Orally Administered Lactoferrin Preserves Bone Mass and Microarchitecture in Ovariectomized Rats1,2

Hui Yuan Guo,3,4 Lu Jiang,3,4 Salam A. Ibrahim,5 Lian Zhang,6 Hao Zhang,3,4 Ming Zhang,3,4 and Fa Zheng Ren3,4,6*

Abstract

Lactoferrin (LF) is reported to stimulate osteoblast proliferation and inhibit osteoclast activity in bone cell culture. However, the effect of oral LF on bone in osteoporosis needs to be explored. Three-month-old female Sprague-Dawley rats (n = 70) were assigned to the following groups: sham-operated, ovariectomized (OVX) untreated, OVX + bovine serum albumin (BSA; 85 mg/kg body weight), OVX + LF (0.85 mg/kg, 8.5 mg/kg, and 85 mg/kg body weight), and OVX + 17β-estradiol (E2; 10 μg/kg body weight). After 3 mo of treatment, E2 completely prevented the OVX-induced bone loss. OVX rats treated with LF were protected against the OVX-induced reduction of bone volume, trabecular number, and thickness, and the elevation of trabecular separation was prevented. LF also increased bone mineral density and increased the parameters of mechanical strength at 8.5- and 85-mg/kg doses. Greater bone formation and reduced bone resorption, as assessed by biochemical markers of bone remodeling, occurred in rats administered LF. LF at 8.5- and 85-mg/kg concentrations caused a significant decrease in serum calcium, but this reduction did not occur in rats fed 0.85 mg/kg LF. In addition, serum tumor necrosis factor-α and interleukin-6 production were suppressed and serum calcitonin was elevated significantly in LF-fed rats at all 3 doses. These findings indicated that oral LF not only preserved bone mass but also improved bone microarchitecture. The absorption of LF peptides and their effects on bone cells could to some extent account for the osteogenic function of oral LF. J. Nutr. 139: 958–964, 2009.

Introduction

Osteoporosis is a disease defined by decreased bone mass and altered microarchitecture, resulting in increased bone fragility and susceptibility to fracture (1). The bone loss is caused by an imbalance between the activities of osteoclasts and osteoblasts and the uncoupling of bone resorption and formation. For the aging population, osteoporosis is a major health and economic burden (2). Current drugs for the prevention and treatment of osteoporosis include estrogen, selective estrogen receptor modulators, calcitonin, and bisphosphonates (3). Despite being effective in preventing bone loss for postmenopausal women, some of these drugs produce adverse side effects on the breast and the cardiovascular system and increase the risk of ovarian cancer (4,5). Moreover, because osteoporosis is a common, chronic disease, treatment by natural and nontoxic food proteins would be a better long-term approach than the use of pharmacological developed drug.

Lactoferrin (LF), an 80-kDa iron-binding glycoprotein of the transferrin family, is produced by epithelial cells and neutrophilic leukocyte precursors (6). As a major functional protein in dairy products, LF is widely present in the diet (7). Although orally administered proteins are hydrolyzed rapidly in the gastrointestinal tract and thus may lose their biological activity, oral LF has been shown to exhibit similar physiologic effects in vivo as displayed in vitro, such as its effects on cell proliferation (8) and cytokine production (9).

In addition to its multiple biological functions (10–15), LF has recently been shown to exert an effect on both the activity and development of osteoblasts (16–20) and osteoclasts in vitro.

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Materials and Methods

Materials. Lyophilized bovine LF (95% purity, 20% iron saturation) was provided by the Australian Yosica Holding. LF and bovine serum albumin (BSA) (Sigma Aldrich) were diluted in water and 17β-estradiol (E2) (Sigma Aldrich) was dissolved in corn oil before use.

Rats and treatments. A total of 70 3-mo-old virgin female Sprague-Dawley rats (Vital River Lab Animal Technology) were housed at a constant temperature (22–26°C) under a 12-h-light/dark cycle, fed a nonpurified diet (Standard Rodent Chow Product, Ke Ao Xie Li Feeds, C). After serum collection, each rat was killed; its uterus was removed and weighed and the femur, tibia, and vertebrae were collected and stored in saline-soaked gauze at −20°C until analysis.

