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ORIGINAL ARTICLE

The effect of pharmacological treatment in combination with vitamin K on healing in an experimental rat model of osteoporosis

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Osteoporosis is a systemic disease characterized by the deterioration of bone microarchitecture and compromised bone mass with consequent bone fragility and fracture risk.[1] It is a growing health problem with the aging of the population worldwide.

Osteoporosis is a silent disease that is often unnoticed by patients before fracture occurs. In general, the chief complaint is waist and back pain due to vertebral compression fractures.[2] However, hip fractures are the leading cause of mortality and morbidity.[3]

The main goal of osteoporosis treatment is to reduce fractures. This can be achieved by either

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ABSTRACT

Objectives: This study aims to compare the effects of teriparatide, zoledronic acid, and their combination therapy with vitamin K on osteoporotic rats.

Materials and methods: We divided a total of 50 female Sprague-Dawley rats into five groups: A (the control group), B and D (the teriparatide group), and C and E (the zoledronic acid group). Following ovariectomy and subcutaneous heparin administration at a dose of 2 IU/kg for four weeks, osteoporosis was created. Groups A, B, and C were fed with standard feed, while Groups D and E were fed with vitamin K-rich feed. After four weeks of treatment, sacrification was performed. The right and left femurs were separated for histopathological and biomechanical evaluation, respectively. For histopathological evaluation, the femurs were decalcified, and the sections were stained with hematoxylin-eosin and evaluated under a light microscope. Fracture healing was evaluated using the classification system as described previously. For biomechanical evaluation, the 3-point stress test and torsion stress test were applied to 10 femurs from each group.

Results: Groups B-E were histopathologically and biomechanically superior to Group A in fracture healing of osteoporotic rats; however, it was not statistically significant ($p>0.05$). The group that received additional vitamin K was histopathologically and biomechanically superior to the group which was fed with standard feed, although it was not statistically significant ($p>0.05$).

Conclusion: Our study results indicated that both teriparatide and zoledronic acid had beneficial effects on osteoporotic fractures with comparable histological and biochemical results. Vitamin K promoted teriparatide and zoledronic acid treatment on osteoporotic fracture healing. Based on these findings, combination therapies may yield the most optimal results in biomechanical and histological examinations.

Keywords: Osteoporosis, teriparatide, vitamin K, zoledronic acid.

increasing bone formation or decreasing bone resorption.[4] Bisphosphonate is the current first-line treatment for postmenopausal osteoporosis and zoledronic acid is a potent bisphosphonate associated with a significant reduction of the risk of fractures in postmenopausal osteoporosis.[5] Teriparatide is a bone-forming agent for the treatment of osteoporosis. Similar to bisphosphonate, teriparatide reduces the risk of vertebral and non-vertebral fractures in postmenopausal women.[6]

Vitamin K is a well-known anti-hemorrhagic factor which improves bone microarchitecture and ensures calcium balance to prevent bone loss.[7]

In the present study, we aimed to investigate the effects of the most commonly used agents in osteoporosis treatment - teriparatide, zoledronic acid, and their combination therapies with vitamin K - on the histological and biomechanical properties of bone healing via a fracture model generated on osteoporotic rat femurs.

MATERIALS AND METHODS

The osteoporosis model was created with ovariectomy and heparin administration for four weeks.[8] After osteoporosis was proven, a non-displaced fracture model was created by making holes 0.8 mm in diameter on bilateral femoral diaphysis.[9]

A total of 50 16-week-old female Sprague-Dawley rats were used in this study. The groups were constituted randomly. The rats were maintained in the animal research laboratory under environmentally controlled conditions at 20±1°C room temperature in a 12-h dark/12-h light cycle and with tap water and food *ad libitum*. The rats were kept in polycarbonate cages, with five animals per cage. Numbers were written on the rats' tails, and the numbers were rewritten if wiped out. The rats drank water purified with Hydro-Pac® (Hydration Technology Innovations, NY, USA) and were allowed to acclimate to their environment for one week before the start of the experiments. To ensure that the rats would not develop hypothermia, the operations were performed on a heated surgical table, and warming was continued until postoperative recovery. Bone mineral densities (BMDs) of four randomly chosen rats were calculated twice, before ovariectomy operations and four weeks later after heparin administration by dual-emission bone absorptiometry (Hologic NV/SA, Zaventem, Belgium).

