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Research article

Experimental study on the effects of simvastatin in reversing the femoral metaphyseal defects induced by sodium valproate in normal and ovariectomized rats

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ABSTRACT

Introduction: Long-term treatment with antiepileptic drugs may cause secondary osteoporosis. The present study investigated the influence of simvastatin (SIM) in reversing the effects of valproate on bone defect healing in normal and ovariectomized (OVX) rats.

Methods: Bone defects in femora were established in seven experimental groups of rats: control (vehicle), sodium valproate (SVP; 300 mg/kg/d), SVP plus SIM (25 mg/kg/d), sham control (sham), OVX, OVX SVP and OVX SVP plus SIM. All rats were euthanized at 8 weeks after bone defect creation.

Results: Micro-CT, biomechanical and histological evaluations demonstrated lower bone strength and delayed bone healing in the SVP therapy group compared with the SVP plus SIM therapy group. Biochemical and immunohistochemical results showed that osteocalcin (OCN), collagen I (Col I) and procollagen type I N-terminal propeptide (P1NP) levels decreased, tartrate-resistant acid phosphatase type 5 precursor (TRACP-5b) expression increased, and Dickkopf-1 (DKK-1) and receptor activator of nuclear factor-κ B ligand (RANKL) expression were upregulated in the SVP therapy rats compared with the SVP plus SIM therapy group. Bone loss was exacerbated by OVX, but the effect of SIM in ameliorating bone loss was also more marked in the OVX rats.

Conclusions: This study indicated lower bone strength and delayed healing of bone defects in rats given SVP therapy, especially the OVX SVP treatment group. In contrast, treatment with SIM was effective in enhancing bone strength and promoting bone defect repair and showed significant influence on promoting osteogenesis and inhibiting osteoclastogenesis.

1. Introduction

Secondary osteoporosis and fracture are focal concerns associated with epilepsy and antiepileptic drugs (AEDs) [1]. More than 50% of people with epilepsy are reported to have bone disorders, including reduced bone mass, osteomalacia, osteoporosis, fractures and bone defects [2, 3]. For patients with epilepsy, AED therapy is the preferred treatment, but long-term intake of AEDs intensifies any decrease in bone mineral density (BMD), accelerates abnormal bone turnover and increases bone loss [4, 5, 6]. In existing experimental studies, different bone metabolic changes associated with some classical antiepileptic drug therapy have been reported. The recent research by Kumandas et al. reported that preadolescent patients had reduced BMD in the lumbar spine with at least 2 years of carbamazepine therapy [7]. Cross-sectional reports have shown reduced BMD at the femur and hip in adult epilepsy patients with the phenytoin treatment [8]. Especially in postmenopausal women and older men, long-term use of phenytoin can increase the risk of fracture and have negative effects on bone metabolism [9, 10]. Estimates suggest that 200 million people worldwide are affected by osteoporosis [11]. The adverse influence of AEDs on bone metabolism has attracted attention owing to the difficulty of healing osteoporotic fractures and bone defects when combined with epilepsy.

Bone remodeling is a continuous regeneration process consisting of formation and resorption processes, mediated by osteoblasts and osteoclasts, respectively. This balance follows a specific temporal and spatial sequence that can be affected by factors including BMPs, TGF-β, PTH, Wnt/β-catenin and RANKL/RANK/OPG [12]. Women are more likely to suffer increased bone fragility and delayed fracture healing due to post-menopausal bone loss [13]. The classic AED sodium valproate (SVP), a broad-spectrum antiepileptic drug, is used for partial and

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generalized epileptic seizures. Many studies of long-term valproate treatment have shown decreased bone density in adults and children [7, 14, 15, 16, 17, 18], while others found no significant difference [8, 19, 20]. The length of therapy may account for the difference. Long-term antiepileptic therapy with SVP may give rise to osteoporosis, but there have been no studies to determine whether SVP treatment causes fractures or affects the healing of bone defects.

To stimulate bone formation and restrain bone resorption, various drugs have been used including estrogen, human parathyroid hormone, calcitonin and bisphosphonates [21, 22, 23]. For bone disease in epileptic patients, the main treatment is to encourage active outdoor exercise in combination with calcium and vitamin D supplements [24]. Mikati et al. showed that vitamin D supplementation stabilized spinal and hip bone density in patients taking antiepileptic drugs [25]. For epileptic patients with osteoporosis, in addition to the above treatment methods, but also should be added to anti-osteoporosis drugs. Bisphosphonates are a first-line treatment for primary osteoporosis. In patients over 40 years of age, bisphosphonates can improve bone mineral density in patients with epilepsy [24]. In our research group, Ruotian Zhang et al. found that alendronate significantly increased bone mineral density in ovariectomized rats treated with antiepileptic drugs [26]. For phenytoin users, vitamin K supplementation improves bone density [27]. As lipid-lowering drugs, statins are commonly used to treat hyperlipidemia, and have been reported to be beneficial to bone regeneration in recent years [28, 29]. Various studies have demonstrated that simvastatin (SIM) can improve osteoblast differentiation, upregulate osteocalcin (OCN) and bone morphogenetic protein-2 (BMP-2), and prevent osteoblast apoptosis [30]. Meanwhile, SIM may restrain proliferation and differentiation of osteoclasts to downregulate bone resorption [31, 32]. However, the effectiveness of SIM in treating osteoporosis remains controversial. The effect of SIM on bone repair has not yet been clarified. On this basis, an SVP treatment experimental model in rats was used to assess whether SIM could improve osteoporosis and repair bone defects.

In this study, we evaluated the impact of SVP therapy on bone in normal and ovariectomized (OVX) female Sprague–Dawley (SD) rats. This is based on the fact that women have lower peak bone mass and smaller skeletons than men, and their bone mass declines faster due to ovarian dysfunction during menopause [33]. Apart from that, healthy/ovariectomized rats were used to determine the effects of sodium valproate in the absence/presence of estrogen deficiency [34]. Moreover, we hypothesized that SIM would increase bone strength and promote healing of bone defects in rats treated with SVP for two months.

2. Methods

2.1. Animals and diet

The present study used 80 female SD rats (Central Laboratory of Yijishan Hospital, 3 months of age, weighing 230 ± 30 g). Rats were housed in groups of five in cages in a temperature-controlled environment (25 ± 1 °C 55–65% relative humidity; 12 h of artificial lighting). All rats were raised on pellet feed with standard laboratory diet (Nanjing Qinglongshan animal breeding farm, lq03-0402) and tap water, ad libitum. The surgical operations and treatments were approved by the Animal Research Committee of Wannan Medical College (SYXK(WAN) 2018–004). The principles of the International Health and Medical Research Guidelines for Animal Welfare were followed.

