

Original Article

Bovine lactoferrin improves bone mass and microstructure in ovariectomized rats via OPG/RANKL/RANK pathway

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Aim: Lactoferrin (LF), an 80-kDa iron-binding glycoprotein, is a pleiotropic factor found in colostrum, milk, saliva and epithelial cells of the exocrine glands. The aim of this study was to evaluate the effects of LF on the bones in ovariectomized (Ovx) rats and to identify the pathways that mediate the anabolic action of LF on the bones.

Methods: Female Sprague-Dawley rats (6-month-old) underwent ovariectomy, and were treated with different doses of LF (10, 100, 1000, and 2000 mg·kg⁻¹·d⁻¹, *po*) or with 7 β -estradiol (0.1 mg·kg⁻¹, *im*, each week) as the positive control. By the end of 6 month-treatments, the bone mass and microstructure in the rats were scanned by micro-computed tomography (micro-CT), and the bone metabolism was evaluated with specific markers, and the mRNA levels of osteoprotegerin (OPG) and the receptor-activator of nuclear factor κ B ligand (RANKL) in femur were measured using qRT-PCR.

Results: LF treatment dose-dependently elevated the bone volume (BV/TV), trabecular thickness (TbTh) and trabecular number (TbN), and reduced the trabecular separation (TbSp) in Ovx rats. Furthermore, higher doses of LF (1000 and 2000 mg·kg⁻¹·d⁻¹) significantly increased the bone mineral density (BMD) compared with the untreated Ovx rats. The higher doses of LF also significantly increased the serum levels of OC and BALP, and decreased the serum levels of β -CTx and NTX. LF treatment significantly increased the OPG mRNA levels, and suppressed the RANKL mRNA levels, and the RANKL/OPG mRNA ratio in Ovx rats.

Conclusion: Oral administration of LF preserves the bone mass and improves the bone microarchitecture. LF enhances bone formation, reduces bone resorption, and decreases bone mass loss, possibly through the regulation of OPG/RANKL/RANK pathway.

Keywords: lactoferrin; bone; bone mineral density; OPG/RANKL/RANK pathway; bone turnover

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Introduction

Osteoporosis is a common disease that is characterized by reduced bone mass and altered bone microarchitecture, which results in fragility fractures^[1]. The bone loss is caused by an imbalance between the activities of osteoclasts and osteoblasts, and it leads to the uncoupling of bone resorption and bone formation^[2]. The bone loss found in postmenopausal osteoporosis (PMOP) cases is caused by estrogen deficiency that increases osteoclast activity and bone remodeling – a predominance of bone resorption over bone formation^[3]. The increased rate of bone turnover as observed from markers, such as β -CrossLaps (β -CTx) and collagen type I N-telopeptide (NTX), may be an additional risk factor for fractures by exacerbating bone loss. Furthermore, the identification of additional

bone formation markers, such as osteocalcin (OC) and bone alkaline phosphatase (BALP), may serve to reduce the risk of osteoporosis and lessen the probability of fractures.

Lactoferrin (LF), an 80-kDa iron-binding glycoprotein that belongs to the transferrin family, is a pleiotropic factor found in colostrum, milk, saliva, and epithelial cells of the exocrine glands^[4, 5]. Many studies have demonstrated that lactoferrin has antimicrobial activity and functions as a regulator of immune response^[6, 7]. In recent years, several studies have shown that LF acts as a growth factor and has both *in vitro* and *in vivo* anabolic activity in bone^[8–12]. Lactoferrin induces the proliferation and differentiation of osteoblast-like cells and inhibits osteoclastogenesis *in vitro*^[10, 13]. LF has also been shown to have an inhibitory effect on osteoclast formation^[11]. Locally injecting LF above the hemicalvaria in adult mice for 5 successive days resulted in increased bone formation and bone area compared with the controls^[8]. In addition, oral LF administered to Ovx rats for 3 months protected against Ovx-induced decreases in bone volume and mineral density

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and led to increased mechanical strength parameters^[14]. In a clinical study, 38 healthy, postmenopausal women randomly received either oral RNase-enriched lactoferrin or a placebo, resulting in an obvious reduction in osteoblast and bone resorption markers in the lactoferrin-treated group^[15].

The pathways that are activated by lactoferrin are unknown. Osteoprotegerin (OPG) and the receptor activator of nuclear factor- κ B ligand (RANKL) are cytokines that are necessary for regulating bone formation and bone resorption. The RANKL/OPG ratio is the key element regulating the balance between bone formation and bone resorption, and the shortage of estrogen leads to an imbalance in this ratio. OPG is a naturally occurring inhibitor of RANKL; it binds RANKL with high affinity and prevents its interaction with RANK on osteoclasts^[16]. Therefore, OPG negatively regulates osteoclast formation and prevents osteoporosis^[17]. Studies have shown that excessive osteoclast activity in OPG-knockout mice (OPG^{-/-}) results in high bone turnover rate, disorganized matrix and impaired attachment of new bone to old bone in the cement lines^[18]. The inability of RANK to bind RANKL leads to the inhibition of osteoclast differentiation and maturation, thereby causing a deficiency of mature osteoclasts at the bone surface^[19]. In addition, other cytokines that mediate bone remodeling, such as monocyte-macrophage colony stimulating factor (M-CSF), are also known to promote osteoclastogenesis^[20].