Initial surgical procedure

All rats were operated under 40 mg/kg ketamine hydrochloride (Keta-Control®; Doğa Pharmaceuticals, Istanbul, Türkiye) and 5 mg/kg xylazine (Basilazin®; Bavet Pharmaceuticals, Istanbul, Türkiye) anesthesia. Cephazolin sodium (Sefazol®; Mustafa Nevzat Pharmaceuticals, Istanbul, Türkiye) was administered preoperatively for antibiotic prophylaxis at a dose of 15 mg/kg. Before surgical incision, the abdomen was shaved and prepared with 10% benzalkonium chloride solution (Zefirolum®; Kimpa Pharmaceuticals, Istanbul, Türkiye) and povidone-iodine solution (Poviiodeks®; Kimpa Pharmaceuticals, Istanbul, Türkiye). Ovariectomies were performed by midline incisions (Figure 1). After surgery, the rectus abdominis muscle and peritonea were closed with polyglycolic acid suture

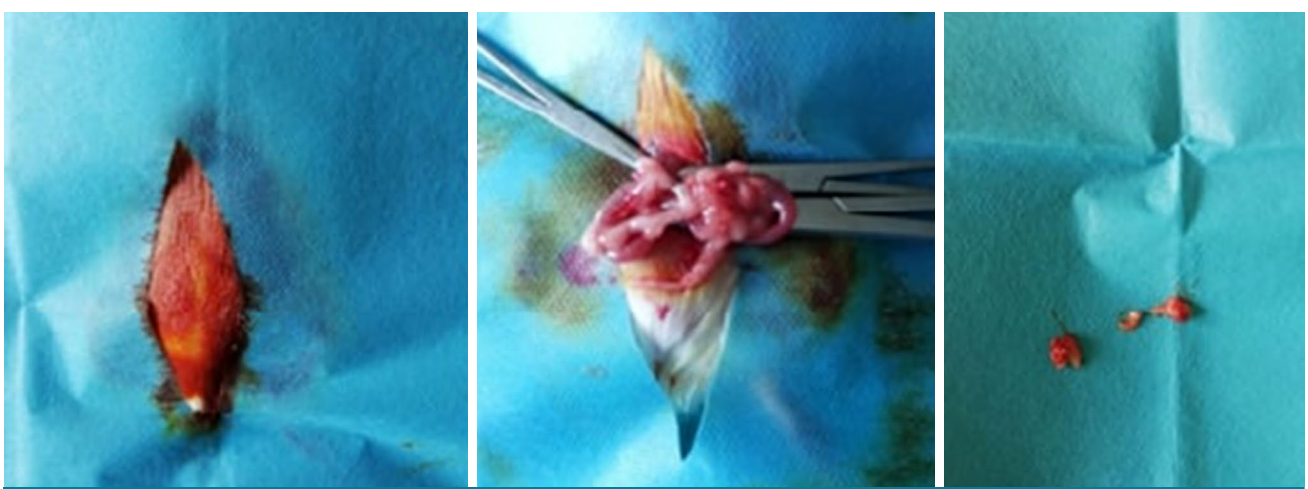


FIGURE 1. Ovariectomy operation.

(Glikosorb®; Boz Medical Consumables, Ankara, Türkiye), and the skin was closed with polypropylene suture (Monoprolen®; Boz Medical Consumables, Ankara, Türkiye). For analgesic treatment, paracetamol (Parol®; Atabay Pharmaceuticals, Istanbul, Türkiye) was administered orally at a dose of 2 mg/mL. Heparin (Nevparin®; Mustafa Nevzat Pharmaceuticals, Istanbul, Türkiye) was administered to all rats for four weeks.

Second surgical orocedure

After osteoporosis was confirmed, including the control group, all rats were operated under 40 mg/kg ketamine hydrochloride (Keta-Control®) and 5 mg/kg xylazine (Basilazin®) anesthesia. Cephazolin sodium (Sefazol®) was administered preoperatively for antibiotic prophylaxis at a dose of 15 mg/kg. Before surgical incision, the bilateral lower limb was shaved and prepared with 10% benzalkonium chloride solution (Zefirolum®) and povidone-iodine solution (Poviiodeks®). A 1-cm incision was made along the femur shaft. When the femur bone was exposed, a non-displaced fracture model was created by drilling a hole with a 0.8-mm Kirchner wire (Figure 2). The muscle and fascia were closed with polyglycolic acid suture (Glikosorb®), and the skin was closed with polypropylene suture (Monoprolen®).