2.2. Study design and surgery

First of all, ovariectomies were performed on all OVX rats. After 12 h of fasting, rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg). After fixation in prone position, an incision was made at 1 cm below the costal margin and 1.5 cm on each side of the spine to locate and remove the ovaries. In the sham group, ovaries were exposed but not removed in the same way. According to a previously-described protocol, the animals were maintained for 12 weeks after bilateral ovariectomy (n = 35) or sham operation (n = 15) for the establishment of osteoporosis before bone defect surgery [35]. Then five randomly-selected OVX rats and five sham-operated rats were sacrificed. Bilateral femora were collected for micro-CT and BMD analysis to verify the establishment of standard osteoporotic animal models.

Once osteoporosis was confirmed, a cylindrical bone defect was created in the femora of the remaining rats, above the epiphyseal line at the distal end. A spherical carbide drill with an external diameter of 2.5 mm was used, driven by an electric motor with a speed of 1500 rpm. Then all animals were randomly divided into seven groups of 10 rats each: controls, SVP (300 mg/kg/d), SVP plus SIM (SIM, 25 mg/kg/d), sham controls, OVX, OVX SVP and OVX SVP plus SIM (OVX SIM). All drugs were administered by oral gavage for eight weeks. Doses of drugs were adjusted from the human doses shown in Table 1 according to the criterion:

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times K_m \text{ ratio}$$

where, HED = human equivalent dose [36], K_m ratio – Animal K_m/Human K_m (correction factor), K_m is evaluated by dividing the average body weight (kg) of a species by its body surface area (m^2).

The calculated doses of SVP and SIM were also in conformity with previous reports [37, 38]. Body weight was measured weekly and the drug doses were adjusted accordingly (Figure 1).

2.3. Sample size

The sample size of the study was calculated based on mean values of biomechanical examination from a previous study [39]. At $\alpha = 0.05$ and 95% confidence interval, the power was resorted to be 95%, the effect size was computed to be 0.318, and the sample size was computed to be 8 rats each group by G-power software version 3.1.90. In this study, 10 rats per group were used.

2.4. Specimen collection

After 8 weeks of treatment, all rats were euthanized using an overdose of mebumal (ACO L € akemedel AB, Solna, Sweden) for specimen collection. Serum specimens were prepared from blood obtained from the abdominal aorta, centrifuged at 3000 rpm for 15 min at 4 °C, and preserved at −80 °C prior to biochemical testing. All femora were harvested and subjected to micro-CT, biomechanical, histological and immunohistochemical analyses.

2.5. Micro-CT

Bone specimens were fixed in 10% neutral-buffered formalin at ambient temperature for 4 days. Formation of new bone in defect areas was evaluated by micro-CT (micro-CT 100, Scanco Medical AG, Bassersdorf, Switzerland). Scanning was performed at 15 μm resolution, at 70 kV and 200 μA, with an integration time of 300 ms, and remodeled with an isotropic voxel size of 5 mm. A 3 mm diameter area in the center of each bone defect was selected as the volume of interest (VOI). The bone morphometric parameters, bone volume per total volume (BV/TV), bone trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular

Table 1. Translation of animal doses from relevant clinical human dose.

DRUG	Human dose	Animal dose
SVP	60 mg/kg	300 mg/kg
SIM	5 mg/kg	25 mg/kg

SVP Sodium valproate (Medchemexpress, USA), SIM Simvastatin (Apexbio, USA).

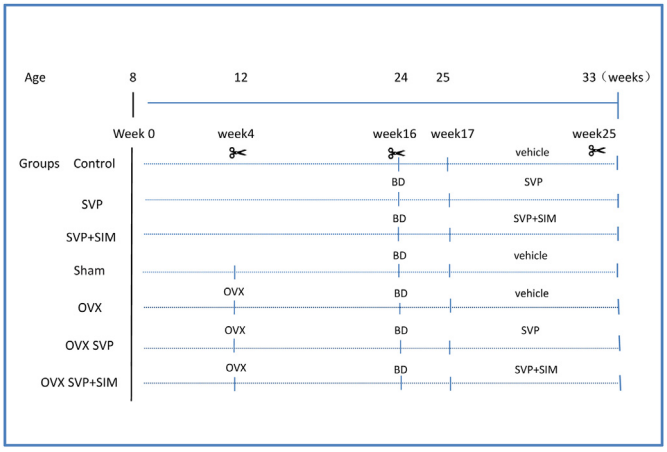

Figure 1. Experimental protocol. Scissors: Animal surgery, which included: OVX: Rats in OVX groups were bilaterally ovariectomized at 4 weeks. Rats in the sham group were sham-operated. BD: All rats in the experimental groups underwent bone defect surgery in bilateral distal femora at 16 weeks. All rats were killed at 25 weeks.

spacing (Tb.Sp), connective density (Conn.D) and BMD were calculated within the VOI zone.

2.6. Biomechanical examination

Compression testing of bone samples of the left legs (n = 10 per group) was performed immediately after micro-CT scan. The distal femoral metaphysis of each femur (near the defect) was placed in a stable three-point contact with an aluminum alloy block attached to a universal material testing system (Instron 5566; Instron, Norwood, MA, USA). The compression load was applied to the femoral shaft at 2 mm/min until failure. Ultimate load (N), elastic modulus (Mpa) and stiffness (N/mm) was calculated from the load deformation curve.

2.7. Histological analysis

The remaining specimens (right legs) were transferred to 10% ethylenediaminetetraacetic acid (pH 7.2) for four weeks. Then, the bone samples were dehydrated in a series of ethanol baths, embedded in paraffin and cut into 5-μm sections along the femoral shaft axis. Specimens were stained with hematoxylin and eosin (H&E) and Masson's trichrome according to a standard protocol, viewed under a light microscope and the stained areas were quantified using a BI-2000 medical image analysis system (Chengdu TME Technology Co, Ltd., Chengdu, China).

2.8. Immunohistochemical analysis

The levels of osteocalcin (OCN), collagen type I (Col I), and tartrate-resistant acid phosphatase type 5 precursor (TRACP-5b) in the sections of the callus were evaluated by immunohistochemical (IHC) staining, as previously described. Briefly, longitudinal sections of the distal femora were placed at ambient temperature for 60 min and treated with xylol and descending gradient ethanol (100–70%) for rehydration. Next, 3% hydrogen peroxide was used to block endogenous peroxidase, and trypsin was used to expose the antigen. The sections were irrigated and incubated overnight with the commercially-available specific antibodies OCN (1:100, Abcam, ab93876, Cambridge, UK), Col I (1:100, Abcam, ab285314, Cambridge, UK) or TRACP-5b (1:300, Abcam, ab238033, Cambridge, UK) at 4 °C. Finally, the slides were incubated with the corresponding goat anti-rabbit secondary antibody for 30 min and counter-stained with diaminobenzidine and hematoxylin. All sections were examined and photographed under a light microscope and analyzed using Image Pro Plus software (Media Cybernetics, Rockville, MD, USA).

2.9. Serum biochemical analysis

The serum levels of procollagen type I N-terminal propeptide (P1NP, Abcam, ab285003, Cambridge, UK), Dickkopf-1 (DKK-1, Abcam, ab113347, Cambridge, UK) and receptor activator of nuclear factor-κ B ligand (RANKL, Abcam, ab239607, Cambridge, UK) were evaluated according to the instructions of the enzyme-linked immunosorbent assay (ELISA) kits (all from R&D System, UK). The above methods followed the manufacturer's protocols.

