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Immunization with FSHβ fusion protein antigen prevents bone loss in a rat ovariectomy-induced osteoporosis model

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ABSTRACT

Osteoporosis, a metabolic bone disease, threatens postmenopausal women globally. Hormone replacement therapy (HTR), especially estrogen replacement therapy (ERT), is used widely in the clinic because it has been generally accepted that postmenopausal osteoporosis is caused by estrogen deficiency. However, hypogonadal α and β estrogen receptor null mice were only mildly osteopenic, and mice with either receptor deleted had normal bone mass, indicating that estrogen may not be the only mediator that induces osteoporosis. Recently, follicle-stimulating hormone (FSH), the serum concentration of which increases from the very beginning of menopause, has been found to play a key role in postmenopausal osteoporosis by promoting osteoclastogenesis. In this article, we confirmed that exogenous FSH can enhance osteoclast differentiation *in vitro* and that this effect can be neutralized by either an anti-FSH monoclonal antibody or anti-FSH polyclonal sera raised by immunizing animals with a recombinant GST-FSHβ fusion protein antigen. Moreover, immunizing ovariectomized rats with the GST-FSHβ antigen does significantly prevent trabecular bone loss and thereby enhance the bone strength, indicating that a FSH-based vaccine may be a promising therapeutic strategy to slow down bone loss in postmenopausal women.

1. Introduction

Postmenopausal osteoporosis, a major prevalent metabolic bone disease, affects all women over the age of 50 with a higher risk of fracture occurrence. Postmenopausal osteoporosis is due to the imbalance between osteoclastic bone resorption and osteoblastic bone formation. Before menopause, bone resorption and formation are in a fine balance. However, during and after menopause, osteoclastic bone resorption increases sharply, causing a decrease in bone density [1].

For years, hypogonadal bone loss has been attributed to declining estrogen levels, and the activation of the estrogen receptor can actually protect bone mass after menopause [2]. As a result, estrogen replacement therapy (ERT) or selective estrogen receptor modulator (SERM) therapy has been used widely in postmenopausal osteoporosis therapy in the clinic. However, the underlying molecular mechanism of how estrogen affects the bone mass is unclear. In 2002, McCauley et al. reported that mice with both hypogonadal α and β estrogen receptors deleted were only mildly osteopenic. Thereafter, several studies demonstrated that hypogonadal mice, with deletion of either the α or β estrogen receptor, have normal bone mass [3–5]. All these results indicated that estrogen may not be the only mediator of postmenopausal osteoporosis.

In the hypothalamic–pituitary–ovarian axis, hypothalamus-derived gonadotropin releasing hormone (GnRH) stimulates the pituitary-secreted follicle-stimulating hormone (FSH), and FSH stimulates the production and secretion of estrogen from ovarian follicle cells. FSH is negative-feedback controlled by the estrogen levels [6,7]. Thus, a decrease in the estrogen levels results in increases in the FSH levels of ten or more times. However, FSH is not related to causing hypogonadal bone loss and is only regarded as a marker for the beginning of menopause. Then, Yeh et al. determined that the bone mass in ovariectomized and hypophysectomized rats were higher than in those with ovariectomy (OVX) alone, suggesting that the hormones generated from the pituitary gland are much more relevant to hypogonadal bone loss;

Abbreviations: HTR, hormone replacement therapy; ERT, estrogen replacement therapy; FSH, follicle-stimulating hormone; SERMs, selective estrogen receptor modulators; GnRH, gonadotropin-releasing hormone; OVX, ovariectomy; LB, Luria Bertani; IPTG, isopropyl β-D-thiogalactopyranoside; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; BMD, bone mineral density; DEXA, dual energy X-ray absorptiometry; VOI, volume of interest; BV/TV, bone volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; CWT, cortical wall thickness; Ca–Co, calcium-cobalt; S.D., standard deviation; AKP, alkaline-phosphatase.

