats (10% Ketazol, Austria). The sham group underwent only laparotomy, while the other groups underwent total abdominal hysterectomy and bilateral ovariectomy. Following ovariectomy, the rats' menstrual bleeding was observed, and two months of treatment with vardenafil, tadalafil, and udenafil was initiated after a six-month period. The inhibitors (10 mg/kg per day) were given via gavage. (10). After administration of PDE5 inhibitors, rats were sacrificed under deep anaesthesia and liver tissues were harvested for study.

**Radiologic examination:** The Hologic QDR-Discovery C DXA device (Hologic, Inc., Waltham, MA) was utilized for bone mineral density (BMD) measurement. The BMD was measured for the whole body of the rats while they were anesthetized. The rats underwent scanning prior to ovariectomy, six months after ovariectomy, and following two months of inhibitor administration.

**Assay of biochemical parameters:** A 1:10 weight-to-volume ratio of 300 mg liver tissue and 3 mL of 50 mM phosphate buffer was used to obtain tissue supernatant. A five-minute homogenization process at 12000 rpm was performed on the mixture using an Ultra Turrax-T25 (Janke and Kunkel, Germany). The homogenized samples were then centrifuged at 300 g and +4°C for 30 minutes, and the supernatant obtained was transferred to clean eppendorf tubes and stored at -80°C until the study. BMP-2, 9 and VEGF levels in the supernatants were determined with commercially available kits working with the enzyme linked immune sorbent assay (ELISA) principle. In addition, the total protein levels were detected using the chemiluminescence immunoparticle method in the routine biochemistry analyser of the Architect System Abbott Plus CI 16200 ® (Abbott Diagnostic Architect Plus CI 16200, USA). Results were expressed as ng/mg protein for BMP 2 and pg/mg protein for BMP 9 and VEGF. High pressure liquid chromatography (HPLC) method was used for MDA measurement in supernatant samples (16). The tissue supernatant was centrifuged at 2500 g for 3 minutes and the clear supernatant was used HPLC device (Agilent 1200 Series, Agilent Technologies, Waldbronn, Germany). The fluorometric detector excitation was set at 527nm and the emission at 551nm wavelength. The analytical coloum was RP-C18 150x4.6 mm, 5µm particle size (ACE, Aberdeen Scotland). The MDA-TBA peak was calibrated using 1,1,3,3 tetraethoxypropane standard solution and sample results were obtained according to the calibration peak. The levels of MDA were expressed as µmol/g protein. We measured the levels of CoQ10 (oxidized) and CoQ10H (reduced) by HPLC method as performed by Mosca at al (17). We employed an electrochemical detector (ECD) (Waters, USA) to detect oxidized CoQ10, with the ECD set at 0.35 V. An analytical column of RP-Supercosil LC 18 (150x4.6 mm, 5 µm i.d., Waters, Inc.) was used for the analysis. The levels of oxidized CoQ10 were quantified and reported as mM/g protein.

**Histopathologic and immunohistochemical examinations:** Liver tissue samples, collected post-necropsy, were fixed for 48 hours in a 10% formalin solution, followed by routine tissue preparation procedures to produce paraffin-embedded blocks. Each block was sectioned to generate 4 µm-thin sections, which were then stained with hematoxylin-eosin (HE) to enable histopathological examination under a light microscope. Severity of any observed histopathological changes in each section was quantified using a grading system varying from 'absent' (-) to 'very severe' (++++), allowing for accurate evaluation of the tissue damage. Immunoperoxidase examination was conducted on all sections taken on adhesive (poly-L-Lysin) slides. The sections were washed with xylol and alcohol and then treated with 3% $H_2O_2$ for 10 minutes to inactivate endogenous peroxidase. Antigen retrieval solution was applied for 2x5 minutes at 500 watts in a microwave oven to reveal the antigen in the tissues, and the primary antibodies were incubated with CD31 (Katolok no: sc-, santa cruz, USA). Immunopositivity was evaluated by assessing sections as absent (-), mild (+), moderate (++), severe (+++), or very severe (++++) after floor staining with hematoxylin. Throughout the process, the immunohistochemistry kit procedure (AbcamHRP/DAB Detection IHC kit) was

followed in order to ensure accuracy and consistency.