Bone mineral density. The femur and lumbar vertebra (L1–L6) were stripped of the musculature and other soft tissues. Total bone mineral density (BMD) (mg/cm²) of femur and lumbar vertebrae from the second to the 6th were measured by DEXA (Prodigy, Lunar) (23).

Micro-computerized tomography analysis. The micro-computerized tomography analysis (μCT) of the right tibial metaphysis was performed using the Explore Locus SP scanner (GE Healthcare Technologies). Quantification of bone volume was performed using MicroView software (GE Healthcare) (24). The tibia was scanned from the proximal growth plate to the distal direction. This region included 500 images obtained from

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**Table 1** Body and uterus weights in SHAM, OVX control, OVX+BSA (85 mg/kg), OVX+LF at 0.85, 8.5, and 85 mg/kg, and OVX+E2 (10 μg/kg) rats.

<table>
<thead>
<tr>
<th>Weight</th>
<th>SHAM</th>
<th>OVX</th>
<th>OVX + BSA</th>
<th>0.85 (mg/kg)</th>
<th>8.5 (mg/kg)</th>
<th>85 (mg/kg)</th>
<th>OVX + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (initial)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body (final)</td>
<td>294 ± 13</td>
<td>287 ± 16</td>
<td>293 ± 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body (final)</td>
<td>338 ± 30*</td>
<td>407 ± 31**</td>
<td>397 ± 37**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterus (final)</td>
<td>0.66 ± 0.09a</td>
<td>0.07 ± 0.01c</td>
<td>0.08 ± 0.01c</td>
<td>0.08 ± 0.02c</td>
<td>0.07 ± 0.01f</td>
<td>0.06 ± 0.02c</td>
<td>0.18 ± 0.03b</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 10. Means in a row with superscripts without a common letter differ, P < 0.05. *Different from initial body weight, P < 0.05.
each tibia using 2048 × 2048 matrix resulting in an isotropic voxel resolution of 14 μm. The volume of interest was selected including as much trabecular envelope as possible but excluding cortical bone. A rectangular prism was created by projecting the region of interest on the adjoining 100 slices below the growth plate. The three-dimensional images were also obtained for visualization and display.

We obtained bone morphometric parameters, including bone volume over total volume (BV/TV), trabecular number (Tb.N), separation (Tb.Sp), and thickness (Tb.Th), by analyzing volume of interest. The operator conducting the scan analysis was unaware of the treatments associated with the specimen.

Biomechanical test. Structural and mechanical properties of femoral midshaft and the 5th lumbar vertebral (L5) were assessed by materials testing system (TMS-Pro, Food Technology). For all tests, loading was done at a constant deformation rate of 0.1 mm/s; force-displacement data were recorded and analyzed using a computerized data acquisition system (Texture Lab Pro version 1.13–002, Food Technology). A 3-point bending test was used to determine the maximal load to failure for femoral midshaft. Bones were placed on supports spaced 20 mm apart and loaded at their midpoint on the anterior cortex. A compression test along the cephalocaudal axis was used to determine the ultimate load of L5. Before testing, a central specimen with a height of ~5 mm was prepared. The cephalic and caudal endplates were removed to provide flat, parallel surfaces for loading.

Bone biochemical markers and cytokines. Serum Ca was analyzed on a Hitachi 717 autoanalyzer (Boehringer). Serum osteocalcin, calcitomin, TNFα, and IL-6 were measured in duplicate with an immunoradiometric assay kit (Immunotopics). Urinary deoxypyridinoline (D-Pyr) was measured using ELISA (Metra Biosystems).

Osteoblast proliferation assays. The pepsin hydrolysate of LF (LFH) was prepared as follows (25): a 5% (wt:v) LF solution was adjusted to pH 2.5 with 1 mol/L HCl and digested with 0.15% (wt:v) porcine pepsin (Sigma Chemical) for 4 h at 37°C. The reaction was terminated by incubation of the solution at 80°C for 15 min and the solution was adjusted to pH 7.0 with 1 mol/L NaOH. The digest was then centrifuged at 17,000 × g, 15 min and the supernatant was lyophilized. Bovine lactoferrin (Lf; amino acid sequence: FCRRWQWRMKKLGAPSTTCRRAF) was synthesized with a purity of >95%.