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thereafter, FSH has been linked to postmenopausal osteoporosis [8,9]. Further studies revealed that FSH can stimulate the RANK expression of human monocytes [10] and stimulate osteoclastic bone resorption [11,12]. Subsequent studies confirmed that monoclonal or polyclonal antibodies against human FSHβ could attenuate osteoclastogenesis [13] and that FSH injections could increase bone loss in ovariectomized rats [14].Moreover, Sun et al. found that FSH can directly regulate the bone mass independent of estrogen [15]. High levels of FSH can directly act on osteoclasts via $G_{i2\alpha}$-coupled FSHR [13,16] and activate the MEK/Erk, NF-κB and Akt pathways to stimulate osteoclastogenesis and bone resorption. Recently, Sun et al. reported that FSH can increase the bone mass in FSHβ haploinsufficient mice [15]. These discoveries suggested that FSH could be the key mediator for postmenopausal osteoporosis.

However, until now, there have been no reports on whether blocking the FSH signaling pathway could slow down osteoporosis in OVX animal models. In this study, we expressed the human recombinant FSHβ protein in *Escherichia coli* and used the recombinant FSHβ protein to immunize OVX rats. We found that hypogonadal bone loss can be reduced after FSHβ immunization, which confirmed that FSH plays an important role in postmenopausal osteoporosis. We also examined the effects of FSH on osteoclast differentiation. Bone marrow differentiation experiments showed that FSH can promote osteoclast differentiation, enhance the function of osteoclasts significantly, and have little effect on osteoblast differentiation. Our results confirmed the discovery of Sun et al. and have provided a novel therapeutic strategy against postmenopausal osteoporosis.

2. Materials and methods

2.1. Expression of FSHβ protein

The gene encoding the mature FSHβ peptide was optimized with *E. coli* preferred codons, synthesized and cloned between the BamH I and EcoR I sites of pGEX-4T-1. The resulting plasmid, pGEX-FSHβ, was transformed into *E. coli* DH5α to express the FSHβ protein. Different isopropyl β-D-thiogalactopyranoside (IPTG) concentrations, durations and temperatures were used to optimize the expression of the GST-FSHβ fusion protein. Solubility analysis was performed according to the method described by Lin et al. [17]. The expression of the recombinant proteins was monitored by SDS–PAGE using the method described by Laemmli UK [18].

2.2. Animals and treatments

Eighteen three-month-old female Sprague–Dawley rats were obtained from the Experimental Animal Center of The Fourth Military Medical University and were divided into three groups of six each. The Sham group was subjected to sham surgery. The OVX group was ovariectomized. The FSH immunization group was also ovariectomized. After 1 month, the rats were immunized subcutaneously with 200 μg of GST-FSHβ (FSH immunization group) or GST (OVX group) emulsified with an equal volume (0.1 ml per rat) of complete Freund's adjuvant (CFA). The GST-FSHβ protein was dissolved in PBS, and the CFA contained heat-killed *Mycobacterium tuberculosis* bacilli. Four weeks after the primary immunization, the first booster immunization was administered with 200 μg of GST-FSHβ or GST emulsified with an equal volume (0.1 ml per rat) of incomplete Freund's adjuvant (IFA). Further booster immunization was administered at 2 week intervals with 200 μg of GST-FSHβ or GST. All animals were treated in accordance with the guidelines provided by the Institutional Ethics Committee of Northwest University.

The body weight of each rat was recorded weekly. All rats were sacrificed at 6 months, and the femurs and lumbar vertebrae (L1) were harvested. The left femurs were fixed in 10% formalin for the micro-CT and histological examinations. The right femurs, after the bone mineral density (BMD) analysis, and L1 were wrapped in saline-soaked gauze bandages and stored at −20 °C for biomechanical testing [19].

2.3. Assessment of antiserum titer by ELISA

An indirect ELISA assay was performed to evaluate the titer of the antiserum. Blood from the FSH immunization group at 10 days after the final booster shot was harvested and centrifuged at 3000 rpm for 20 min. After coating with 100 μl of the FSH protein at a concentration of 5 μg/mL at 4 °C overnight, the 96 well plates were washed with PBS three times and blocked with 1% BSA (W/V) at 37 °C for 1 h. Then, a series of diluted antiserum ranging from 1:10,000 to 1:2,560,000 was added to triplicate wells, and the plates were incubated at 37 °C for 1 h. After washing all wells with PBS three times, goat anti-rat HRP-conjugated IgG was added and incubated at 37 °C for 1 h. Subsequently, the substrate was added to each well, and the OD450 nm value was measured with an enzyme-labeled instrument (Thermo Fisher, Finland).