**Statistical analysis:** Analyse-it software (Analyse-it Software, Ltd., Leeds, United Kingdom) was used for statistical analysis. Normal distribution of data was determined by Kolmogorov–Smirnov test. Comparison of groups was made with one-way ANOVA test. Comparison of repeated measures between groups was made with the repeated measure ANOVA test.A p value <0.05 was considered statistically significant.

**Ethics approval:** This study was performed in line with the principles of the Declaration of Helsinki. Ethics Committee permission was obtained from Van Yuzuncu Yil University Experimental Animals Local Ethics Committee with the decision number 05 dated 31.05.2018.

## Results

BMD was measured three times in all groups: before ovariectomy, after ovariectomy and after treatment. It was determined that BMD levels decreased significantly after 6 months in rats that underwent ovariectomy. In the measurements performed two months after the inhibitor application, it was determined that the BMD values increased. All BMD results are summarized in Table 1 and Figure 1.

**Table 1:** The BMD levels comparisons in all groups.

| Groups | BMD1 (g/cm²) (mean±SD) | BMD2 (g/cm²) (mean±SD) | BMD3 (g/cm²) (mean±SD) |
|---|---|---|---|
| Sham | 0.181±0.021a | 0.215±0.013b | 0.221±0.011b |
| OVEX | 0.199±0.006a | 0.179±0.006b | 0.171±0.008c |
| OVEX+Tadalafil | 0.205±0.008a | 0.183±0.005b | 0.197±0.006a |
| OVEX+Udenafil | 0.186±0.007a | 0.172±0.009b | 0.191±0.007a |
| OVEX+VArdenafil | 0.198±0.011a | 0.179±0.004b | 0.192±0.008a |

**BMD1:** Before ovariectomy, **BMD2:** After ovariectomy and **BMD3:** After inhibitors administration. Different letters on the same row show a statistically significant difference (p<0.05).

