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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