2.10. Statistical analysis

All data are shown as mean ± standard deviation, analyzed using SPSS ver. 19.0 software (IBM SPSS Statistics for Windows, Armonk, NY, USA). One-way analysis of variance (ANOVA) was used for multiple between-group comparisons followed by Tukey's post hoc test. The independent t-test was used for comparisons of normal groups and OVX groups. A value of $P \leq 0.05$ was considered to reflect significance.

3. Results

3.1. Confirmation of osteoporotic rat model

The increase in body weight showed a significant difference from 33.478% in the sham group to 62.288%, 67.382%, and 64.601% in the OVX, OVX SVP and OVX SIM groups, respectively ($P < 0.05$, Figure 2C). The mean BMDs of the femoral shaft metaphysis were assessed via micro-CT, and revealed that BMDs of the sham group were 27.778% higher than the OVX groups ($P < 0.05$), confirming the successful establishment of a rat model of osteoporosis (Figure 2A–D).

3.2. Micro-CT

The reconstructed three-dimensional (3D) images of new trabecular bone in the distal femoral defect are presented in Figure 3A, and quantitative microstructure parameters were calculated including BV/TV, Tb.Th, Tb.N, Tb.Sp, Conn.D and BMD (Figure 3B–G). At 8 weeks after SVP treatment, the BMD, BV/TV, Tb.Th, Tb.N, and Conn.D were all lower, while Tb.Sp was higher than the control group ($P < 0.05$). However, in the groups receiving SIM therapy, the BMD, BV/TV, Tb.Th, Tb.N, and Conn.D were significantly higher, and Tb.Sp was significantly lower than control ($P < 0.05$). After ovariectomies, the bone microscopic parameters of OVX group were further deteriorated compared with that of Sham group, and after SVP treatment, the bone microscopic parameters of OVX SVP were also worse than that of OVX group. The microcosmic parameters of bone were improved after SIM treatment. In addition, in all SVP groups, bone loss was exacerbated by OVX, while in all SIM groups, improvements in bone quality were more marked in OVX rats.

3.3. Biomechanical test

The ultimate load, elastic modulus (Mpa) and stiffness (N/mm) were acquired by biomechanical testing of the femoral condyles. After 8 weeks of treatment, the biomechanical strength decreased in SVP group, and increased in the SIM group ($P < 0.05$). Ovariectomy exacerbates the loss of bone biomechanical strength. The bone biomechanics strength was decreased after ovarian removal, and was also lower in the OVX SVP group than in the OVX group, whereas improved in the OVX SVP plus SIM group compared to the OVX SVP group. Furthermore, the ultimate load, elastic modulus and stiffness were significantly higher in the SVP group compared to the OVX SVP group, and in the SIM group compared to the OVX SIM group ($P < 0.05$) (Figure 4A–C).

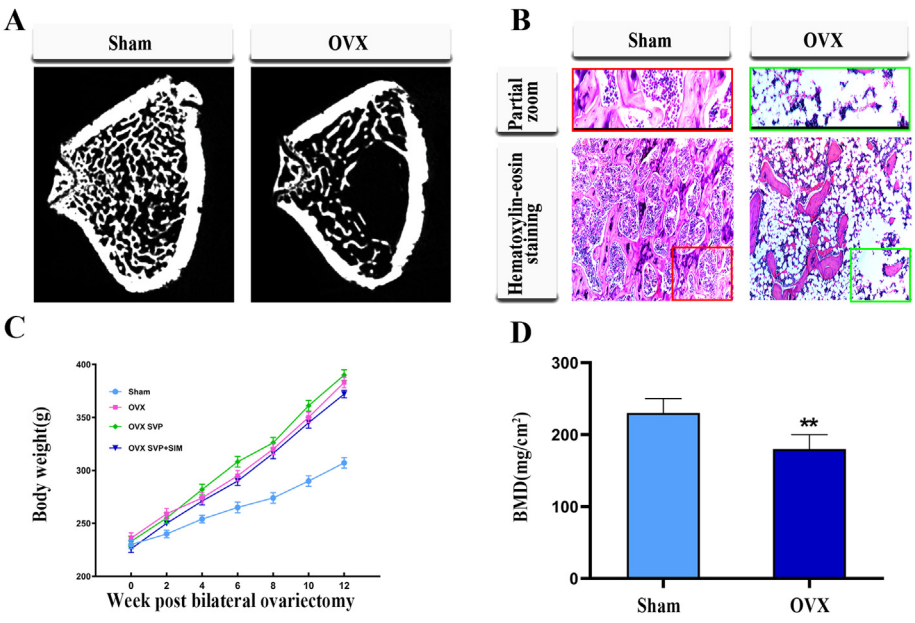

Figure 2. (A) Two-dimensional micro-CT representation of normal bone in sham-operated rats and osteoporotic bone in OVX groups (bar = 2 mm). (B) Representative H&E staining images of the normal and osteoporotic femur sections (magnification × 20). (C) Body weight measured weekly after OVX. Data are expressed as mean ± SD; n = 5, $P < 0.05$ for OVX groups vs. sham group. (D) The BMD of trabecular bone in the OVX group was significantly lower than that of the sham group. Data are expressed as mean ± SD; n = 5, $^{**}P < 0.01$ vs. sham group.

3.4. Histological evaluation

Histological images and histomorphometric analysis of decalcified sections are shown in Figure 5A–D ($P < 0.05$). The bone defects in the SVP group were filled with loose connective tissue and contained less new bone compared with the control (Figure 5A). The Osteoblast surface/bone surface (ObS/BS) was decreased and the osteoclast surface/bone surface (OcS/BS) was increased in bone defect areas compared the SVP group to the control. Besides, compared the OVX group to the sham group and the OVX SVP to the OVX group, the defect areas contained minimal new bone, thinner connective tissue, lower ObS/BS and higher OcS/BS. Compared the OVX SVP plus SIM to the OVX SVP, new bone tissues in defects was improved, ObS/BS was higher and OcS/BS was lower. However, the SVP plus SIM group exhibited maximum new bone formation and more advanced bone reconstruction and consolidation than the other groups (Figure 5B–D).

3.5. Immunohistochemical evaluation

The protein expression in the femur sections of all groups were shown in Figure 6A. After treatment for 8 weeks, OCN and Col I expressions were lower and TRACP-5P was higher in the SVP group than in the control group, while the opposite results were observed in the SVP plus SIM group. Compared the OVX group to the sham group and the OVX SVP to the OVX group, OCN and Col I expressions were lower and TRACP-5P was higher, while the opposite results were observed compared the OVX SVP plus SIM group to the OVX SVP group. However, OCN and Col I staining were significantly greater and TRACP-5P was much lower in the normal groups than in the OVX groups (Figure 6B–D).