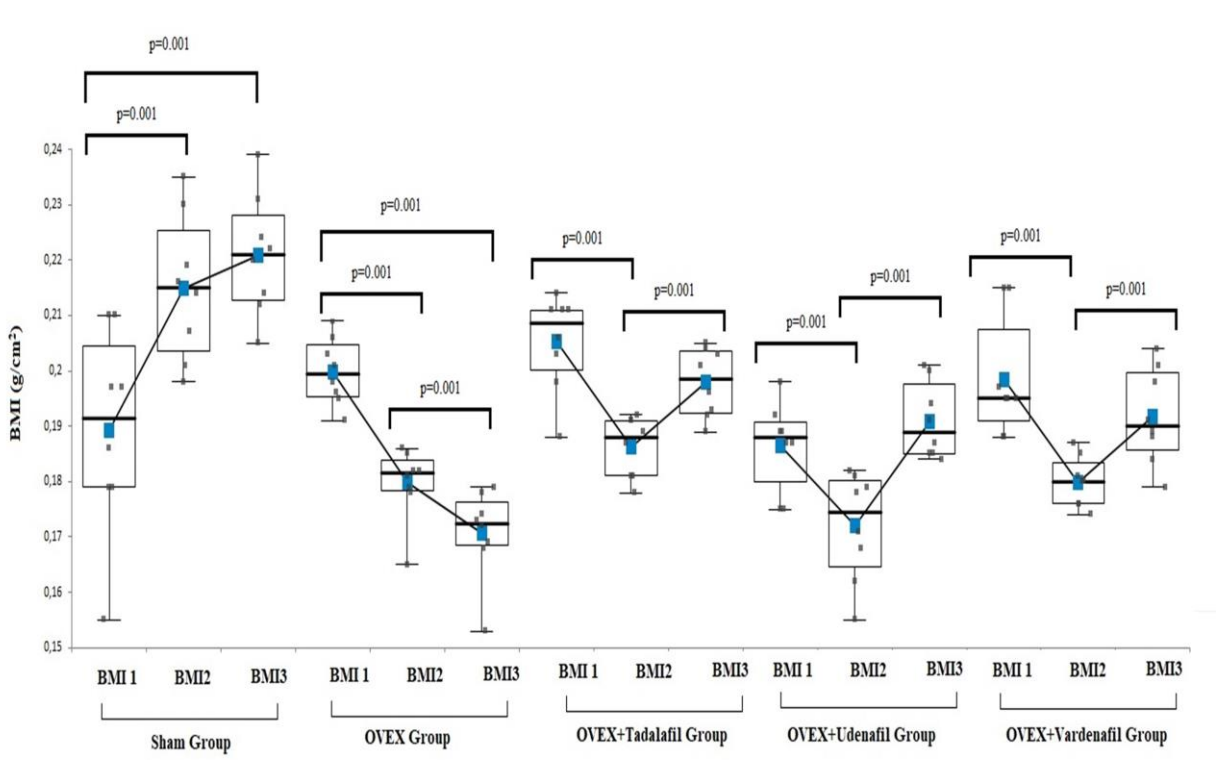


**Figure 1.** Box plot presentation of the BMD levels in all groups. BMD1: Before ovariectomy, BMD2: After ovariectomy and BMD3: After inhibitors administration.

**Table 2:** The result of biochemical parameters

| | BMP-2 (ng/g protein) | BMP-9 (pg/g protein) | VEGF (pg/g protein) | MDA (µmol/g protein) | CoQ10 (µmol/g protein) |
|---|---|---|---|---|---|
| Sham | 2.62±0.16[a] | 49.3±3.94[a] | 44.5±1.44[a] | 22.2±4.06[a] | 7.11±1.66[a] |
| OVEX | 2.73±0.14[a] | 52.4±2.61[a] | 47.9±2.69[a] | 49.6±3.11[b] | 14.5±1.22[b] |
| OVEX+vardenafil | 3.51±0.19[b] | 61.1±5.55[b] | 60.7±3.31[b] | 38.1±1.88[c] | 9.15±1.42[a] |
| OVEX+Tadalafil | 4.32±0.33[c] | 70.3±6.43[c] | 73.1±4.45[c] | 36.5±3.05[c] | 9.89±0.87[c] |
| OVEX+udenafil | 3.63±0.34[b] | 62.9±4.81[b] | 62.1±4.41[b] | 37.6±2.12[c] | 10.5±1.57[c] |

Different letters in the same column indicate a statistically significant difference between groups. Results are given as mean ± standard deviation.

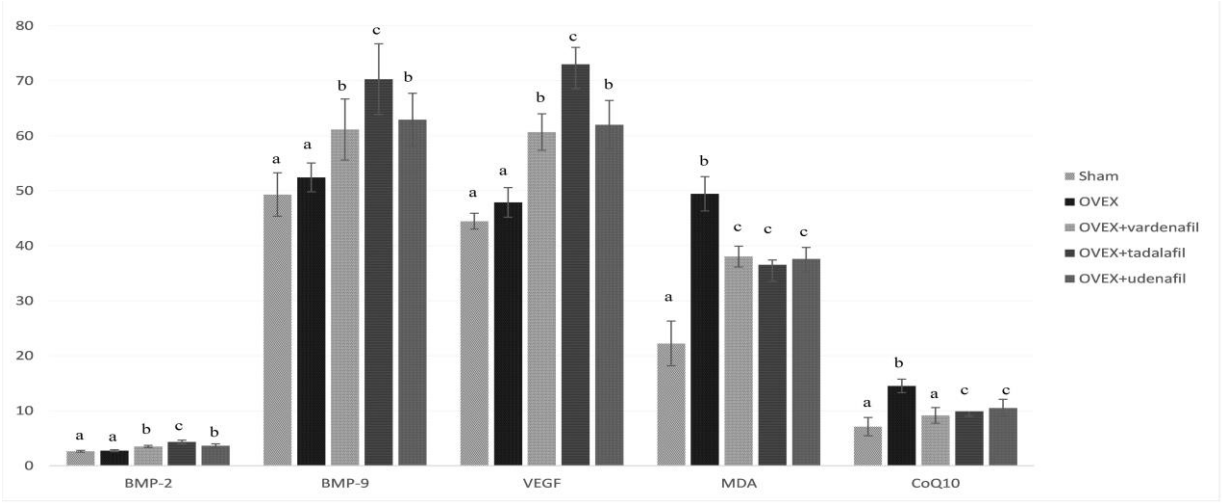


**Figure 2.** Comparison of the results of biochemical parameters between groups. Different letters for the same variable indicate statistical significance.