In a number of recent studies, osteoblast cultures treated with LF showed positive effects on bone *in vitro*. It is important to study the effects of oral LF in an animal osteoporosis model as observed on bone mass and bone microarchitecture. Moreover, to utilize LF as a therapeutic agent for treating bone loss, a better understanding has to be obtained of the pathways involved in LF's anabolic effects in the bone and its effects on the biochemical markers of bone turnover. Herein, we explored in further details the pathways by which lactoferrin stimulates osteoblast growth.

Materials and methods

Materials

The bovine LF (95% purity with 20% iron saturation) used in this study was produced in Australia. Prior to use, the LF was diluted in distilled water, and the 17 β -estradiol (E₂; Shanghai General Pharmaceutical Co, LTD; batch number: 090801) was dissolved in corn oil. All of the rats were treated in accordance with "Fujian experimental animal management measures" and with the approval of the Fujian Provincial Hospital, Fuzhou, China.

Ovx rat model

A total of seventy 6-month-old virgin female Sprague-Dawley rats weighing 300 \pm 20 g were purchased from Shanghai SCXK Experimental Animals Co, LTD (qualified number: SCXK (Shanghai) 2007-0005). The rats were housed in individual stainless-steel cages with a controlled temperature of 20 \pm 3 $^{\circ}$ C, a relative humidity of 40%–60% and a 12-h light-dark cycle. All of the rats were given free access to a standard pellet diet and water during the 6-month feeding period. After 1 week

of adaptability feeding, 10 rats were randomly assigned to the sham-operated (Sham) group, and the remaining 60 rats were assigned to the ovariectomized (Ovx) group. The Ovx rats were anesthetized with ketamine (100 mg/kg), and both ovaries were removed. The Sham rats had surgery that only resected the fat tissue near the ovaries. After surgery, penicillin was administered intramuscularly at 50 000 U per day, with each dose lasting for three days. One week after the surgery, the Ovx rats were randomly divided into six groups of 10 rats each, as follows: Ovx untreated (Con); Ovx+17 β -estradiol 0.1 mg \cdot kg⁻¹ \cdot week⁻¹, the positive control (E₂); Ovx+LF 10 mg \cdot kg⁻¹ \cdot d⁻¹ (LF₁); Ovx+LF 100 mg \cdot kg⁻¹ \cdot d⁻¹ (LF₂); Ovx+LF 1.0 g \cdot kg⁻¹ \cdot d⁻¹ (LF₃); and Ovx+LF 2.0 g \cdot kg⁻¹ \cdot d⁻¹ (LF₄). The doses of LF were chosen according to previous studies in the literature^[21].

Treatment

In the Sham and Con groups, the rats were orally intubated with 0.9% physiological saline at a dose of 2 mL/kg. For the Ovx+E₂, the E₂ was dissolved in corn oil and injected into muscle at a dose of 0.1 mg/kg body weight on a weekly basis. The LF was dissolved in distilled water and given orally every day at the appropriate dose. The doses were readjusted every week to accommodate changes in body weight.

Sample collection

After 6 months of treatment, each rat was euthanized, its uterus and subcutaneous fat were removed and weighed, and the femur and the L2-4 vertebrae of each rat were separated from the surrounding muscle. Venous blood was drawn into a Vacutainer (BD Biosciences, NJ, USA) and the serum was separated by centrifugation. The serum specimens were stored at -80 $^{\circ}$ C until analysis. The right femur was collected in a tube, frozen in liquid nitrogen and stored at -80 $^{\circ}$ C until analysis. The left femur and L2-4 vertebrae were preserved in 4% paraformaldehyde for the micro-computed tomography scans.

Micro-computed tomography (micro-CT)

Scanning parameters

Each specimen consisted of the proximal femur and L2-4 vertebrae and was evaluated using a microfocus CT system (ZKKS-SHARP Institute of Chinese Science Research). Each sample was scanned at 10-mm intervals at a voltage of 70 kVp, amplitude of 200 mA, scanning resolution of 50 μ m, rotating angle of 360 degrees, power of 40 W, frame average of 6 and pixel combination of 1 \times 1.

Three dimensional (3D) reconstruction and bone parameter analysis

The trabecular bone microarchitecture of the proximal femur and L2-4 vertebrae was analyzed with mCT at a 50- μ m isotropic image resolution. The trabecular region was isolated from the cortical region in each 2D image by a manual contouring analysis. Model-independent 3D measurement methods were used to reconstruct and define the trabecular bone volume of interest (VOI) for the cancellous bone, and the following

parameters were calculated: trabecular bone volume fraction (BV/TV, %), trabecular thickness (TbTh, mm), trabecular number (TbN, mm⁻¹), trabecular separation (TbSp, mm), and bone mineral density (BMD).