3.6. Serum levels of bone markers

The serum levels of P1NP decreased with SVP treatment, whereas the levels of DKK-1 and RANKL increased compared with control. However, P1NP levels further increased, while DKK-1 and RANKL decreased with SIM treatment compared with control. After ovariectomies, the P1NP decreased and the DKK-1 and RANKL increased compared with that of Sham group. In OVX animals, a significant reduction in P1NP levels and increases in DKK-1 and RANKL levels were observed upon treatment with SVP post-OVX, and an additional increase in P1NP levels and decreases in DKK-1 and RANKL levels were observed upon SIM treatment post-OVX (Figure 7A–C).

4. Discussion

In this preclinical study, a standard osteoporotic animal model was established via bilateral ovariectomy. Bone-forming capacity was evaluated in rats treated with SVP or SVP plus SIM in clinically-relevant doses for 8 weeks after distal femur defect creation. The present study provides evidence, by ELISA, micro-CT, biomechanical, histological, and immunohistochemical analyses, confirming the adverse effects on bone of SVP therapy in female rats. In addition, the adverse effects on osteoporotic status and bone formation after bone injury were more obvious in OVX rats compared with normal rats. After treatment with SIM, osteogenic ability and biomechanical parameters were significantly improved in all groups. Therefore, our findings indicated that SIM reversed the effects of SVP on osteoporosis, strengthened the mechanical properties of bone and enhanced the repair of femoral metaphyseal defects in normal and OVX rats.

Bone strength is connected with bone mass and bone quality. In this study, all animals treated with SVP for 8 weeks exhibited striking decreases of BMD at distal femora. Meanwhile, the trabecular bone microarchitectural parameters BV/TV, Tb.N and Tb.Th were decreased, Tb.Sp was increased, and ultimate load was reduced. Our results were similar to those of a preclinical study showing reductions in BMD and total bone mineral content with administration of SVP for 3 months to female Wistar rats [40]. One study showed that after more than 2 years of valproate (VPA) treatment, the overall stature, bone formation and bone mass of children with epilepsy were reduced [41]. Similar results were reported showing lower femoral and lumbar BMD in adults and children after long-term VPA treatment [42, 43, 44, 45, 46, 47]. However, Kim and co-workers discovered no significant change in bone density of adults after SVP treatment for over 6 months [8]. Prolonged VPA therapy in our and previous research seemed to be the cause of BMD reduction. This study revealed that the effect of SVP on bone strength may be characterized by changes in bone mass, bone microstructure and mechanical properties.

As a biologically-active tissue, bone is continually reconstructed. The old bone is resorbed while at the same time new bone is continuously formed, hence maintaining a dynamic balance of bone mass. Bone reconstruction relies on osteoblasts and osteoclasts, and the balance of the two is of great importance for stable bone metabolism. In our study, we observed that the recovery rate of bone injury in SVP-treated rats was slower than that of the controls. The results of staining showed that collagen fibers and new bone were decreased in SVP-treated rats. In

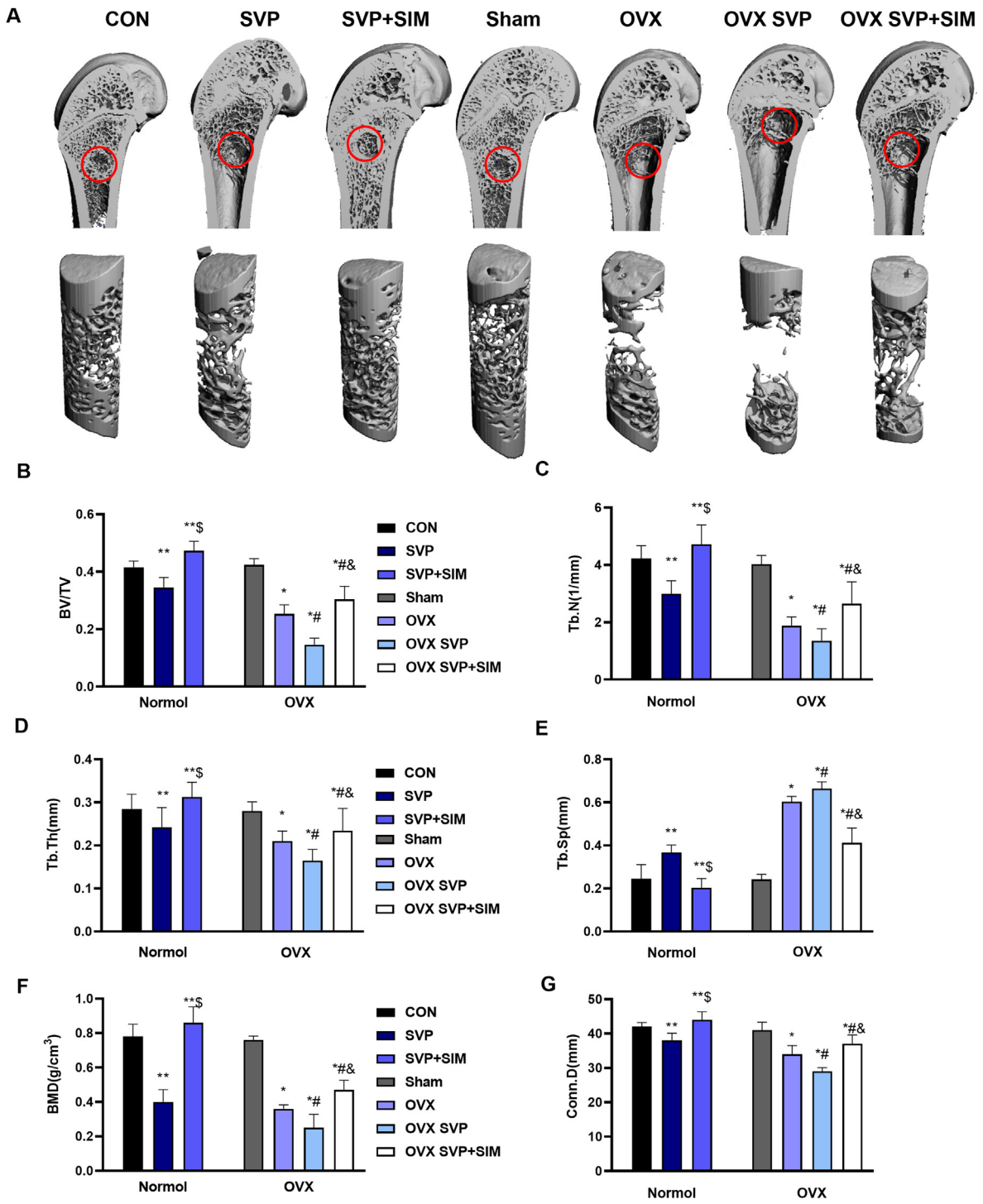

Figure 3. (A) Three-dimensional reconstructions of micro-CT of femora and bone tissue in the bone defects, the red circle shows the bone defect area. (B–G) Quantitative results of micro-CT analysis expressed as BV/TV, TB.N, Tb.Th, Tb.Sp, BMD and Conn.D. Data are expressed as mean ± SD; n = 5, $^{**}P < 0.05$ vs. CON group, $\$P < 0.05$ vs. SVP group, $^{*}P < 0.05$ vs. sham group, $\#P < 0.05$ vs. OVX group, $\&P < 0.05$ vs. OVX SVP group.