In our study, the lowest BMP 2 levels were detected in the sham group. Although BMP 2 levels in the sham group were lower than those in the OVEX group, this was not statistically significant (p=0.934). BMP 2 levels of the groups treated with inhibitor were statistically significantly higher than the levels in both the sham and OVEX groups (p<0.001). There was no significant difference between OVEX+vardenefail and OVEX+udenafil groups in terms of BMP 2 levels (p=0.995). BMP 2 levels of OVEX+tadalafil group were significantly higher than all other groups (p<0.001). When BMP 9 levels were examined, the lowest BMP-9 levels were detected in the sham group. Although BMP 9 levels in the Sham group were lower than those in the OVEX group, this difference was not significant (p=0.601). BMP 9 levels of inhibitor-treated groups were significantly higher than both sham and OVEX groups. There was no significant difference between the groups, although the highest level was from the OVEX+tadalafil group among the inhibitor applied groups. The highest VEGF levels were detected in the OVEX+tadalafil group, and VEGF values of this group were significantly higher than all other groups. Similar to BMP 2 and 9, although VEGF values of the sham group were lower than those of the OVEX group, this difference was not significant. Also, no significant difference was observed between OVEX+vardenafil and OVEX+udenafil groups. When MDA and CoQ10 levels, which are oxidative damage markers, were examined, the lowest MDA and CoQ10 levels were found in the sham group, and this decrease was statistically significant (p<0.05). The MDA levels of the inhibitor applied groups were significantly lower than the MDA levels of the OVEX group (p<0.05). There was no significant difference in MDA levels between the inhibitor-treated groups. CoQ10 levels of the Sham group were significantly lower than both the inhibitor-treated groups and the OVEX group. CoQ10 levels of the inhibitor applied groups were

significantly lower than the OVEX group ($p<0.05$). All biochemical data are summarized in Table 2 and Figure 2.

**Histopathological and immunohistochemical results:** The liver tissues of the rats in the sham group had a normal histological appearance (Figure 3-A). The liver tissue samples of the rats in the OVEX group had mild dilatation of the sinusoids and mild hydropic degeneration of the hepatocytes were observed (Figure 3-B). The group of rats that received OVEX+Tadalafil treatment exhibited mild dilatation in the liver tissues and sinusoids, and also demonstrated dilatation and hyperemia in the interstitial vessels (as illustrated in Figure 3-C). Significantly increased number of vessels were observed in this group as compared to the sham and OVX groups ($p<0.05$). Histopathological examination of the OVEX+Vardenafil group revealed sinusoidal dilatation and hyperaemia in the interstitial vessels (Figure 1-E). The number of these veins was significantly higher in this group as compared to the sham and other experimental groups ($p<0.05$). All data were given in Table 3. The immunohistochemical examination of liver tissues from rats in different groups showed varyin levels of CD31 expression in the vascular endothelium. In the sham group, there was very little CD31 expression observed in the vascular endothelium (Figure 4-A). However, in the OVEX group, mild CD31 expression was observed in the interstitial vessel endothelium in the liver parenchyma (Figure 4-B). In the OVEX+tadalafil group, moderate CD31 expression was observed in the liver interstitial vessel endothelium, which was significantly higher than that observed in the sham and OVEX groups ($p<0.05$) (Figure 4-C). Similarly, in the OVEX+udenafil group, moderate CD31 expression was observed in the liver interstitial vessel endothelium, which was significantly higher than that observed in the sham and OVEX groups ($p<0.05$) (Figure 4-D). In the OVEX+vardenafil group, intense CD31 expression was observed in the interstitial vessel endothelium, and CD31 expression levels were significantly higher than those observed in other groups ($p<0.05$) (Figure 4-E). The immunohistochemical findings is presented in Table 3.