Serum E₂ level and biochemical markers of bone turnover

Total estradiol (E₂), osteocalcin (OC), bone alkaline phosphatase (BALP), β-CrossLaps (β-CTX), and collagen type I N-telopeptide (NTX) levels were determined in serum samples obtained at the time of bone biopsy. E₂, OC, BALP, and β-CTX were measured using an immunoradiometric assay autoanalyzer (R&D, USA). NTX levels were determined by electrochemiluminescence (ECL) assay (Roche Elecsys 2010, Switzerland). The sensitivities of these assays were 1 pg/mL and 50 pg/mL, and their inter-assay variation coefficients were less than 10%.

Quantitative real-time reverse transcription-PCR (qRT-PCR)

The local expression of the RANKL and OPG genes in the trabecular bone of the femur was analyzed by qRT-PCR. A small cube of trabecular bone (proximal femur) was homogenized, and the total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA)^[22]. The extracted RNA was dissolved in RNase-free distilled water. The quality and quantity of the RNA samples were determined by spectrophotometry, with the ratios of absorbance at 260 nm and 280 nm ranging from 1.8 to 2.0. Next, 5 mg of total RNA was first reverse transcribed into cDNA using a High-Capacity cDNA Kit (DRR037, TaKaRa, Japan) according to the manufacturer's instructions.

The quantitative PCR for the local RANKL and OPG gene expression was performed in 96-well plates; the reaction volume was 25 mL/well, which included the SYBR PremixEx Taq II (2×) (DRR081A, TaKaRa, Japan), diluted gene primers, and cDNA. The primer sequences are listed in Table 1. The qRT-PCR was performed using a Thermal Cycler Dice™ Real Time (TP800, TaKaRa). The standard curve method was used to calculate the relative expression levels, with the levels of each gene normalized to β-actin. The data from the qRT-PCR analyses are presented in relative mRNA units. The RANKL/OPG mRNA ratio was calculated from the RANKL/actin and OPG/actin mRNA values.

Statistical analysis

The results are reported as the mean±standard deviation (SD). The data analyses were performed using the Statistical Package for the Social Sciences (SPSS version 16.0) software package. The differences among the 7 groups were analyzed using a one-way ANOVA model followed by *post-hoc* tests. The changes in body weight within a group over time were compared using the paired-samples *t*-test. The variables with unequal variances were analyzed using the Kruskal-Wallis H test. Differences were considered significant at *P*<0.05.

Results

General index changes

The body weights of the rats were not significantly different from each other before the treatment (*P*>0.05, Table 2). By the end of the 6-month experiment, all of the rats had increased

Table 1. The primer sequences for quantitative real-time PCR.

Genes	GenBank numbers	Primer sequence	Product size (bp)
OPG	NM-012870	Forward 5'-ACAATGAACAAGTGCTGTGCTG-3' Reverse 5'-CGGTTTCTGGGTCATAATGCAAG-3'	109
RANKL	NM-057149	Forward 5'-GCAGCATCGCTCTGTTCTCTGTA-3' Reverse 5'-GCATGAGTCAGGTAGTGCTTCTGTG-3'	164
β-Actin	NM-031144.2	Forward 5'-GGAGATTACTGCCCTGGCTCCTA-3' Reverse 5'-GACTCATCGTACTCTGCTTGTCTG-3'	150

Table 2. The body, uterus, and subcutaneous fat weights in the Con, Sham, E₂, and LF groups.

Weights	Numbers		Body (g)		Uterus (g)	Subcutaneous fat (g)
	Initial	Final	Initial	Final	Final	Final
Con	10	7	289.9±19.2	434.1±46 ^e	0.15±0.09	20.1±1.3
Sham	10	7	298.0±18.0	358.9±40.5 ^{be}	0.91±0.85	12.3±0.6
E ₂	10	8	295.6±13.8	370±42.4 ^{be}	0.85±0.78	13.5±0.7
LF ₁	10	7	300.9±14.4	426.1±43.3 ^e	0.36±0.55	18.8±1.5
LF ₂	10	10	290.1±15.0	393.6±29.4 ^e	0.45±0.49	17.5±1.0
LF ₃	10	9	294.5±13.8	393.1±35.2 ^e	0.60±0.51	15.3±0.8
LF ₄	10	8	297.02±18.8	395.3±288 ^e	0.86±0.59	13.8±0.7

Con: ovariectomized untreated; Sham: sham-operated; E₂: Ovx+E₂ 0.1 mg·kg⁻¹·week⁻¹; LF₁: Ovx+LF 10 mg·kg⁻¹·d⁻¹; LF₂: Ovx+LF 100 mg·kg⁻¹·d⁻¹; LF₃: Ovx+LF 1.0 g·kg⁻¹·d⁻¹; LF₄: Ovx+LF 2.0 g·kg⁻¹·d⁻¹. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 compared with the Con group; ^e*P*<0.05 compared with the initial body weight.

body weight. The Con rats tended to gain more weight than the Sham rats ($P < 0.05$). The final body weights of the LF-treated rats tended to be lower than those of the Con group; however, these differences were not statistically significant ($P > 0.05$, Table 2). In addition, estrogen treatment completely prevented the Ovx-induced weight gain.