2.4. Cell culture and differentiation

The differentiation of bone marrow osteoclasts was induced with 10^{-8} M 1.25 VD_3 in α-MEM supplemented with 10% fetal bovine serum (HyClone, USA) and a concentration series of FSH at 0, 3, 10 and 30 ng/mL(Groups A–D). Group E was treated with 30 ng/mL FSH and 10 μl of antiserum obtained from the immune rats; Group F was treated with 30 ng/mL FSH and the monoclonal antibodies. After culturing for 7 days, TRAP staining was performed, and the number of $TRAP^+$ cells was counted [20].

Bone marrow osteoblast differentiation was induced with 0 ng/mL, 10 ng/mL and 30 ng/mL FSH in DMEM containing 15% FBS, 10^{-8} M dexamethasone, 200 μM ascorbic acid and 10 mM β-glycerophosphate [21]. After 10 days, the cells were stained with von Kossa and Gomori calcium–cobalt (Ca–Co) [22,23].

2.5. BMD analysis

The BMD of the right femurs was measured in a blinded fashion using the Lunar Prodigy Advance by dual energy X-ray absorptiometry (DEXA) (GE Healthcare, USA) in the small-subjects mode [24].

2.6. Biomechanical testing

The mechanical properties of the bone were determined by three-point bending of the right femurs and compression of the lumbar vertebral body (L1). Three-point bending was performed in a blinded fashion on a material-testing machine (Instron Universal, model3365). The load was applied midway at a span of 20 mm, and the deformation rate was 2 mm/min. The maximum load, stiffness, maximum stress and Young's modulus were obtained from the three-point bending test of the right femurs [25].

The bone was loaded using a material-testing machine along the axis of the lumbar vertebra at a displacement rate of 1 mm/min. The corresponding loads and displacements were continuously recorded until the sample failed [24,26].

2.7. Micro-CT evaluation

The left femurs were scanned by micro-CT (Siemens, Germany) at an energy level of 80 kV and intensity of 500 μA with a high resolution of 10 μm in all three spatial dimensions. The volume of interest (VOI) was selected at 0.8 mm to 1.5 mm from the growth plate at the proximal end of the femur. The 3D images of the

trabecular architecture were reconstructed with Siemens COBRA EXXim software using a 1024 × 1024 matrix. The relative bone volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th) were obtained by analyzing the VOI using the analysis software (Siemens Inveon Research Workplace v2.2.0). The cortical wall thickness (CWT) was measured from the VOI at 3 mm [25,27].

2.8. Histological examination

After the micro-CT evaluation, the left femurs were decalcified with EDTA, dehydrated in a graded series of ethanol, and embedded in paraffin. These decalcified sections of all groups were stained with hematoxylin-eosin.

After the three-point bending test, the right femurs were fixed, dehydrated and embedded without decalcification in methyl methacrylate for hard tissue slicing. Metaphyseal sections were cut at a thickness of 100 μm. These sections were processed with von Kossa staining.

2.9. Statistical analysis

All data were expressed as mean values ± standard deviation (S.D.) for each group. Group differences were assessed using one-way ANOVA ($n = 6$). All statistical analyses were performed using the statistics package SPSS19.0 (SPSS Inc., Chicago, USA), and differences were considered significant for $P < 0.05$ [26–28].

3. Results

3.1. Expression of the GST-tagged FSHβ protein

The constructed expression plasmid, pGEX-FSHβ, was digested by BamH I and EcoR I, and the band size consistent with the expected size (Fig. 1A). SDS–PAGE showed an approximately 38 kDa band, which was consistent with the expected molecular weight of GST-tagged FSHβ (Fig. 1B, lane 3). The optimized studies revealed that the highest expression condition for FSHβ was incubation at 37 °C with 0.5 mM IPTG for 4 h. The solubility study showed that the induced FSHβ protein was produced in the form of inclusion bodies (Fig. 1B, lane 5).

3.2. Antibody titer

The results of the indirect ELISA revealed that positive reactions were detected at the titer of 1:128,0000. This result shows that the immunization of OVX rats with recombinant FSHβ protein can obtain a higher antibody titer and thus successfully block FSH.

3.3. Cell culture

The osteoclast differentiation experiment showed that FSH can promote osteoclast formation. There was an expected increase in the $TRAP^+$ cell number and osteoclast nuclei after FSH treatment (Fig. 2A–D, G and H). Additionally, the anti-FSH monoclonal antibodies or antisera produced by the immunized rats had a similar blocking effect on the FSH-induced osteoclast differentiation (Fig. 2E and F). Moreover, FSH had a little effects on osteoblastic bone formation, as shown by von Kossa staining (Fig. 2I–K and O) and alkaline-phosphatase (AKP) staining (Fig. 2L–N and P).