**Table 3: Histopathological and immunohistochemical findings in liver tissues**

| | Sham | OVEX | OVEX+Tadalafil | OVEX+Udenafil | OVEX+Vardenafil |
|---|---|---|---|---|---|
| Degeneration in Hepatocytes | - | ++ | - | - | - |
| Sinusoidal Dilation | - | + | ++ | ++ | +++ |
| Hyperemia in the veins | - | + | ++ | ++ | +++ |
| CD31 Expression | + | + | ++ | ++ | +++ |

**Note:** The intensity of staining was scored as no staining (-), low (+), moderate (++), marked (+++).

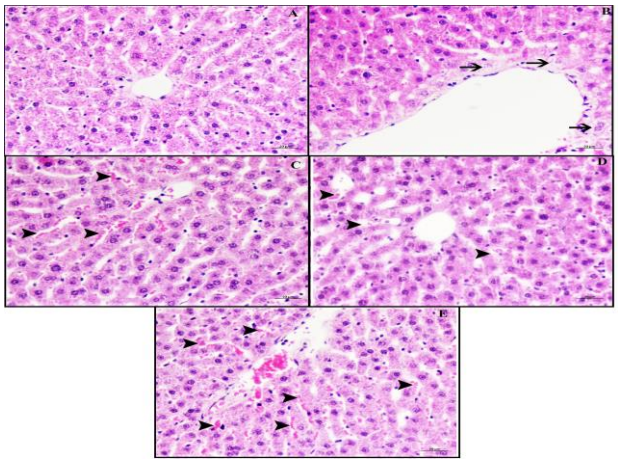


**Figure 3. A**: Sham group, liver tissue, normal histological appearance, **B**: OVEX group, liver tissue, mild hydropic degeneration (Arrows), sinusoidal dilatation, **C**: OVEX+Tadalafil group, liver tissue, moderate sinusoidal dilatation and vascular hyperemia (Arrowheads), **D**: OVX+Udenafil group, liver tissue, moderate sinusoidal dilatation and vascular hyperemia (Arrowheads), **E:** OVX+Vardenafil group, liver tissue, severe sinusoidal dilatation and vascular hyperemia (Arrowheads), H&E, Bar: 20 μm.

## Discussion

Liver tissue has important effects on bone tissue by secreting proteins such as BMP. Therefore, it is important that the agents to be used in the treatment of osteoporosis do not cause liver toxicity. In previous studies, it has been reported that medications such as bisphosphonates used in