The Con rats had uterine tissue atrophy, indicating the success of the surgical procedure (Table 2). The estrogen partly prevented the Ovx-induced uterine atrophy, whereas the LF had no detectable effect on the uterus. The subcutaneous fat weight of the Con rats was significantly greater than that of the Sham rats, but this difference was not statistically significant ($P > 0.05$, Table 2).

Serum E₂ level

At the completion of the experiment, the levels of serum estradiol were comparable between the E₂ and the Sham groups ($P > 0.05$), whereas the Con and the different LF groups had decreased estradiol levels as compared to the Sham group ($P < 0.01$) (Figure 1).

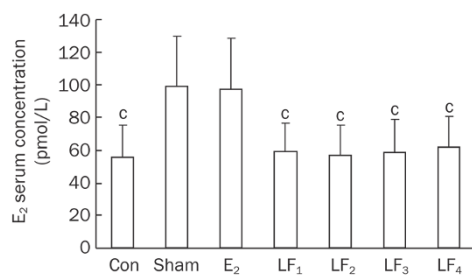


Figure 1. The serum E₂ level in the Con, Sham, E₂, and LF groups at the end of the experiment. Mean \pm SD. ^c $P < 0.01$ compared with the Sham group.

Micro-computed tomography (micro-CT)

Microstructural parameters of the trabecular bone

As shown in Figures 2 and 3, the ovariectomies significantly reduced BV/TV, TbTh, TbN values and increased the TbSp values in both femur and L2-4 vertebrae of the rats. Treatment with LF or estrogen protected the rats from the Ovx-induced effects on the above biomarker levels.

Bone mineral density (BMD)

At the end of the experiment, the Con rats had markedly reduced BMD in both their femurs and L2-4 vertebrae compared with the Sham and E₂ groups ($P < 0.01$). The BMD values in the rats treated with 1.0 g·kg⁻¹·d⁻¹ and 2.0 g·kg⁻¹·d⁻¹ LF were greater than those in the Ovx control ($P < 0.05$ and $P < 0.01$). However, the lower LF doses (10 mg·kg⁻¹·d⁻¹ and 100 mg·kg⁻¹·d⁻¹) did not significantly alter the BMD ($P > 0.05$, Figure 4). The BMD differences among the groups followed a similar pattern for both L2-4 lumbar vertebrae and femur.

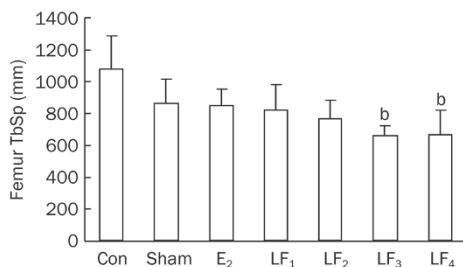
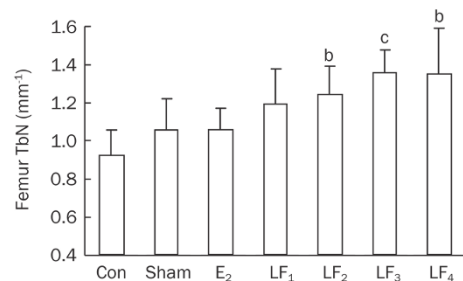
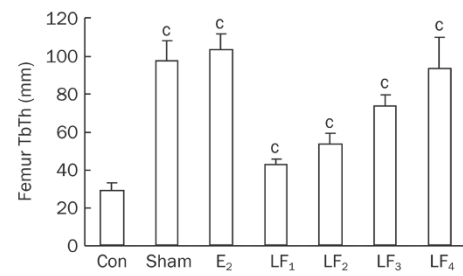
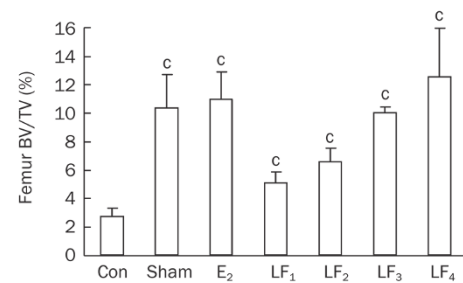


Figure 2. The trabecular bone microstructural parameters of the femurs in the Con, Sham, E₂, and LF groups at the end of the experiment. ^b $P < 0.05$, ^c $P < 0.01$ compared with the Con group. BV=bone volume; TV=tissue volume; TbTh=trabecular thickness; TbN=trabecular number; TbSp=trabecular separation.