3.4. Body weight

All three groups of rats showed a similar initial body weight. By the end of the study, even though all the rats pair fed, the FSH immunization group had a significantly higher weight than the Sham group ($P < 0.01$) but had no significantly different body weight compared with the OVX group ($P > 0.05$) (Fig. 3A).

3.5. BMD analysis of distal femur

The BMD in the right femur was measured. The BMD in both the FSH immunization and Sham groups was significantly higher than the OVX group ($P < 0.01$), but there was no difference between the FSH immunization and Sham groups ($P > 0.05$) (Fig. 3B).

3.6. Biomechanical testing

The results of the three-point bending test indicated that compared with the OVX group, the femurs in the FSH immunization group possessed a higher maximum load and stiffness (Table 1) ($P < 0.05$) and showed significantly increases in Young's modulus and maximum stress ($P < 0.01$). However, the FSH immunization group showed no differences compared with the Sham group.

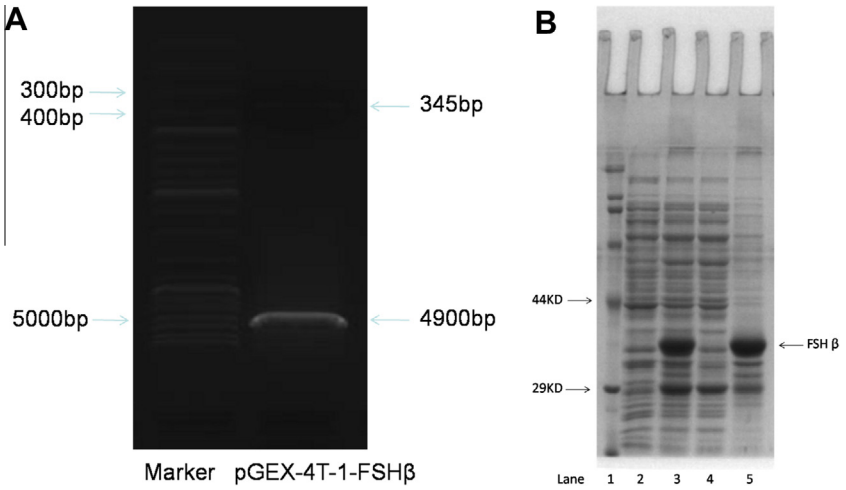

Fig. 1. Double digestion and expression of pGEX-FSHβ. (A) pGEX-4T-1-FSHβ digested by BamH I and EcoR I. (B) Expression and purification of GST-tagged FSHβ as shown by SDS–PAGE. The position of the FSHβ protein is indicated by an arrow. Lane 1, marker; lane 2, before induction; lane 3, after induction; lane 4, soluble fraction; lane 5, insoluble fraction.