Study groups

Group A (control group) was given 1 mL/kg serum saline subcutaneously three days a week for the placebo effect and fed with standard feed. Group B was treated with 6 μg/kg intraperitoneal teriparatide three days a week and fed with standard feed. Group C was treated with 0.1 mg/kg subcutaneous zoledronic acid three days a week and fed with standard feed. Group D was treated with 6 μg/kg intraperitoneal teriparatide three days a week and fed with 30 mg/kg vitamin K-rich feed. Group E was treated with 0.1 mg/kg subcutaneous zoledronic acid three days a week and fed with 30 mg/kg vitamin K-rich feed. After four weeks of treatment, the animals were sacrificed using the cervical dislocation method under 40 mg/kg ketamine hydrochloride (Keta-Control®) and 5 mg/kg xylazine (Basilazin®) anesthesia. Histological evaluation was performed on the right femurs, and biomechanical evaluation was performed on the left femurs.

Histological examination

A total of 50 femurs were kept in 10% formaldehyde for four days and, then, in 10% formic acid solution for 15 days for decalcification. All femurs were embedded in paraffin. Sections of 4 μm were obtained from the paraffin blocks and stained with hematoxylin-eosin for light microscope evaluation. Fracture healing was analyzed using the classification system described by Allen et al.[10] (Figures 3 and 4).

Biomechanical examination

Biomechanical tests were applied to 50 femurs. Five randomly selected femurs from each group were allocated for a 3-point stress test, and the remaining five femurs were allocated for torsion tests. Both sides of the femurs were embedded in polyurethane material so that both ends could be connected to the jaws, and the middle parts of the bones were placed on the rotational axes of the jaws. Torsion testing was assessed using the Instron™ 55MT2 Electromechanical Testing Machine, Partner Torsional Stiffness Software (Instron Co., Norwood, MA, USA). Torsion force was applied to all specimens at a rate of 2.5 degrees per sec. A 225-Joule capacity load cell reaction torque sensor 2110-2k (Lebow, Troy, MI, USA)



FIGURE 2. Drilling hole in femur operation.

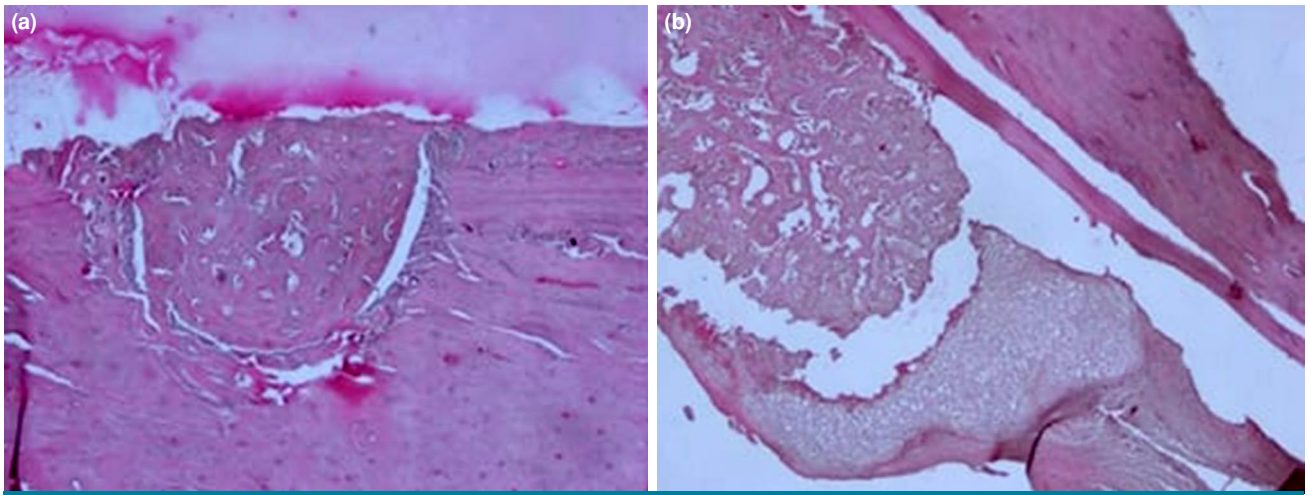


FIGURE 3. According to Allen et al.'s scoring system[10] (a) score 5 example, magnified ×2 times, (b) score 4 example, magnified ×4 times.