Bone turnover markers

The serum levels of OC, BALP, β -CTx, and NTX in the Con group were higher compared with those in the Sham group ($P < 0.01$), with the levels of β -CTx and NTX being noticeably higher than those of OC and BALP. The serum levels of OC, BALP, β -CTx, NTX increased by 17.9%, 14.0%, 47.7%, and 31.7%, respectively, in the Con group compared with the Sham. Although the serum levels of OC and BALP in the different LF groups were higher compared with the Con

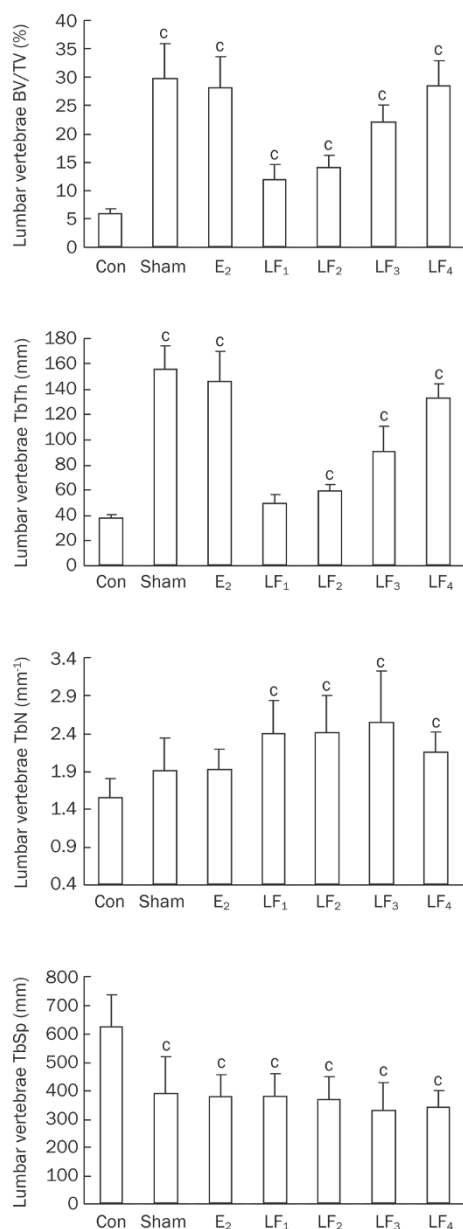


Figure 3. The trabecular bone microstructure parameters of the L2-4 vertebrae in the Con, Sham, E₂, and LF groups at the end of the experiment. ^b $P < 0.05$, ^c $P < 0.01$ compared with Con group.

group, these increases were only statistically significant for LF₃ (1 g·kg⁻¹·d⁻¹ LF treatment) and LF₄ (2 g·kg⁻¹·d⁻¹ LF treatment) groups. Moreover, the serum levels of β-CTx and NTX were lower in the different LF groups compared with the Con group, and these decreases were significant for the LF₃ and LF₄ groups. The serum levels of OC, BALP, β-CTx, and NTX were similar between the E₂ and Sham groups ($P > 0.05$, Figures 5 and 6).

The local expression of RANKL and OPG genes in femur

As shown in Figure 7, the local expression of the RANKL gene was higher in the Con group than in the Sham and E₂

groups ($P < 0.01$). The groups with 1 g·kg⁻¹·d⁻¹ and 2 g·kg⁻¹·d⁻¹ LF treatments had significantly lower RANKL mRNA levels in the proximal femur compared with the Con group ($P < 0.01$ and $P < 0.05$). However, the groups with 10 mg·kg⁻¹·d⁻¹ and 100 mg·kg⁻¹·d⁻¹ LF treatments did not show significant alterations of the RANKL mRNA levels ($P > 0.05$). The local OPG mRNA expression increased in the Sham and E₂ groups compared with the Con group ($P < 0.01$). The groups with 1 g·kg⁻¹·d⁻¹ and 2 g·kg⁻¹·d⁻¹ LF treatment had significantly higher OPG levels compared with the Con group ($P < 0.01$ and $P < 0.05$); however, the two lower doses of LF did not show significant effects ($P > 0.05$). The ratio of RANKL/OPG mRNA in the Con group was significantly higher than that in the Sham and E₂ groups ($P < 0.01$). Moreover, there was a significant decrease ($P < 0.01$) in the RANKL/OPG mRNA ratio in the proximal femur following treatment with 100 mg·kg⁻¹·d⁻¹, 1 g·kg⁻¹·d⁻¹, and 2 g·kg⁻¹·d⁻¹ LF compared with the Con group; however, 10 mg·kg⁻¹·d⁻¹ LF treatment did not show any significant change ($P > 0.05$) for the same.