Primary osteoblasts were prepared from the calvaria of newborn Sprague-Dawley rats (26). The osteoblasts were cultured in a-minimum essential medium (Sigma) containing 10% fetal calf serum (Gibco, Invitrogen) until the cells became 90% confluent. Cells were then seeded into 96-well plates in 10% fetal bovine serum albumin/MEM for 24 h. Cells were grown in fresh media for 24 h and then experimental compounds (LF, LFH, and Lfcin) at various concentrations were added for a further 24 h. Osteoblast proliferation was measured by MTT assay (27).

Statistical analysis. The results were expressed as mean ± SD. Data analysis was carried out using SPSS software, version 12.0. Differences among 7 groups and body weight changes within a group over time were compared using ANOVA followed by Duncan’s post hoc test. Variables with unequal variances were analyzed using log-transformed numbers. Differences were considered significant at P < 0.05.

Results

Body and uterine weights. All rats had increased body weight by the end of the experiment (Table 1). The final body weight of the OVX control rats was significantly higher than that of the SHAM rats. Estrogen completely prevented the OVX-induced weight gain. However, treatment with LF and BSA had no such influence on the body weight. Final body weights of LF-treated rats at 85 mg/kg tended to be lower than that of the OVX control group (P = 0.07). As expected, OVX caused atrophy of uterine tissue, indicating the success of the surgical procedure (Table 1). Estrogen prevented the OVX-induced uterine atrophy partly, whereas LF had no detectable effect on the uterus like estrogen. Food intakes due to diet did not differ among the 7 groups (data not shown).

BMD measurements. OVX treatment significantly reduced BMD in both femur (Fig. 1A) and the lumbar vertebra (Fig. 1B). OVX + BSA did not counteract the BMD loss induced by OVX. BMD in the OVX + E2 group was much greater than that in the OVX control. Substantial protection against BMD loss was obtained by treatment with LF at 8.5 and 85 mg/kg, but the

TABLE 2  Microstructural parameters of proximal tibia in SHAM, OVX control, OVX+BSA (85 mg/kg), OVX+LF at 0.85, 8.5, and 85 mg/kg, and OVX+E2 (10 μg/kg) rats at the end of the 3-mo treatment

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>OVX</th>
<th>OVX + BSA</th>
<th>OVX + LF 0.85</th>
<th>OVX + LF 8.5</th>
<th>OVX + LF 85</th>
<th>OVX + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV, %</td>
<td>36.35 ± 2.57a</td>
<td>3.46 ± 1.68a</td>
<td>4.62 ± 1.50a</td>
<td>7.31 ± 1.61d</td>
<td>10.07 ± 2.15d</td>
<td>12.12 ± 2.67d</td>
<td>18.29 ± 3.05d</td>
</tr>
<tr>
<td>Tb.Th, mm</td>
<td>0.10 ± 0.019b</td>
<td>0.04 ± 0.005b</td>
<td>0.04 ± 0.006b</td>
<td>0.05 ± 0.003b</td>
<td>0.05 ± 0.005b</td>
<td>0.05 ± 0.003b</td>
<td>0.05 ± 0.005b</td>
</tr>
<tr>
<td>Tb.N, mm−1</td>
<td>5.81 ± 0.43c</td>
<td>1.00 ± 0.12c</td>
<td>0.97 ± 0.14a</td>
<td>1.58 ± 0.27d</td>
<td>2.12 ± 0.36c</td>
<td>2.46 ± 0.34c</td>
<td>3.41 ± 0.44c</td>
</tr>
<tr>
<td>Tb.Sp, mm</td>
<td>0.07 ± 0.01c</td>
<td>0.96 ± 0.12a</td>
<td>1.01 ± 0.16a</td>
<td>0.52 ± 0.09b</td>
<td>0.44 ± 0.11bc</td>
<td>0.36 ± 0.05bc</td>
<td>0.24 ± 0.04c</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 10. Means in a row with superscripts without a common letter differ, at P < 0.05.
Ovariectomy markedly reduced the BV/TV, Tb.Th, and Tb.N of the lumbar vertebra (L2–L5). Compared with the OVX control group, the plate-like structure mostly resolved into a rod-like structure, with lots of the connecting rods missing, whereas in the OVX + LF group, this loss of trabecular bone mass and connectivity was prevented (Fig. 2). Microstructure, which deteriorated substantially after OVX, was markedly improved by the treatment of LF.