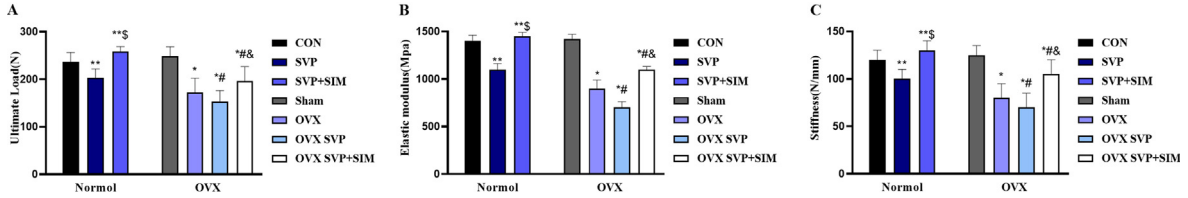

Figure 4. Biomechanical results expressed as the ultimate load, elastic modulus and stiffness. Data are expressed as mean ± SD; n = 5, $^{**}P < 0.05$ vs. CON group, $\$P < 0.05$ vs. SVP group, $^{*}P < 0.05$ vs. sham group, $\#P < 0.05$ vs. OVX group, $\&P < 0.05$ vs. OVX SVP group, $^{***}P < 0.05$ vs. SVP group, $\#\#P < 0.05$ vs. SVP + SIM group.

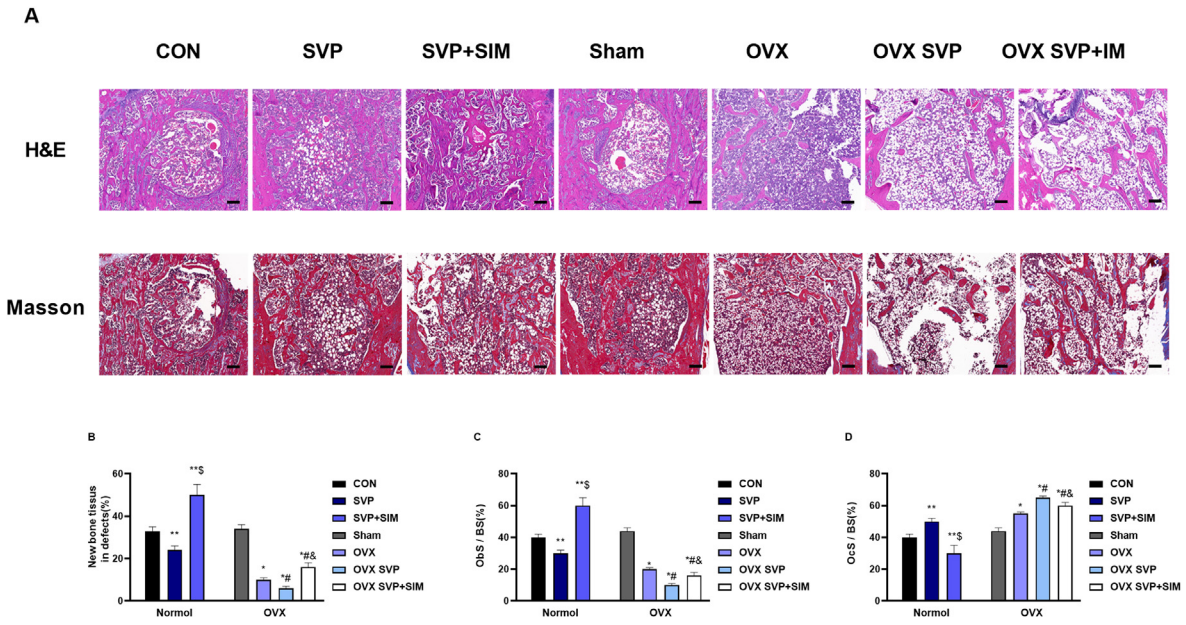

Figure 5. (A) Representative H&E and Masson's trichrome staining images of distal femora (magnification × 4, bar = 200 μm). (B–D) Quantitative results of histomorphometric analysis expressed as new bone fill in defects, osteoblast surface/bone surface (ObS/BS), Osteoclast surface/bone surface (OcS/BS). Data are expressed as mean ± SD; n = 5, $^{**}P < 0.05$ vs. CON group, $\$P < 0.05$ vs. SVP group, $^{*}P < 0.05$ vs. sham group, $\#P < 0.05$ vs. OVX group, $\&P < 0.05$ vs. OVX SVP group, $^{***}P < 0.05$ vs. SVP group, $\#\#P < 0.05$ vs. SVP + SIM group.

addition, OCN, Col I and P1NP levels were reduced and TRACP-5b expression increased in SVP-treated rats, suggesting that SVP therapy exerted an osteoporotic effect by promoting bone resorption and inhibiting bone formation. The Wnt/β-catenin signal is closely related to stimulation of osteoblast proliferation and differentiation, as well as reduced osteoclast differentiation [48] and prevention of osteoporosis [49]. DKK1 is an inhibitor of Wnt signaling which can affect bone formation [50]. Our results showed that SVP may upregulate DKK1 expression and inhibit the Wnt/β-catenin signaling pathway, consequently resulting in reduced osteogenesis. In addition to its effect on osteoblasts, SVP may also upregulate RANKL which mediates osteoclastogenesis, thus activating bone resorption mediated by RANKL/cathepsin K [51].

As a lipid-lowering medicine, SIM has been found to be beneficial to skeletal metabolism in the last two decades [52]. Simvastatin promotes osteogenesis by increasing the activity and differentiation of osteoblasts.