Discussion

Previous studies have shown that lactoferrin has an anabolic effect on osteoblasts and osteoclasts *in vitro*^[8,9,11]. The aim of this study was to evaluate the changes induced by lactoferrin in the Ovx rat model and to identify the pathways that mediate the anabolic action of lactoferrin in bone. We found that LF at concentrations of 1 g·kg⁻¹·d⁻¹ and 2 g·kg⁻¹·d⁻¹ significantly increased the bone mass and microarchitecture compared with the untreated Ovx rats that suffered from increasing bone loss, severe osteopenia and increased bone resorption. Compared with the untreated Ovx rats, the LF treatment resulted in increased bone formation in the femur and lumbar vertebrae, and these changes were associated with higher bone volume (BV/TV) and trabecular number (TbN). LF also improved the trabecular microarchitecture, which is consistent with the increase in bone mineral density (BMD). Moreover, LF seemed to partially restore trabecular connectivity by increasing trabecular thickness (TbTh) while reducing trabecular separation (TbSp), suggesting that LF could prevent the loss of bone mass after Ovx. These results were confirmed in a series of experiments on a mixed primary culture of murine bone cells. In our study, significant microarchitectural differences were observed between the rats given the LF treatment and the untreated Ovx controls. We need to pay attention to that what in the Guo's study, LF could increase bone mineral density at 8.5- and 85- mg/kg doses^[14]. However, similar increases did not occur at the 10 mg·kg⁻¹·d⁻¹ and 100 mg·kg⁻¹·d⁻¹ LF doses that were used in our study. A possible explanation could be the differences in density and purity of different LF preparations in these studies. As a new anti-osteoporosis agent, LF has been reported to exert beneficial effects on osteoblast proliferation and differentiation as well as inhibit osteoclast activity in bone cell cultures^[9,10].

Bone mass depends on the balance between bone resorption and bone formation within a remodeling unit, and it varies with the number of remodeling units that are activated within