Ovariectomy markedly reduced the BV/TV, Tb.Th, and Tb.N and increased the Tb.Sp of rats (Table 2). Compared with the OVX control rats, these parameters did not differ in rats treated with OVX + BSA. In contrast, LF or estrogen protected rats from the OVX-induced reduction of BV/TV, Tb.Th, and Tb.N and the elevation of Tb.Sp. Cortical thickness of tibia diaphysis did not differ among groups (data not shown).

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>OVX</th>
<th>OVX + BSA</th>
<th>OVX + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Ca, mmol/L</td>
<td>2.45 ± 0.07</td>
<td>2.25 ± 0.06</td>
<td>2.26 ± 0.03</td>
<td>2.28 ± 0.08</td>
</tr>
<tr>
<td>Serum osteocalcin, nmol/L</td>
<td>1.78 ± 0.14</td>
<td>2.19 ± 0.18</td>
<td>2.22 ± 0.21</td>
<td>2.45 ± 0.19</td>
</tr>
<tr>
<td>Urine D-Pyr/Cr, mmol/mmol</td>
<td>30.23 ± 3.84</td>
<td>50.10 ± 5.87</td>
<td>49.27 ± 5.68</td>
<td>40.06 ± 4.47</td>
</tr>
<tr>
<td>Urine Cr, mmol/L</td>
<td>5.2 ± 0.4</td>
<td>6.1 ± 0.5</td>
<td>6.4 ± 0.4</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>Urine D-Pyr, nmol/L</td>
<td>157 ± 5</td>
<td>307 ± 37</td>
<td>316 ± 40</td>
<td>228 ± 16</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 10. Means in a row with superscripts without a common letter differ, P < 0.05.
gen-dependent bone loss and biomechanical deterioration in OVX rats.

The mechanical properties of bone are influenced by bone mass as well as bone size, bone quality, and cancellous bone architecture (28). In this study, LF treatment resulted in an increase of BV/TV consistent with the improvement in BMD, suggesting it could prevent the loss of bone mass after OVX. LF administration improves trabecular architecture as it increases Tb.Th. In addition, LF seems to partially restore trabecular connectivity by increasing Tb.N and reducing Tb.Sp.

LF was superior to OVX controls at increasing maximum load in lumbar vertebrae but not very markedly in femoral diaphyses. The femoral diaphysis is mainly composed of cortical bone, whereas lumbar vertebrae are composed of both cortical and cancellous bone. Considering the previous results in this study showed no alternation in cortical thickness after LF treatment, we think that LF could be a more effective protecting agent against estrogen-dependent bone loss on the trabecular than on the cortical bone. This could also account for the discrepancy between the moderate influence of LF treatment on BMD and the marked effect on bone microstructure at 0.85 mg/kg. BMD analysis was conducted on samples formed by both cortical and cancellous bone, whereas µCT analysis was only performed on cancellous bone. These observations suggest that LF might improve bone strength by increasing both BMD and intrinsic properties of bone.