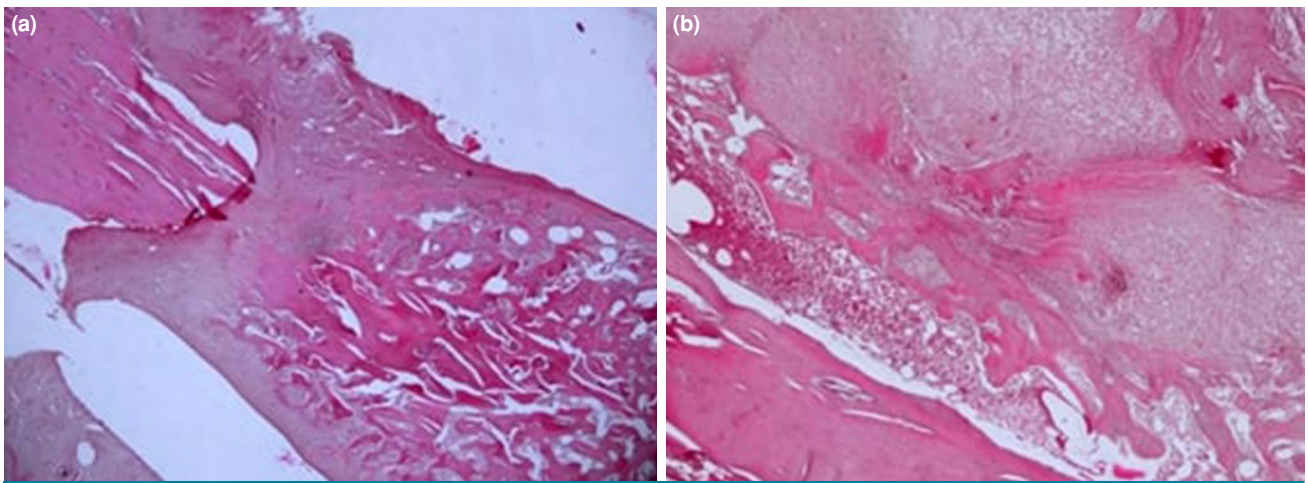


FIGURE 4. According to Allen et al.'s scoring system[10] **(a)** score 2 example, magnified ×2 times, **(b)** score 3 example, magnified ×4 times.

was used to measure torque. The maximum torque required for fracture and the degree of torsion up to fracture were calculated (Figure 5).

To calculate the mechanical properties of the bones, 3-point bending tests were performed on the 30 kN capacity machine 3367J5963 Electromechanical Testing Machine (Instron Co., Norwood, MA, USA). This machine recorded load displacement. The load velocity was 0.5 mm/min and the data acquisition rate was 0.5 kH. The testing machine supporter diameters and distances were 5 mm and 20 mm, respectively. The maximum load and tensile strength were calculated (Figure 6).

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 25.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were presented in median and interquartile range (IQR) or number and frequency, where applicable. The Kruskal-Wallis test was used to assess non-parametric variables. A p value of <0.05 was considered statistically significant.

RESULTS

We measured BMD before the ovariectomy operation and four weeks after heparin administration in four randomly chosen rats from each group.

The BMD values were assessed by dual-emission bone absorptiometry. The first median BMD was 0.266 g/cm² and the second measurement was 0.213 g/cm². There was no significant difference among the groups in the first and second measurements (p=0.770 and p=0.633, respectively). The amount of decrease in BMD among the groups was similar (p=0.980). The amount of decrease in BMD was approximately 20% and was statistically significant (p<0.001).

The biomechanical properties of 25 femurs were prepared to be assessed using a 3-point stress test. The tensile strength and maximum load scores of the control group were lower than those of the treatment groups. There was no superiority between the teriparatide and zoledronic acid groups. The groups in which vitamin K was added to teriparatide and zoledronic acid were found to be superior, although this was not statistically significant (maximum load p=0.032, tensile strength p=0.028) (Figures 7 and 8).

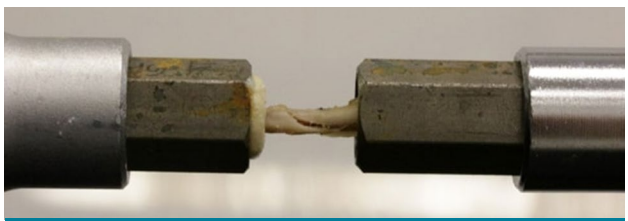


FIGURE 5. Torsion stress test, spiral fracture line.