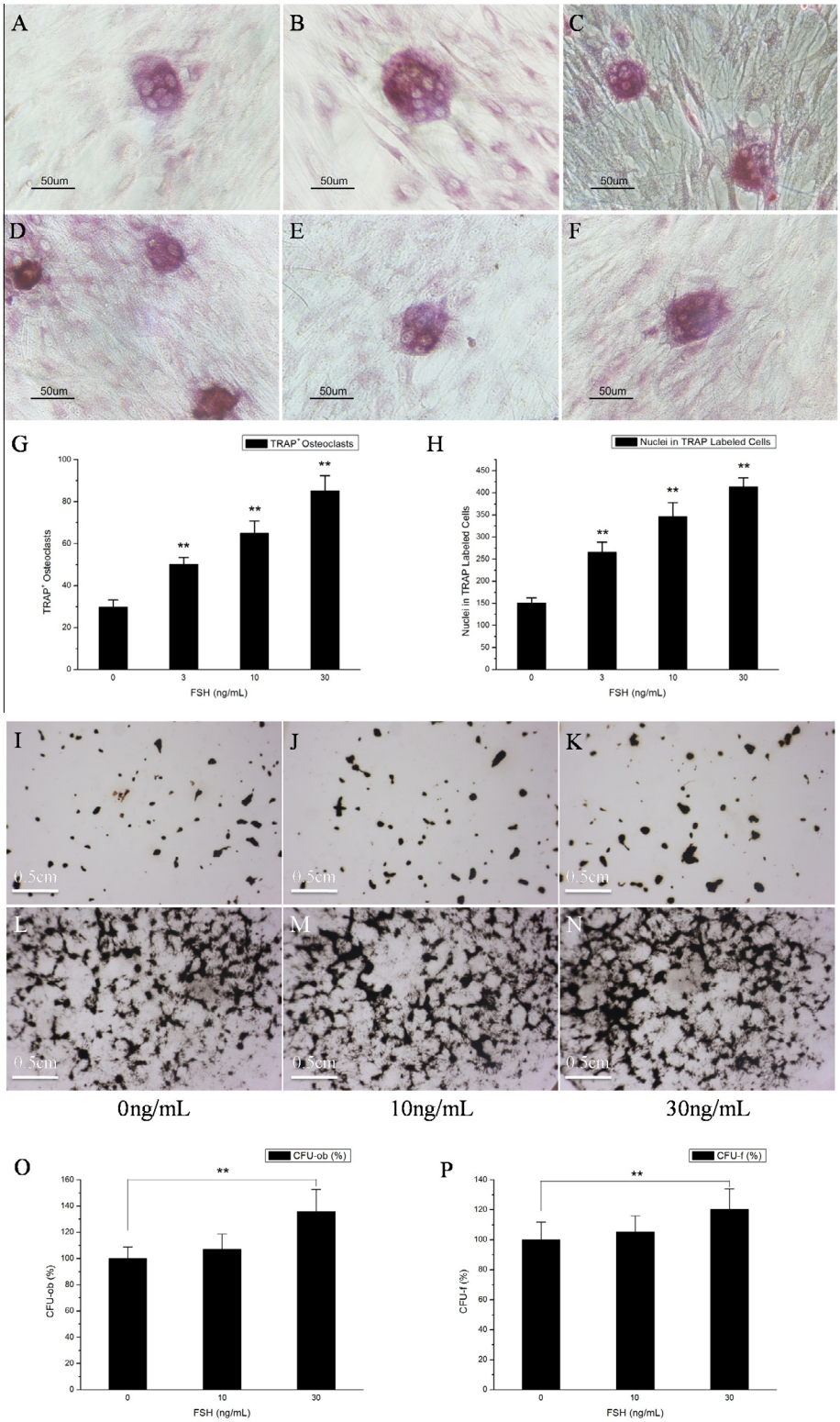

Fig. 2. (A–H) Staining and statistical analysis of osteoclasts differentiated from bone marrow. The bone marrow cells were cultured in α-MEM supplied with 10^{-8}M 1.25 VD_3 plus FSH (A) 0 ng/mL, (B) 3 ng/mL, (C) 10 ng/mL, (D) 30 ng/mL, (E) 30 ng/mL and 10 μl of antiserum, or (F) 30 ng/mL and FSH monoclonal antibodies. (G) $TRAP^+$ osteoclast numbers. (H) Nuclei in TRAP-labeled cells. (I–P) Osteogenic activity detection. (I–K) Von Kossa staining of bone marrow after osteogenic differentiation for 10 days, CFU-ob. (L–N) AKP-positive CFU-f stained with Gomori Ca–Co. (O) Statistical analysis of the CFU-ob area. (P) Statistical analysis of the CFU-f area.

These results indicated that FSH significantly improved the mechanical stability of the femurs in the OVX rats.

The lumbar compression test showed that the FSH immunization group had improvements in the maximum load, stiffness, Young's modulus and maximum stress compared with the OVX group, although these changes did not reach the normal level (Table 2). These results indicated that FSHβ immunization led to more stable lumbar vertebrae in the OVX rats.