A sharp decrease in serum Ca occurred in OVX rats treated with LF with 8.5- and 85-mg/kg doses for 3 mo. Interestingly, in these rats, the serum calcitonin was elevated significantly at the same time. Calcitonin is a peptide with hypocalcemic and hypophosphatemic activities, which plays a major role in Ca homeostasis by inhibiting osteoclast-mediated bone resorption through the regulation of both the number and activity of osteoclasts and by increasing urinary Ca excretion (29). It is likely that the elevated production of calcitonin by LF reduced the Ca release from the skeleton via osteoclastic bone resorption, which in turn accounts for the decrease of serum Ca. However, other factors are likely to be involved here, because higher concentrations of calcitonin were observed in E2-replete treatment groups, but the serum Ca concentration was within the normal range. This was reasonable because estrogen has a physiological role in promoting intestinal Ca absorption by upregulating the intestinal Ca transporter genes (30), but LF does not. These results suggest that if LF were put into clinical use, it should be given in combination with a Ca supplement to avoid hypocalcemia. LF may have a significant, although narrow, therapeutic window in terms of bone efficacy compared with hypercalcemia. Because urine Ca is a more sensitive parameter for changes in Ca homeostasis, it will need to be assessed in future studies to determine this therapeutic window.

To address the issue of the mechanisms involved, we measured markers of bone remodeling such as osteocalcin and D-Pyr. To the OVX control group, the bone resorption marker D-Pyr was elevated without a commensurate change of the bone formation marker osteocalcin. This could be interpreted as an uncoupling between resorption and formation, leading to accelerated bone loss. LF may stimulate bone formation and attenuate bone resorption, as indicated by the substantial increase in osteocalcin and the accompanying decrease in D-Pyr. In addition, we measured 2 osteoclastogenic cytokines and found that LF downregulated the production of TNFα and IL-6.
under our experimental conditions. TNFα, along with IL-6, plays a critical causal role in the rapid bone loss associated with estrogen deficiency. They promote bone resorption by affecting osteoclast differentiation and activity (31,32). Results of previous work suggested that orally administered bovine Lactoferrin (bLF) induced both an intestinal mucosal and systemic immune response in mice (33,34) and decreased serum TNFα and IL-6 concentrations (35–37) and spontaneous production of TNFα and IL-6 by peripheral blood cells in humans (22). Our results agree well with these previous reports, indicating that this combination of marker and cytokine changes may be the underlying mechanism for the observation of the BMD increase and bone architectural modifications in LF-treated rats.

So far, most of the published studies about LF and bone properties come from Cornish et al. (16–20). Studies on the LF and bone carried out in those set of experiments allowed direct contact of LF with the bone cells (16–21). Because bLF was not detectable in the portal blood of mice given oral bLF at a dose of 300 mg/kg (38), it was thought that LF would be less likely to be transported from the intestine into the blood and interact with the bone-related cells directly when administered orally.

As reported, ingested LF was not extensively degraded and a substantial amount of functional fragments survived proteolytic degradation in the small intestine (39). Among these functional fragments, the most promising is Lfcin. It is a peptide released through acidic pepsin hydrolysis containing the N-terminal sequence of LF. Lfcin encompasses a large portion of the functional domain of the intact LF and it not only retains the activities of LF, but in many cases is more potent than the parent protein. Substantial levels of Lfcin generated from ingested LF in the human stomach (40) and surviving in the small adult rats’ intestine (39) have also been documented.

To investigate the osteogenic function of degraded LF in the digestive system when administered orally, we evaluated the osteoblast-stimulating ability of LFH and Lfcin in vitro. It was proven that even when digested, peptides of LF still exhibited osteogenic properties. And Lfcin may be the main provider of osteogenic activity in LHF. Supposing that there is some absorption of LF fragments, especially Lfcin, through the gut wall into the circulation, interactions between the LF fragments that had survived digestion and bone cells could have some relevance in terms of the osteogenic function of oral LF in addition to indirect effects via cytokines.

In summary, this study showed that orally administered LF prevented bone loss of both femoral and lumbar vertebrae, preserved trabecular microarchitecture, and improved bone biomechanical properties in estrogen-deficient rats. In addition, LF reduces body weight and, to some degree, without affecting the uterus. Furthermore, LF decreased serum Ca, TNFα, and IL-6 but elevated calcitonin production. Its effects on the activity and development of bone cells may be indirect and mediated by its peptides surviving in the intestine. Therefore, LF might be a safe and orally active protein for prevention and treatment of estrogen-dependent bone loss.

Acknowledgments
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Literature Cited