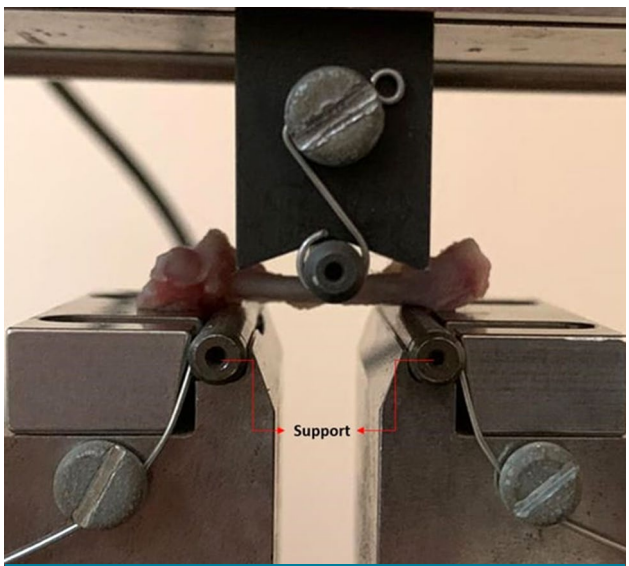


FIGURE 6. 3-point stress test.

The biomechanical properties of 25 femurs were prepared for assessment using a torsion test. Rotational stiffness, maximum failure angle, and peak torque score of the control group were lower than those of the treated groups. There was no superiority between the teriparatide and zoledronic acid groups. The groups in which vitamin K was added to teriparatide and zoledronic acid were found to be superior, although the difference was not statistically significant (rotational stiffness p=0.017, maximum failure angle p=0.005, peak torque p=0.011) (Figures 9-11).

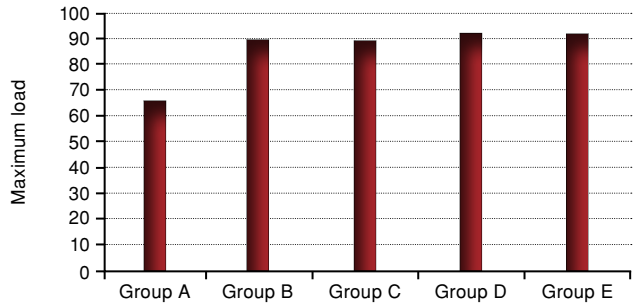


FIGURE 7. 3-point stress test, maximum load scores.

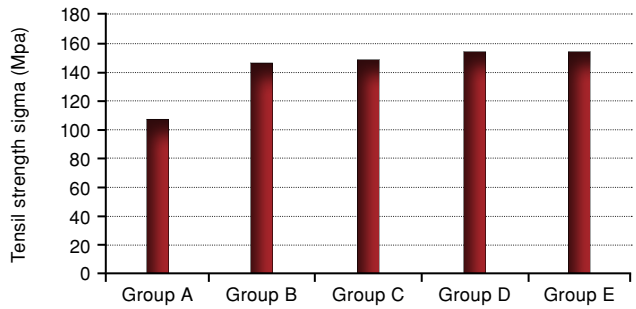


FIGURE 8. 3-point stress test, tensile strength scores.

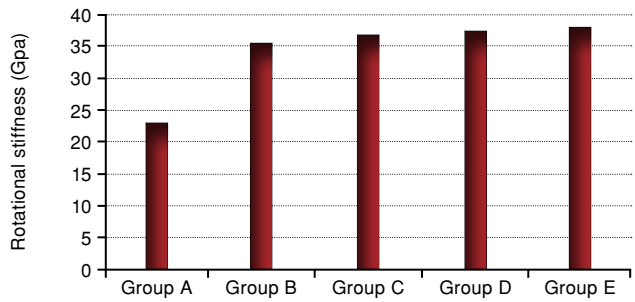


FIGURE 9. Torsion stress test, rotational stiffness scores.