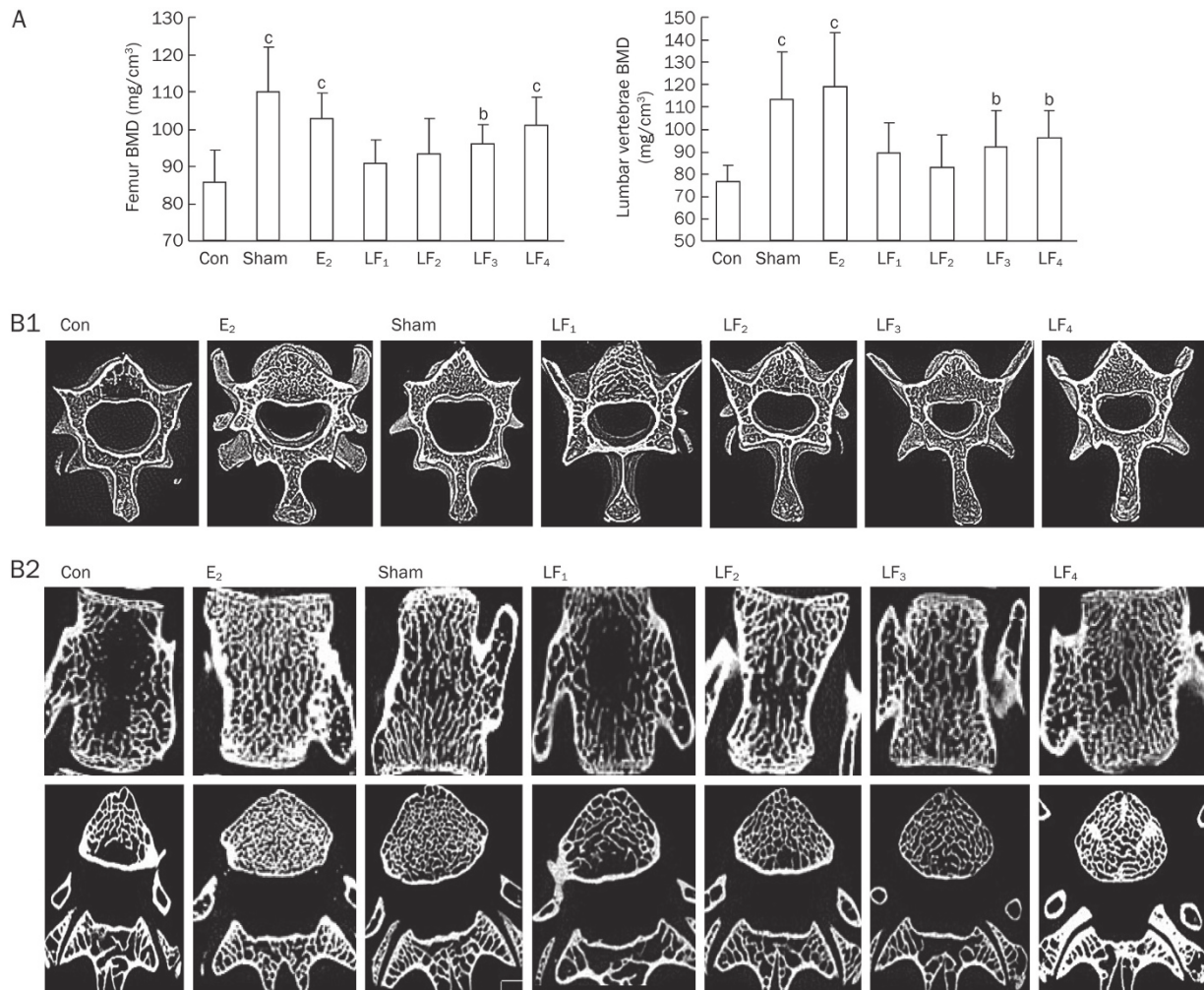


Figure 4. (A) The BMD of the femurs and L2-4 vertebrae in the Con, Sham, E₂, and LF groups at the end of the experiment. ^b*P*<0.05, ^c*P*<0.01 compared with Con group. (B1) The trabecular bone microstructure of the L3 vertebrae in the Con, Sham, E₂, and LF groups at the end of the experiment. B1. A representative 3D reconstruction of a cross-section of the bone microstructure in the L3 vertebrae. (B2) Representative 2D images of a coronal section and a cross-section of the bone microstructure in the L3 vertebrae separately from the micro-CT scans. These pictures show that the Con group has severe osteopenia, main bone loss with trabecular thinning, and fewer areas of trabecular bone, which were highly disconnected and partly fractured. However, the LF groups display orderly trabecular arrangements, with more areas of trabecular bone and better connectivity.

a given period of time in a defined area of bone. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Biochemical markers of bone turnover are indicative of events occurring during the bone remodeling cycle. These biochemical markers are divided into two categories: markers of bone formation and markers of bone resorption. These markers reflect the metabolic conversion of bone, predict osteoporosis and indicate the risk of bone fractures. The levels of biochemical markers of bone turnover, both for formation and resorption, continue to increase with age following menopause^[23]. In this study, all of the markers displayed this tendency. The serum levels of OC, BALP, β -CTx, and NTX in the Con group were increased after ovariectomy compared with the Sham group. In addition, β -CTx and NTX levels demonstrated stronger changes than OC and BALP levels, suggesting that at 6-months post-

ovariectomy, there is enhanced bone resorption resulting in bone loss. However, LF prevented the Ovx-induced bone loss by increasing bone formation over bone resorption.

We also observed that treatment with 1 g·kg⁻¹·d⁻¹ and 2 g·kg⁻¹·d⁻¹ LF increased the local OPG gene expression in the proximal femur. These elevations in OPG can contribute to a reduced local RANKL/OPG ratio, suggesting that LF may decrease osteoclast differentiation.

Previous studies have shown that osteogenesis is determined by the duration of ovariectomy and diet, factors that regulate the expression of genes involved in bone formation. This regulation specifically allows bone loss to occur following ovariectomy^[24, 25] until the end of the osteogenic process. Therefore, the ovariectomy procedure induced negative long-term regulation of bone formation results in the predominance of bone resorption over bone formation. An increase in bone

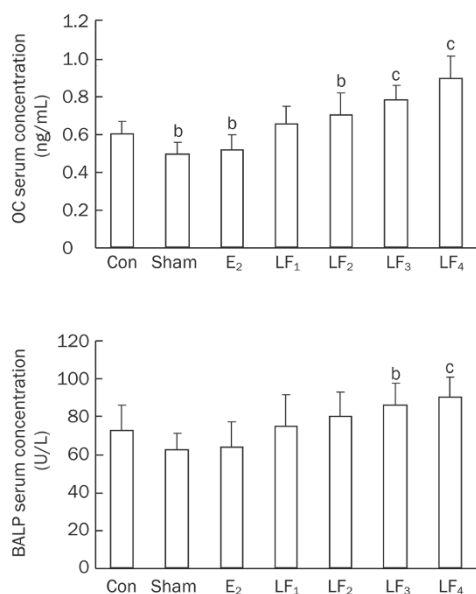


Figure 5. The bone formation makers of serum in the Con, Sham, E₂, and LF groups at the end of the experiment. ^bP<0.05, ^cP<0.01 compared with the Con group. OC=Osteocalcin; BALP=bone alkaline phosphatase.

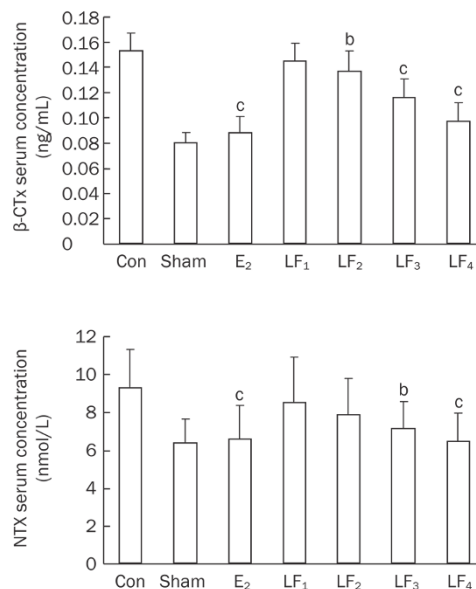


Figure 6. The bone absorption makers of serum in the Con, Sham, E₂, and LF groups at the end of the experiment. ^bP<0.05, ^cP<0.01 compared with the Con group. β-CTX=β-CrossLaps; NTX=collagen type I N-telopeptide.