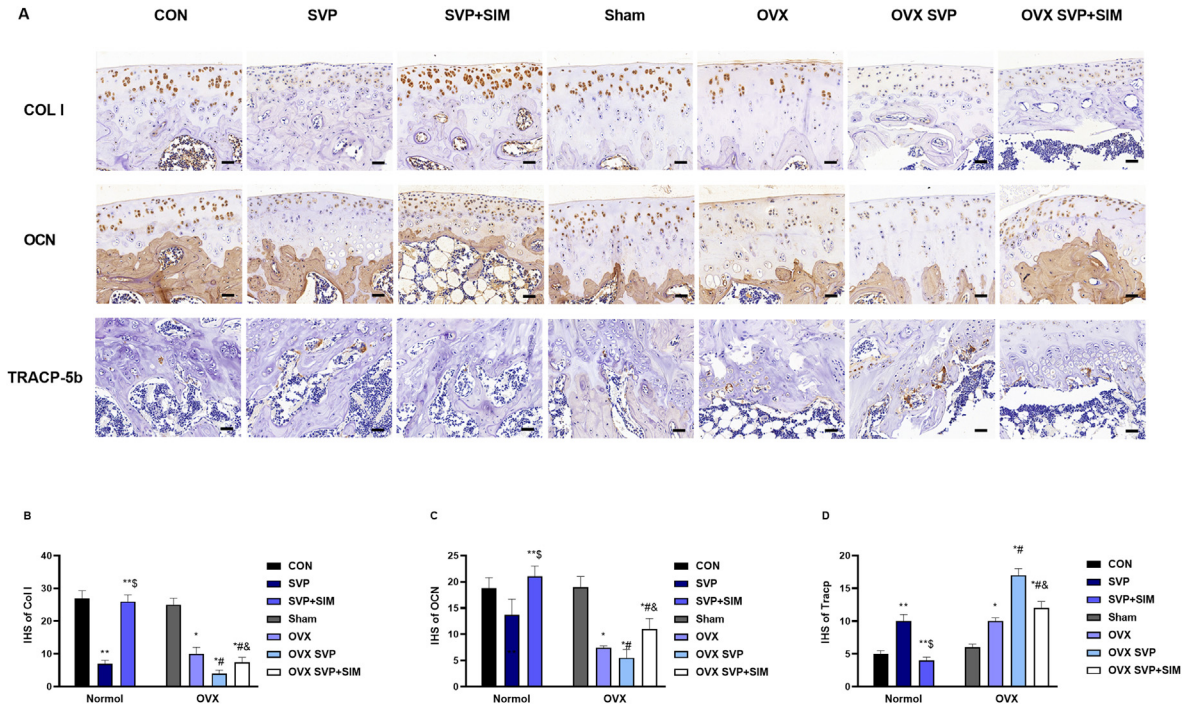

Figure 6. (A) Immunohistochemical staining for OCN, Col I and TRACP-5b in femora (magnification ×20, bar = 200 μm). (B–D) Quantitative results of immunohistochemical analyses for OCN, Col I and TRACP-5b. Data are expressed as mean ± SD; n = 5, $^{**}P < 0.05$ vs. CON group, $\$P < 0.05$ vs. SVP group, $^{*}P < 0.05$ vs. sham group, $\#P < 0.05$ vs. OVX group, $\&P < 0.05$ vs. OVX SVP group, $^{***}P < 0.05$ vs. SVP group, $\#\#P < 0.05$ vs. SVP + SIM group.

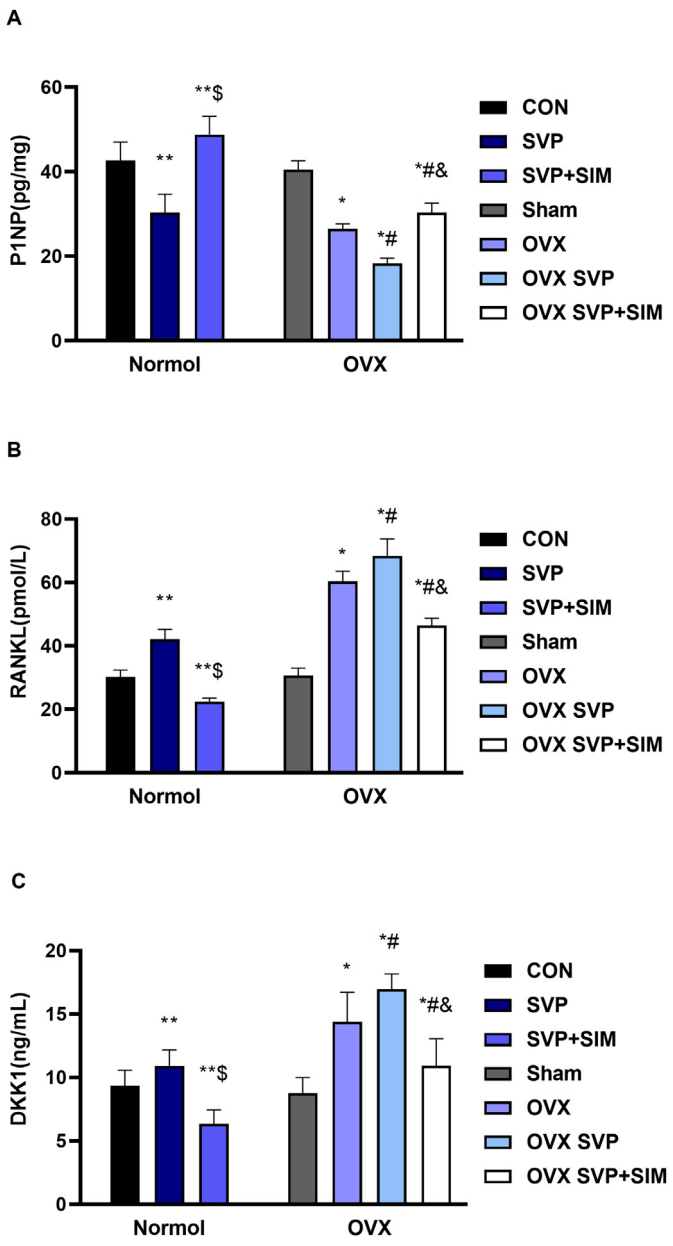

Figure 7. Serum levels of bone markers expressed as P1NP, DKK1, and RANKL. Data are expressed as mean ± SD; n = 5, $^{**}P < 0.05$ vs. CON group, $\$P < 0.05$ vs. SVP group, $^{*}P < 0.05$ vs. sham group, $\#P < 0.05$ vs. OVX group, $\&P < 0.05$ vs. OVX SVP group, $^{****}P < 0.05$ vs. SVP group, $\#\#P < 0.05$ vs. SVP + SIM group.

Simvastatin enhances BMP-2 promoter, luciferase activity and up-regulates BMP-2, alkaline phosphatase, type I collagen, osteopontin and salivation protein expression through Ras/PI3K/Akt/Erk/MAPK/BMP-2 pathway. In addition, simvastatin protects osteoblasts from apoptosis in a dose-dependent manner through the TGFb/Smad3 signaling pathway. Simvastatin increased the expression of OPG mRNA, decreased the expression of RANKL mRNA, and blocked the osteoclast differentiation induced by RANKL. Although many studies have assessed the beneficial effects of SIM on bone regeneration, some disputed consequences can be found in previous studies. In addition, the roles of SIM in bone defect repair and ameliorating AED-induced bone loss have not been determined yet. After treatment with SIM, bone strength significantly improved and more new bone was created in bone defects. The serum biochemical and immunohistochemical analysis results showed that SIM downregulates DKK1 and decreases RANKL expression, which activates osteogenesis and inhibits osteoclastogenesis. This was in contrast to the results after valproate treatment, although it cannot be said that simvastatin can specifically inhibit the negative effects of valproate on bone, it does play a certain role in improving the negative effects of valproate on bone metabolism. Our experiments show that SIM could ameliorate SVP-induced bone loss by improving bone formation and reducing bone resorption.