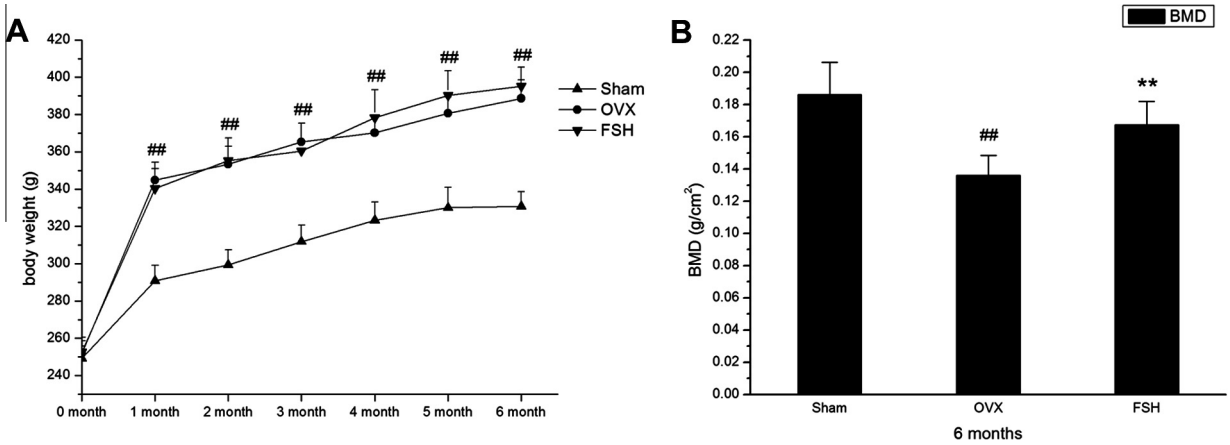

Fig. 3. Assessment of the body weight and BMD of the rats. (A) Effects of FSH on the body weight of the OVX rats. (B) BMD analysis of the distal femur. The values are the mean ± S.D., $^{**}P < 0.01$ vs. OVX; $^{\#\#}P < 0.01$ vs. Sham as evaluated by one-way ANOVA.

Table 1
Three-point bending test of the femoral diaphysis.

Parameters	Sham	OVX	FSH
Maximum load(N)	156.41 ± 5.66	138.27 ± 6.6[c]	150.28 ± 10.16[a]
Stiffness(N/mm)	265.42 ± 9.22	221.86 ± 18.48[c]	245.39 ± 21.33[a]
Young's modulus(MPa)	8199.58 ± 210.45	5999.62 ± 145.54[c]	8119.51 ± 305.08[b]
Maximum stress(MPa)	144.31 ± 11.45	97.19 ± 8.52[c]	140.25 ± 7.95[b]

Note. Values are the mean ± S.D.
[a] $P < 0.05$ vs. OVX.
[b] $P < 0.01$ vs. OVX.
[c] $P < 0.01$ vs. Sham.

Table 2
Compression test of the lumbar.

Parameters	Sham	OVX	FSH
Maximum load (N)	415.35 ± 25.283	293.66 ± 14.04[c]	338.27 ± 24.97 [bc]
Stiffness (N/mm)	257.74 ± 20.199	143.63 ± 14.39[c]	218.14 ± 20.33 [bc]
Young's modulus (MPa)	78.87 ± 6.48	51.11 ± 4.55[c]	59.63 ± 5.86 [ac]
Maximum stress (MPa)	11.8 ± 1.98	6.85 ± 0.96 [c]	10.14 ± 1.05 [b]

Note. Values are the mean ± S.D.
[a] $P < 0.05$ vs. OVX.
[b] $P < 0.01$ vs. OVX.
[c] $P < 0.01$ vs. Sham.

Table 3
Micro-CT analysis of the distal femur.

Parameters	Sham	OVX	FSH
BV/TV(L)	0.395 ± 0.023	0.207 ± 0.031[c]	0.282 ± 0.021[bc]
Tb.N(1/mm)	3.125 ± 0.213	2.441 ± 0.376[c]	2.883 ± 0.158[a]
Tb.Th(mm)	0.123 ± 0.011	0.084 ± 0.008[c]	0.1 ± 0.012[ac]
Tb.Sp(mm)	0.205 ± 0.014	0.344 ± 0.029[c]	0.268 ± 0.023[bc]
CWT(mm)	0.5 ± 0.055	0.495 ± 0.03	0.538 ± 0.026

Note. Values are the mean ± S.D.
[a] $P < 0.05$ vs. OVX.
[b] $P < 0.01$ vs. OVX.
[c] $P < 0.01$ vs. Sham.