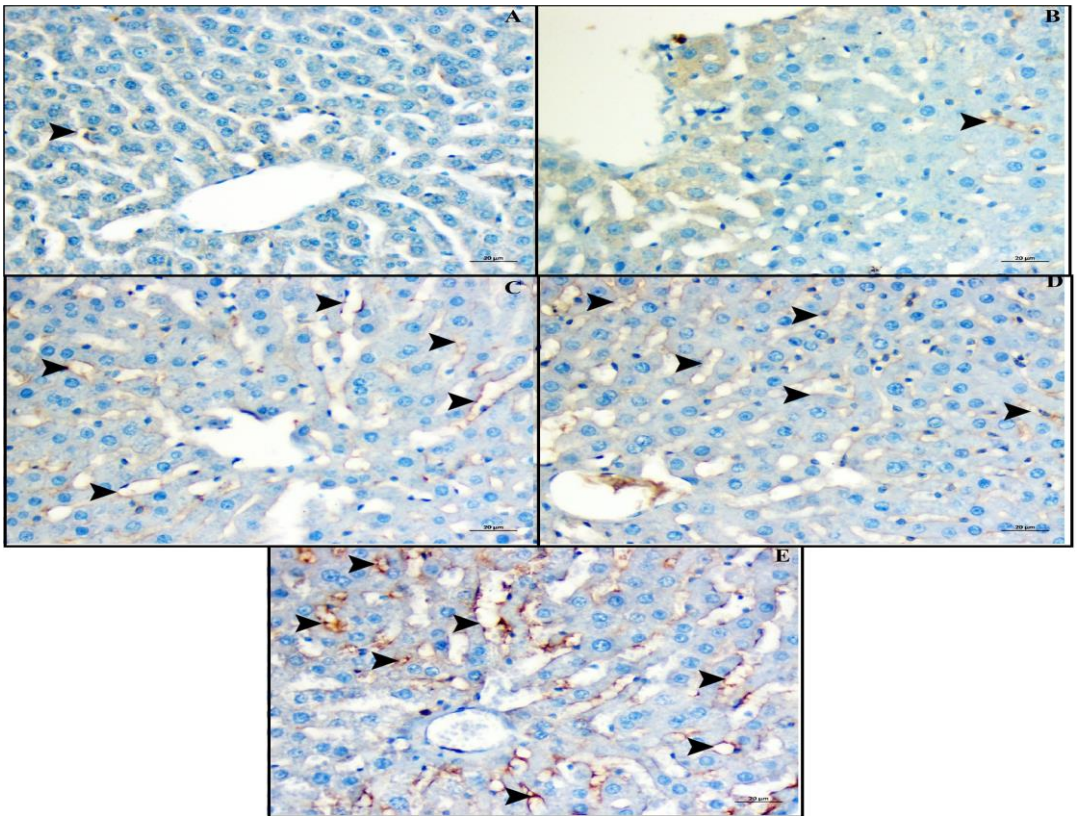


**Figure 4.** A: Sham group, liver tissue, very mild CD31 expression (Arrowheads), B: OVX group, liver tissue, mild CD31 expression (Arrowheads), sinusoidal dilatation, C: OVX+Tadalafil group, liver tissue, moderate CD31 expression (Arrowheads), D: OVX+Udenafil group, liver tissue, moderate CD31 expression (Arrowheads), E: OVX+Vardenafil group, liver tissue, severe CD31 expression (Arrowheads), IHC-P, Bar: 20 µm.

**Table 4:** Immunohistochemical findings in liver tissues

| | Sham | OVEX | OVEX+Tadalafil | OVEX+Udenafil | OVEX+Vardenafil |
|---|---|---|---|---|---|
| Degeneration in Hepatocytes | - | + | - | - | - |
| Sinusoidal Dilation | - | + | ++ | ++ | ++ |
| Hyperemia in the veins | - | + | ++ | ++ | +++ |
| CD31 Expression | + | + | ++ | ++ | +++ |

**Note:** The intensity of staining was scored as no staining (-), low (+), moderate (++), marked (+++).

the treatment of osteoporosis may affect liver function and thus the efficacy of these medications may decrease (18). Therefore, agents to be used in the treatment of osteoporosis should not have a negative effect on important proteins secreted from the liver. This consideration has led researchers to look for different agents for the treatment of osteoporosis and NO may be a good alternative. However, a situation that should not be ignored is that NO easily reacts with the superoxide radical to form products such as peroxynitrite anion, which can cause oxidative damage. Considering the positive effects of NO on bone formation, it has been hypothesised that PDE5 inhibitors can be used to increase the positive effects of NO on bone via the cGMP/PKG pathway without external NO supplementation. This hypothesis was supported by previous experimental studies completed by our study group. One study demonstrated the positive effects of PDE5 inhibitors (udenafil, tadalafil and vardenafil) on bone formation in rats with ovariectomy-induced osteoporosis (10). Our group was also able to demonstrate the positive effects of avanafil and zaprinast, which are also PDE5 inhibitors, on bone formation in male rats with glucocorticoid-induced osteoporosis (19). In this study, we investigated the effects of