On the one hand, estrogen loss can induce expression of the pro-inflammatory cytokines midkine and interleukin-6, reducing the differentiation ability of the osteogenic system during the early phase after fracture [53], while on the other hand, the reduction of estrogen promotes the production of interleukin-7, which subsequently stimulates T cells and delivers interferon and RANKL, thereby restraining osteoclastogenesis [54], and ultimately leading to delayed fracture healing. Our results suggest that women in postmenopause receiving SVP therapy are likely to be more sensitive to negative effects on bone metabolism. The effect of SIM therapy on postmenopausal women with SVP-associated bone defects may be decreased, but it is likely to remain effective.

Based on what we know, this is the first report describing the effects of simvastatin and valproate on femoral metaphyseal defects of female SD rats, which is a significant issue, because in clinical, it is not known whether long-term use of antiepileptic drugs will cause bone disease, or whether statins should be added when bone metabolic disease occurs. Of course, this study also has several shortcomings. First of all, the lack of positive control is a limitation of this study. Secondly, we chose only one antiepileptic drug to participate in the trial, which does not mean that all antiepileptic drugs have these effects. We only chose two months of treatment time, while ignoring whether the drugs will have different results depending on the duration of treatment. Moreover, different doses of drugs may also cause different consequences. In the following time, we will explore whether other antiepileptic drugs can cause negative effects on bone and other anti-osteoporosis drugs can reverse these impacts.

5. Conclusions

In summary, our study suggests that 8 weeks of therapy with SVP at clinically-relevant dosage leads to negative effects on bone metabolism and affects the healing of bone defects in female SD rats. Further, the adverse impacts on bone are more obvious in OVX rats. These effects might be on account of the promotion of bone resorption and the inhibition of bone formation as well as the modulation of Wnt inhibitors. Finally, our study confirmed that SIM reversed the effects of SVP on femoral metaphyseal defect repair in normal and OVX rats, and thus could have the potential to be an effective therapeutic strategy for AED-associated osteoporosis and bone defects in the clinic.

Declarations

Author contribution statement

Yang Li: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Min Yang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Ruotian Zhang: Performed the experiments; Analyzed and interpreted the data.

Hedong Liu and Maoxian Ren: Analyzed and interpreted the data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