3.7. Micro-CT analysis of the distal femur

The micro-CT analysis revealed significant improvements in the trabecular parameters in the FSH immunization group compared with the OVX group, including BV/TV and Tb.Sp (Table 3) ($P < 0.01$). Tb.Th and Tb.N in the FSH immunization group were also increased ($P < 0.05$). These results indicated that blocking the FSH signaling pathway can significantly improve the mechanical and structural parameters in OVX rats. The only data showing no differences were the cortical wall thickness measurements ($P > 0.05$). The three-dimensional images of the femoral metaphysic and maximum cross-section confirmed these improvements, as represented in Fig. 4.

3.8. Histological examination of the distal femur

The histological observations revealed that compared with the OVX group, the Tb.N had a notable increase in the FSH immunization group, as shown by the hematoxylin-eosin staining (Fig. 4J–L) and von Kossa staining (Fig. 4M–O). These results were consistent with the biomechanical testing and micro-CT analysis.

4. Discussion

Osteoporosis threatens the health of postmenopausal women. Hence, there is an urgent need to identify the causes and explore the possible therapies for this disease. Based on the recent finding that FSH from the pituitary gland can regulate bone mass in mice independent of estrogen [15], we first proposed a novel type of vaccine against postmenopausal osteoporosis in this article.

Previous reports have proven that FSH can promote osteoclastogenesis by enhancing TNF production both *in vitro* and *in vivo* and thereby lead to bone loss [29]. Our results also show that exogenous FSH stimulates osteoclastogenesis in bone marrow differentiation experiments in a dose-dependent manner. In addition, both anti-FSH monoclonal antibodies and polyclonal sera prepared by immunizing rats with a recombinant GST-FSHβ fusion protein can antagonize the effects of exogenous FSH. Breaking the tolerance to self-antigens may help treat some diseases, such as tumors [30,31]. We postulated that inducing anti-FSH immunity *in vivo* by immunization with a recombinant FSHβ antigen might be therapeutic for postmenopausal osteoporosis.

After the immunization with the recombinant FSHβ fusion protein, the titer of the anti-FSH poly sera was very high, approximately 1:1,280,000, demonstrating that anti-FSH antibodies were successfully induced. However, the mean body weight of the FSH immunization group showed little difference from that of the OVX group but was significantly higher than that of the Sham group, implying that lipogenesis was enhanced due to estrogen