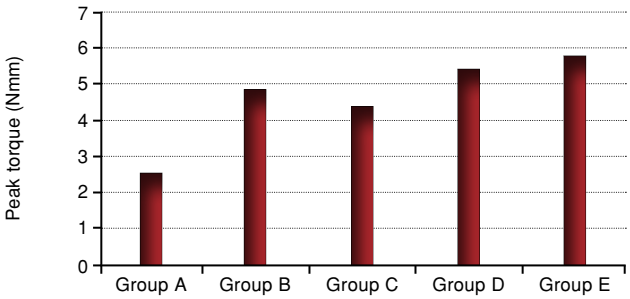


FIGURE 10. Torsion stress test, peak torque scores.

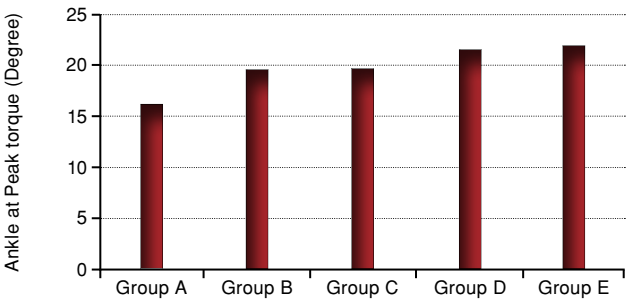


FIGURE 11. Torsion stress test, angle at peak torque scores.

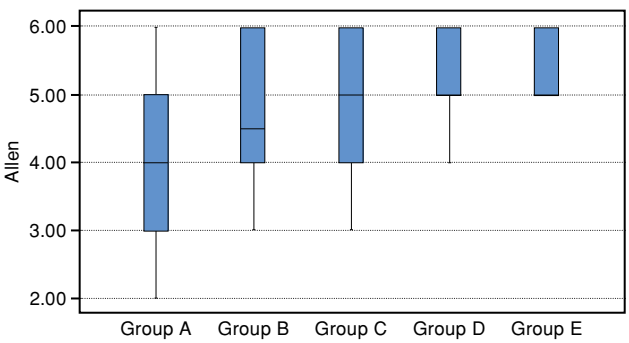


FIGURE 12. Histological scores according Allen et al.[10]

A total of 50 femurs were prepared for assessment by histological examination. Histological examination was performed based on the classification system described by Allen et al.[10] The fracture healing score of the control group was lower than that of the treatment groups. There was no superiority between the teriparatide and zoledronic acid groups. Adding vitamin K to teriparatide and zoledronic acid was found to be superior, although it was not statistically significant (p=0.084) (Figure 12).

DISCUSSION

In this study, we compared the effects of the anabolic agent teriparatide, the antiresorptive agent zoledronic acid, and their combination therapy with vitamin K, which is frequently used in osteoporosis treatment, particularly in the Asian population, on biomechanical and histological examinations in osteoporotic rats.

The use of rats as a model of ovariectomized bone loss was first reported by Miller et al.[11] Ovariectomized rats are widely used as an experimental model of osteoporosis. This model has a high turnover rate and accelerated bone formation and degradation. In this study, we created an osteoporosis model by administering heparin after ovariectomy operation. Osteoporosis was confirmed by comparing BMD measurements before the ovariectomy operation and after the ovariectomy and the administration of heparin injections for four weeks.

Osteoporosis is a serious public health problem that causes mortality and morbidity due to associated fractures. The most optimal pharmacological treatment for osteoporosis is still unclear. Osteoporosis and its treatment have been discussed extensively in the literature.[12] Huang[13] excised the ovaries of Sprague-Dawley rats to construct a rat osteoporosis model. Teriparatide or combined therapies with vitamin K were compared on bone metabolism and biomechanics in osteoporotic rats. Combination therapy was found to be superior to monotherapy. Nagura et al.[14] created an osteoporosis model by performing ovariectomy in Sprague-Dawley rats and compared teriparatide and combined therapies with vitamin K. Combination therapy was found to be superior to monotherapy in biomechanical and histopathological examinations. In our study, teriparatide treatment was found to be superior to the control group. Vitamin K and teriparatide combination therapy was found to be superior to teriparatide monotherapy treatment. However, it was not statistically significant. Teriparatide treatment in osteoporosis has been discussed extensively in the literature and found to be effective in reducing osteoporotic fracture risk, accelerating fracture healing and increasing BMD.[15]

Hornby et al.[16] evaluated the effects of long-term administration to ovariectomized Sprague-Dawley rats. Zoledronic acid caused an increase in BMD and bone strength in biomechanical tests in a dose-dependent manner. Gasser et al.[17] administered a single dose of zoledronic acid to ovariectomized