vardenafil, tadalafil and udenafil on BMP2, BMP9 and VEGF levels in the liver tissue of rats with ovariectomy-induced osteoporosis. In addition, the effects of these inhibitors on oxidative damage in liver tissue were also investigated. Although BMP2 is an FDA-approved agent for clinical use in the treatment of bone fractures, it is still unclear whether it is effective enough to remain a treatment option in orthopaedics (20). In addition to the osteogenic properties of NO's pathways, its secondary messenger properties suggest that NO may also have a relationship with BMP2. The cGMP secondary messenger has a crucial role in BMP-related signalling. Therefore, the NO/cGMP/PKG signalling pathway may be closely related to BMP receptors. Yang et al. reported that sildenafil enhanced canonical BMP signalling via cGMP and cGMP-dependent protein kinase I in vitro and in vivo (21). In our study, it was determined that PDE5 inhibitors increased BMP2 and BMP9 levels in the liver tissue of rats with ovariectomy-induced osteoporosis. These results show that the PDE5 inhibitors vardenafil, udenafil and tadalafil are closely related to BMP2 and BMP9 expression. These results support recent work by our study group. Huyut et al. investigated BMP2 and BMP4 levels in the kidney tissue of rats with ovariectomy-induced osteoporosis and reported that BMP2 and BMP4 levels in the vardenafil, udenafil and tadalafil group were higher than those in the sham and ovariectomised groups (12). Again, in the study conducted by Huyut et al., the levels of BMP2 and BMP4 in the kidney tissue of rats with ovariectomy-induced osteoporosis using avanafil and zaprinast, which are PDE5 inhibitors, were examined, and they reported that BMP2 and BMP4 levels in the inhibitor-administered groups were higher than those in the sham and ovariectomised groups. (22). One of the most important growth factors required for vascular development and angiogenesis is VEGF. In addition to these roles, VEGF also plays an important role in bone repair and regeneration (23). Senel et al. reported that VEGF levels decreased in postmenopausal women with osteoporosis (24). There is also a relationship between VEGF and BMPs. BMPs have been reported to increase VEGF expression in osteoblasts (25). In our study, we found that VEGF levels in the vardenafil, udenafil and tadalafil group were higher than those in the ovariectomised group. These results were also supported by histopathological and immunohistochemical analysis. Huyut et al. investigated VEGF levels in the kidney tissue of ovariectomy-induced rats and reported that VEGF levels were significantly higher in the groups treated with vardenafil, udenafil, tadalafil, avanfil and zaprinast (12, 22). These results show that angiogenesis plays an important role in the positive effects of PDE5 inhibitors on bone and that this may be mediated by VEGF. MDA and CoQ10 are important biomarkers for oxidative stress. There is evidence that PDE5 inhibitors reduce oxidative stress. Özer et al. investigated the effects of some PDE5 inhibitors on oxidative stress in different rat tissues and reported that while MDA levels decreased in the group administered sildenafil, glutathione peroxidase and superoxide dismutase levels increased (26). Fan et al. examined the effects of vardenafil on oxidative stress in patients with pulmonary arterial hypertension and reported that superoxide dismutase levels increased (27). However, Savaş et al. investigated the effects of tadalafil on oxidative damage in men with erectile dysfunction and reported that the total oxidant status decreased, and the total antioxidant status increased (28). As a result, our biochemical, histopathological and immunohistochemical analyses show that the PDE5 inhibitors vardenafil, tadalafil and udenafil increase VEGF, BMP2 and BMP9 levels in the liver tissue of rats with ovariectomy-induced osteoporosis. This suggests that VEGF, BMP2 and BMP9 mediated angiogenesis may play a role in the positive effects of PDE5 inhibitors on the bone tissue of rats with osteoporosis. In addition, these inhibitors may have positive effects on oxidative damage. PDE5 inhibition may be a useful target in the treatment of osteoporosis.

**Study limitations:** The study did not investigate the effects of different doses or dosing regimens of the phosphodiesterase 5 inhibitors. Variation in dosing could potentially affect the observed outcomes and their effectiveness. Further studies exploring different doses and treatment durations are needed to determine the optimal therapeutic approach.

**Ethics approval:** This study was performed in line with the principles of the Declaration of Helsinki. Ethics Committee permission was obtained from Van Yuzuncu Yil University Experimental Animals Local Ethics Committee with the decision number 05 dated 31.05.2018.

**Competing interests:** The authors have no relevant financial or non-financial interests to disclose.

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