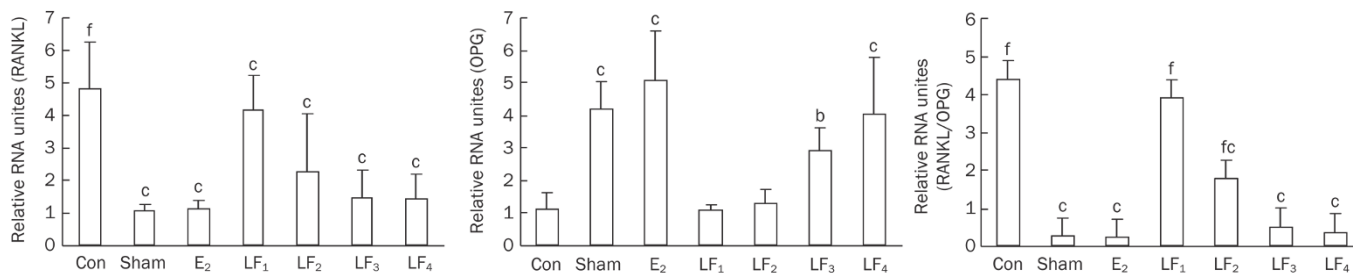


Figure 7. The effects of LF on the local expression of RANKL and OPG mRNA and the RANKL/OPG mRNA ratio in the bone trabecula of the proximal femur. The RANKL and OPG mRNA expression in the proximal femur was determined by qRT-PCR, and the RANKL/OPG mRNA ratio was calculated. Mean±SD. ^bP<0.05, ^cP<0.01 compared with the Con group; ^fP<0.01 compared with the E₂ group.

resorption is a general characteristic of osteoporosis and is primarily regulated by the relative balance between RANKL and OPG levels. The LF treatment induced a bone formation marker expression pattern similar to that observed in the untreated ovariectomized rats. The LF induced a decline in RANKL gene expression in the trabecular bone of the Ovx rats, suggesting that LF prevents bone loss by reducing the expression of RANKL in osteoblasts. The LF treatment did not cause differentiation of the bone marrow stromal cells to an osteoblastic lineage in this ovariectomized rat model. According to previous studies^[3], however, the inhibition of bone resorption markers by oral LF suggests that LF may prevent Ovx-induced bone loss via a preferential anti-osteoclastogenic pathway.

RANKL has been shown to have a significant role in the generation, activity, and survival of osteoclasts^[26,27]. OPG is an endogenous antagonist of RANKL and inhibits RANKL action by acting as a soluble decoy receptor, thus balancing bone

turnover^[28]. In bone, RANKL is expressed most abundantly in MSCs, osteoblasts and T cells^[29]. Despite OPG being a known inhibitor of RANKL, its biological effect may depend on the molar ratio between RANKL and OPG^[30]. In this study of ovariectomized rats, we demonstrated that although LF treatment seems to have an inhibitory effect on RANKL, it enhances OPG. The RANKL/OPG ratio may be a major mechanism for the increased bone formation and decreased bone resorption observed in the LF treated rats, and this finding suggests that LF decreases osteoclast differentiation.

Nevertheless, this study has several limitations worth discussing. First, the number of rats in this study was small, and there was much variability in the measurements; therefore, additional studies should be conducted to confirm these results. Second, we did not assess the biochemical markers of mineral homeostasis (estrogen, calcium, phosphate and parathyroid hormone); therefore, we cannot evaluate their impact

in this study. Finally, this study did not include information on mechanical bone properties or histological data; thus, we cannot determine whether the routine of daily LF treatment impaired the osteoid mineralization rate or individual osteoblast function.

In conclusion, this study used an ovariectomy-induced osteoporosis rat model to show that orally administered LF preserves bone mass and bone microarchitecture and prevents bone loss in both the femoral and lumbar vertebrae. We also demonstrated that LF has dual effects on both osteoblasts and osteoclasts as well as on the OPG/RANKL/RANK pathway. In summary, LF may be a safe and effective treatment for estrogen-dependent bone loss, and it is possible that OPG/RANKL/RANK signaling may be involved in the LF-induced antiresorptive effect. Further investigations need to be performed on the relevance of LF treatment strategy for postmenopausal bone loss in humans with implications on the anabolic effects of LF on bone metabolism.

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Author contribution

Jian-ming HOU, Ying XUE, and Qing-ming LIN designed the research project; Jian-ming HOU supervised the project; Ying XUE performed the animal assays, serum assays, analyzed the data and wrote the manuscript; and Jian-ming HOU, Ying XUE, and Qing-ming LIN revised the manuscript.

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