[1] A. Verrotti, G. Coppola, P. Parisi, et al., Bone and calcium metabolism and antiepileptic drugs, Clin. Neurol. Neurosurg. 112 (1) (2010) 1–10.
[2] S.S. Diemar, A.S. Sejling, P. Eiken, et al., Hyponatremia and metabolic bone disease in patients with epilepsy: a cross-sectional study, Bone 123 (2019) 67–75.
[3] G. Coppola, D. Fortunato, G. Auricchio, et al., Bone mineral density in children, adolescents, and young adults with epilepsy, Epilepsia 50 (9) (2009) 2140–2146.
[4] B. Miziak, B. Blaszczyk, M. Chroscinska-Krawczyk, et al., The problem of osteoporosis in epileptic patients taking antiepileptic drugs, Expet Opin. Drug Saf. 13 (7) (2014) 935–946.
[5] S. Paticheep, C. Chotipanich, K. Khusiwilai, et al., Antiepileptic drugs and bone health in Thai children with epilepsy, J. Med. Assoc. Thai. 98 (6) (2015) 535–541.
[6] Y.A. Park, A.K. Subasinghe, B.S. Ahmad, et al., Associations between serum sodium concentration and bone health measures in individuals who use antiepileptic drugs: a pilot study, J. Clin. Densitom. (2019).
[7] S. Kumandas, E. Koklu, H. Gumus, et al., Effect of carbamezapine and valproic acid on bone mineral density, IGF-I and IGFBP-3, J. Pediatr. Endocrinol. Metab. 19 (4) (2006) 529–534.
[8] S.H. Kim, J.W. Lee, K.G. Choi, et al., A 6-month longitudinal study of bone mineral density with antiepileptic drug monotherapy, Epilepsy Behav. 10 (2) (2007) 291–295.
[9] K.B. Kristensen, S.A. Pedersen, S.A.J. Schmidt, et al., Use of antiepileptic drugs and risk of skin cancer: a nationwide case-control study, J. Am. Acad. Dermatol. 82 (2) (2020) 326–335.
[10] G. Farhat, B. Yamout, M.A. Mikati, et al., Effect of antiepileptic drugs on bone density in ambulatory patients, Neurology 58 (9) (2002) 1348–1353.
[11] K. Mukherjee, N. Chattopadhyay, Pharmacological inhibition of cathepsin K: a promising novel approach for postmenopausal osteoporosis therapy, Biochem. Pharmacol. 117 (2016) 10–19.
[12] A. Al Saedi, N. Stupka, G. Duque, Pathogenesis of osteoporosis, Handb. Exp. Pharmacol. (2020).
[13] M.U. Keen, A.K.R. Reddivari, Osteoporosis in Females. StatPearls. Treasure Island (FL), 2020.
[14] I. Villegas-Martinez, I. de-Miguel-Elizaga, R. Carrasco-Torres, et al., The COL1A1 SP1 polymorphism is associated with lower bone mineral density in patients treated with valproic acid, Pharmacogenetics Genom. 26 (3) (2016) 126–132.
[15] M. Papassava, I. Nakou, E. Siomou, et al., Vitamin D supplementation and bone markers in ambulatory children on long-term valproic acid therapy. A prospective interventional study, Epilepsy Behav. 97 (2019) 192–196.
[16] H.M. Serin, Z.P. Koc, B. Temelli, et al., The bone mineral content alterations in pediatric patients medicated with levetiracetam, valproic acid, and carbamazepine, Epilepsy Behav. 51 (2015) 221–224.
[17] N. Oner, M. Kaya, S. Karasalihoglu, et al., Bone mineral metabolism changes in epileptic children receiving valproic acid, J. Paediatr. Child Health 40 (8) (2004) 470–473.
[18] E. Erbayat Altay, A. Serdaroglu, L. Tumer, et al., Evaluation of bone mineral metabolism in children receiving carbamazepine and valproic acid, J. Pediatr. Endocrinol. Metab. 13 (7) (2000) 933–939.
[19] H. Tekgul, G. Serdaroglu, A. Huseyinov, et al., Bone mineral status in pediatric outpatients on antiepileptic drug monotherapy, J. Child Neurol. 21 (5) (2006) 411–414.
[20] A.M. Pack, M.J. Morrell, A. Randall, et al., Bone health in young women with epilepsy after one year of antiepileptic drug monotherapy, Neurology 70 (18) (2008) 1586–1593.
[21] S. Tuck, E.A. Little, T.J. Aspray, Implications of guidelines for osteoporosis and its treatment, Age Ageing 47 (3) (2018) 334–339.
[22] P. Dovjak, [Diagnostics and treatment of osteoporosis in patients over 65 years old : current status and future perspectives], Z. Gerontol. Geriatr. 52 (5) (2019) 421–427.
[23] K.G. Cotts, A.S. Cifu, Treatment of osteoporosis, JAMA 319 (10) (2018) 1040–1041.
[24] A.A. Lazzari, P.M. Dussault, M. Thakore-James, et al., Prevention of bone loss and vertebral fractures in patients with chronic epilepsy–antiepileptic drug and osteoporosis prevention trial, Epilepsia 54 (11) (2013) 1997–2004.
[25] M.A. Mikati, L. Dib, B. Yamout, et al., Two randomized vitamin D trials in ambulatory patients on anticonvulsants: impact on bone, Neurology 67 (11) (2006) 2005–2014.
[26] R. Zhang, M. Yang, Y. Li, et al., Effect of alendronate on the femoral metaphyseal defect under carbamazepine in ovariectomized rats, J. Orthop. Surg. Res. 16 (1) (2021) 14.
[27] K. Onodera, A. Takahashi, S. Sakurada, et al., Effects of phenytoin and/or vitamin K2 (menatetrenone) on bone mineral density in the tibiae of growing rats, Life Sci. 70 (13) (2002) 1533–1542.
[28] A. Oryan, A. Kamali, A. Moshiri, Potential mechanisms and applications of statins on osteogenesis: current modalities, conflicts and future directions, J. Contr. Release 215 (2015) 12–24.
[29] V. Moraschini, D.C.F. Almeida, J.A. Calasans-Maia, et al., The ability of topical and systemic statins to increase osteogenesis around dental implants: a systematic review of histomorphometric outcomes in animal studies, Int. J. Oral Maxillofac. Surg. 47 (8) (2018) 1070–1078.
[30] A. Oryan, S. Alidadi, A. Moshiri, et al., Bone regenerative medicine: classic options, novel strategies, and future directions, J. Orthop. Surg. Res. 9 (1) (2014) 18.
[31] W.A. Grasser, A.P. Baumann, S.F. Petras, et al., Regulation of osteoclast differentiation by statins, J. Musculoskelet. Neuronal Interact. 3 (1) (2003) 53–62.
[32] A. Hughes, M.J. Rogers, A.I. Idris, et al., A comparison between the effects of hydrophobic and hydrophilic statins on osteoclast function in vitro and ovariectomy-induced bone loss in vivo, Calcif. Tissue Int. 81 (5) (2007) 403–413.
[33] J. Dasarathy, H. Labrador, Bone health in women, Prim Care 45 (4) (2018) 643–657.
[34] S.J. Petty, T.J. O'Brien, J.D. Wark, Anti-epileptic medication and bone health, Osteoporos. Int. 18 (2) (2007) 129–142.
[35] J.H. Xu, M. Yao, J. Ye, et al., Bone mass improved effect of icariin for postmenopausal osteoporosis in ovariectomy-induced rats: a meta-analysis and systematic review, Menopause 23 (10) (2016) 1152–1157.
[36] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, FASEB J. 22 (3) (2008) 659–661.
[37] M.J. Anwar, K.V. Radhakrishna, A. Sharma, et al., Raloxifene preserves phenytoin and sodium valproate induced bone loss by modulating serum estradiol and TGF-beta3 content in bone of female mice, Eur. J. Pharmaceut. Sci. 62 (2014) 219–226.
[38] X.F. Li, C.B. Lin, F.R. Xie, et al., Effects of simvastatin and combination of simvastatin and nylestriol on bone metabolism in ovariectomized rats, Am. J. Therapeut. 23 (6) (2016) e1630–e1636.
[39] F. Faul, E. Erdfelder, A.G. Lang, et al., *Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences, Behav. Res. Methods 39 (2) (2007) 175–191.
[40] L.S. Nissen-Meyer, S. Svalheim, E. Tauboll, et al., How can antiepileptic drugs affect bone mass, structure and metabolism? Lessons from animal studies, Seizure 17 (2) (2008) 187–191.
[41] C.Y. Guo, G.M. Ronen, S.A. Atkinson, Long-term valproate and lamotrigine treatment may be a marker for reduced growth and bone mass in children with epilepsy, Epilepsia 42 (9) (2001) 1141–1147.
[42] C.M. Lin, H.C. Fan, T.Y. Chao, et al., Potential effects of valproate and oxcarbazepine on growth velocity and bone metabolism in epileptic children- a medical center experience, BMC Pediatr. 16 (2016) 61.
[43] A. Rahimdel, A. Dehghan, M.A. Moghadam, et al., Relationship between bone density and biochemical markers of bone among two groups taking carbamazepine and sodium valproate for epilepsy in comparison with healthy individuals in yazd, Electron. Phys. 8 (11) (2016) 3257–3265.
[44] Y. Sato, I. Kondo, S. Ishida, et al., Decreased bone mass and increased bone turnover with valproate therapy in adults with epilepsy, Neurology 57 (3) (2001) 445–449.
[45] S.M. Senn, S. Kantor, I.J. Poulton, et al., Adverse effects of valproate on bone: defining a model to investigate the pathophysiology, Epilepsia 51 (6) (2010) 984–993.
[46] X.Q. Song, Z.P. Wang, K.R. Bao, et al., [Effect of carbamazepine and valproate on bone metabolism in children with epilepsy], Zhonghua Er Ke Za Zhi 43 (10) (2005) 728–732.
[47] T. The, R. Kolla, F. Dawkins, et al., Pure red cell aplasia after 13 years of sodium valproate, and bone marrow suppression after 17 years of carbamazepine, PLoS Med. 1 (2) (2004) e51.
[48] A.N. Shuid, E. El-arabi, N.M. Effendy, et al., Eurycoma longifolia upregulates osteoprotegerin gene expression in androgen- deficient osteoporosis rat model, BMC Compl. Alternative Med. 12 (2012) 152.
[49] Y. Wang, Y.P. Li, C. Paulson, et al., Wnt and the Wnt signaling pathway in bone development and disease, Front. Biosci. (Landmark Ed.). 19 (2014) 379–407.
[50] R. Baron, M. Kneissel, WNT signaling in bone homeostasis and disease: from human mutations to treatments, Nat. Med. 19 (2) (2013) 179–192.
[51] J.H. Kang, H. Jung, M. Yim, 3',4',7,8-Tetrahydroxyflavone inhibits RANKL-induced osteoclast formation and bone resorption, Pharmazie 72 (3) (2017) 161–166.
[52] G. Mundy, R. Garrett, S. Harris, et al., Stimulation of bone formation in vitro and in rodents by statins, Science 286 (5446) (1999) 1946–1949.
[53] M.S. Goseki, N. Omi, A. Yamamoto, et al., Ovariectomy decreases osteogenetic activity in rat bone, J. Nutr. Sci. Vitaminol. 42 (1) (1996) 55–67.
[54] S. Khanna, K.K. Pillai, D. Vohora, Insights into liaison between antiepileptic drugs and bone, Drug Discov. Today 14 (7-8) (2009) 428–435.