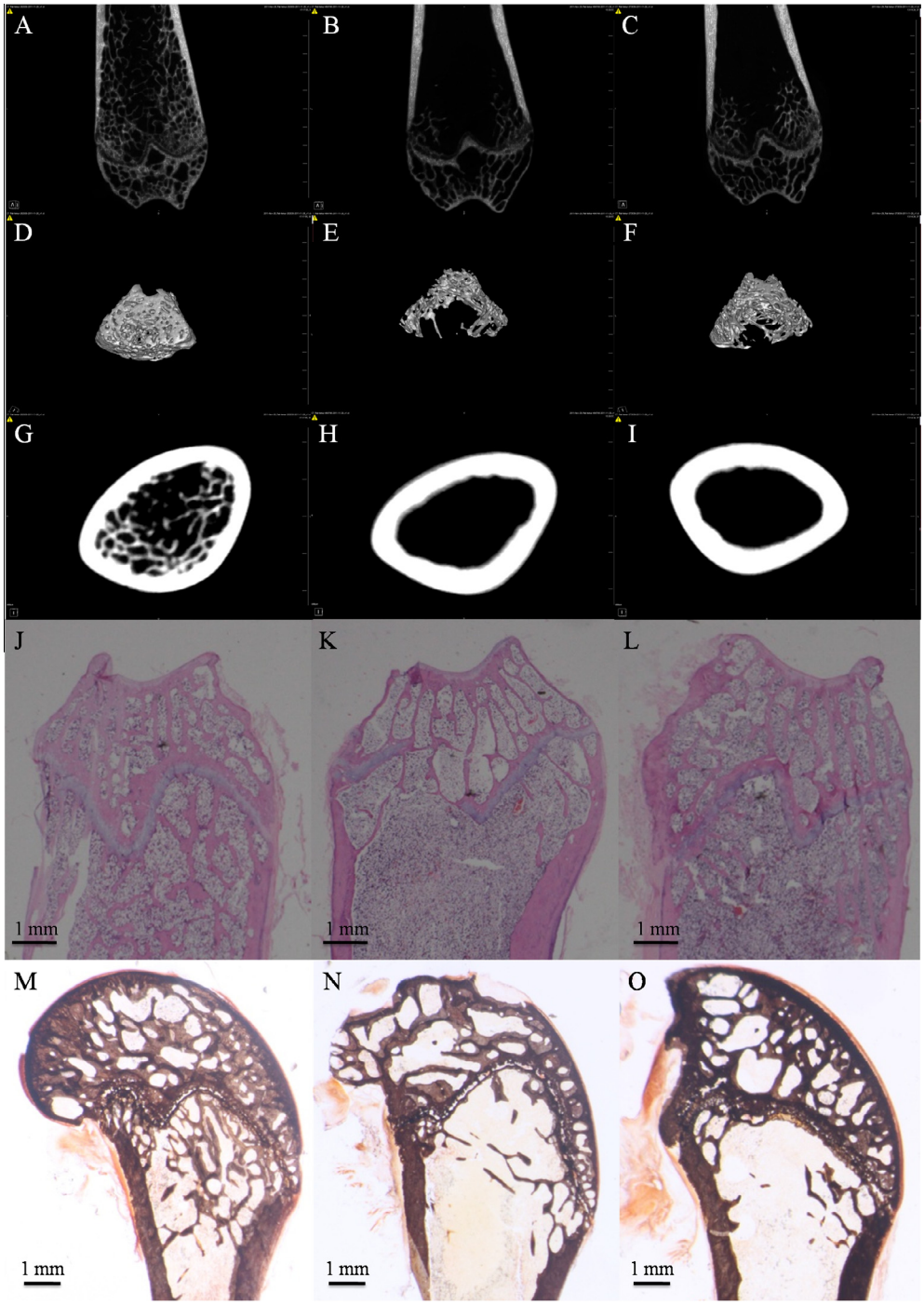

Fig. 4. Micro-CT analysis and histological examination of the distal femur. (A–C) Maximum cross-section of the distal femur. (E–F) Three-dimensional images of the femoral metaphysic. (G–I) Cortical wall distance of the VOI area at 3 mm. (J–L) Hematoxylin-eosin-staining. (M–O) Von Kossa staining.

deficiency and that neutralization of FSH cannot inhibit lipogenesis.

As BMD is the most important indicator for evaluating the degree of osteoporosis, we compared the BMD data from each group and found that the BMD of the FSH immunization group was significantly higher than that of the OVX group. These results indicated that neutralizing FSH could significantly prevent bone loss. We also found that FSH immunization can enhance osteogenesis slightly, as shown by the cortical bone mass of the FSH immunization group being slightly but not significantly higher than that of the Sham group ($P > 0.05$) when the BMD values of both groups were compared. This result was consistent with the previous finding that FSH can promote osteogenesis [29] but is inconsistent with the observation that the BMD of the FSH$\beta^{+/-}$ mouse was slightly higher than that of the FSH$^{+/+}$mice [15].

We also performed biomechanical testing, including both the femur three-point bending test and lumbar compression test, to detect bone stability [32,33] and found that most of the parameters were significantly improved in the FSH immunization group, demonstrating that bone strength was enhanced. The analysis of bone internal structural changes showed that BV/TV and Tb.N were significantly improved in the FSH immunization group, which is consistent with the results of the BMD analysis and biochemical tests. Bone microstructural changes were confirmed by the histological

examination of both decalcified and undecalcified bone tissues. However, the CWT showed little differences among three groups, demonstrating that the OVX-induced osteoporosis was mainly due to trabeculae decreases and that blocking the FSH signaling pathway can increase the trabecular bone without affecting the CWT.

Recently, Mone ZaiDi et al. reported that injecting OVX mice with a 13-amino-acid-long FSHβ subunit polyclonal antibody significantly attenuated bone loss not only by inhibiting bone resorption but also by stimulating bone formation [13]. These findings agreed with our results, and thus both studies provide a basis for using FSHβ antigen immunization as a highly effective immunotherapy strategy to treat osteoporosis.

In conclusion, immunization with a recombinant FSHβ fusion protein antigen can raise a high-titer anti-FSH polyclonal antibody that can neutralize FSH both *in vitro* and *in vivo*. Additionally, FSH immunization can not only prevent bone loss but also enhance bone strength in a rat ovariectomy-induced osteoporosis model, demonstrating that an anti-FSHβ vaccine has great potential in treating postmenopausal osteoporosis.

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