Wistar rats. Compared to alendronate, it was 10 times more potent in preventing bone loss in assessment at Week 32. Zhao et al.[18] established an animal model in rats via ovariectomy. Prior administration of vitamin K to zoledronic acid improved BMD and bone strength on biomechanical tests. It was found that vitamin K pre-treatment partially prevented the inhibition of bone formation caused by zoledronic acid. Li et al.[19] investigated zoledronic acid, teriparatide, and combination treatment on ovariectomized Sprague-Dawley rats with osteotomized tibia. Zoledronic acid and teriparatide combination therapy was found to be superior on fracture healing, but this effect was specific to callus formation. In our study, the combination of teriparatide and zoledronic acid was found to be superior to the control group. However, neither was superior to the other. Zoledronic acid combination therapy with vitamin K was found to be superior to monotherapy, but it was not statistically significant. In several studies, zoledronic acid treatment was shown to be effective in osteoporotic fracture prevention, increasing BMD and reducing mortality, particularly in postmenopausal women.[20]

Yishake et al.[21] compared the effects of single and combined zoledronic acid and teriparatide treatments on lumbar fusion in ovariectomized Sprague-Dawley rats. They found that teriparatide monotherapy and combination therapy with teriparatide and zoledronic acid increased bone fusion mass and bone fusion rate. Zoledronic acid monotherapy did not alter the bone fusion rate. Cosman et al.[22] conducted a multi-center study to compare combination therapy with teriparatide and zoledronic acid. They concluded that zoledronic acid increased hip BMD more than teriparatide, while teriparatide increased spine BMD more than zoledronic acid, and combination therapy increased both spine and hip BMD more than monotherapies. Tsubouchi et al.[23] compared teriparatide and zoledronic acid combination therapy on a rat femoral fracture model. Their experiment showed that the administration of low-dose teriparatide and zoledronic acid combination therapy increased callus volume and enhanced callus formation. In many meta-analyses, teriparatide and zoledronic acid were compared and shown to reduce the risk of fragility in osteoporosis.[24] In our study, we found no superiority of either the bone-forming agent teriparatide or the anti-resorptive agent zoledronic acid treatment in biomechanical and histological examinations.

Furthermore, Kobayashi et al.[7] investigated the effects of ovariectomy and vitamin K on calcium balance in Fischer rats. Calcium balance was poorer in the ovariectomized groups. Vitamin K treatment improved calcium balance and prevented bone loss. Akiyama et al.[25] performed ovariectomy in Fischer and Sprague-Dawley rats and created an osteoporosis model. Vitamin K administration improved BMD values and was found to be superior in biomechanical testing compared to the control groups. In addition, Hara et al.[26] carried out a prednisolone-induced bone loss model in male Fischer rats and showed that vitamin K treatment inhibited bone loss through the enhancement of bone formation. Vitamin K plays an important role in osteoporosis treatment according to some meta-analyses.[27] On the other hand, some meta-analyses have shown that studies may be biased and further studies are required.[28] In our study, vitamin K treatment was combined with teriparatide and zoledronic acid. Combination therapies were found to be superior to monotherapies in biomechanical and histological examinations.

Nonetheless, there are some limitations to this study. First, we did not find the vitamin K combination therapy group to be superior to the monotherapy groups, although it was not statistically significant. A statistically significant difference may be found with a larger number of subjects. Second, analysis with micro-computed tomography may have provided a more objective radiological evaluation than plain radiography and the drill holes could have been analyzed in detail. Further studies are needed to confirm these findings.

In conclusion, our study results indicate that both teriparatide and zoledronic acid have beneficial effects on osteoporotic fractures with comparable histological and biomechanical results. Although we did not find superiority of one over the other in our study, vitamin K can enhance teriparatide and zoledronic acid treatment on osteoporotic fracture healing. Based on these findings, combination therapies may yield the most optimal results in biomechanical and histological examinations.

Ethics Committee Approval: This study was approved by the Local Ethics Committee of Animal Experiments (date: 08/01/2020; no: 08) and conducted at Experimental Medicine Research and Application Centre laboratories.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Writer of the manuscript: İ.Ö.; Designer of the study: A.K.; Co-writer: Y.L.A.; Data collection: D.K.; Reviewer of the manuscript: B.M.Ç.; Data collection: H.Ç.; Manuscript editing: H.P.

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