Alfacalcidol Enhances Collagen Quality in Ovariectomized Rat Bones

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Received 14 May 2013; accepted 10 April 2014
Published online 9 May 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22642

ABSTRACT: The aim of this study was to investigate the effects of alfacalcidol (1α(OH)D3: ALF) on bone collagen employing an ovariectomized rat model. Thirty-five 16-week-old female Sprague-Dawley rats were divided into five groups: SHAM (sham-operated + vehicle), OVX (ovariectomy + vehicle), and three ALF-treated groups, that is, ovariectomy + 0.022 μg/kg/day ALF, ovariectomy + 0.067 μg/kg/day ALF, and ovariectomy + 0.2 μg/kg/day ALF. After 12 weeks of treatment, tibiae were subjected to histological, biochemical and immunohistochemical analyses. Collagen matrices in OVX bone appeared as immature and poorly organized; however, with the ALF treatment, it was improved in a dose-dependent manner. Contents of collagen and pyridinoline cross-link were decreased in OVX compared with SHAM, but they increased to the level comparable to SHAM in ALF-treated groups. The total aldehyde, that is, a sum of free and those involved cross-links, in the highest dose of ALF was significantly higher than the rest of the groups (p < 0.05). In addition, the expression of lysyl oxidase was increased in the all ALF-treated groups compared with OVX (p < 0.05). In conclusion, ALF increases not only the amount of collagen but also enhances the maturation of collagen in ovariectomy-induced osteoporotic bones, which likely contributes to the improvement of bone quality. © 2014 The Authors. Journal of Orthopaedic Research. Published by Wiley Periodicals, Inc. J Orthop Res 32:1030–1036, 2014.

Keywords: vitamin D; alfacalcidol; collagen; collagen cross-link; ovariectomized rats

Bone is a complex mineralized tissue, the principal function of which is to resist mechanical forces. Bone strength depends not only on the quantity of bone tissue but also on its quality, which is characterized by the geometry and shape of bones, the microarchitecture of the trabecular bones, the mineral deposition, and the collagen quality.1–3 Fibrillar type I collagen, the predominant organic matrix component in bone, functions as a 3-dimensional stable template to spatially regulate mineralization.4 Crucial to this function is the formation of covalent intermolecular collagen cross-linking as it is the basis for the stability of the fibrils. The type and quantity of collagen cross-links are primarily determined by the extent of two post-translational modifications, that is, hydroxylation and oxidative deamination of the specific lysine (Lys)/hydroxyllysine (Hyl) residues, catalyzed by lysyl hydroxylases (LHs; LH1-3) and lysyl oxidases (LOXs: LOX, LOXL1-4), respectively.5 The maturation of collagen cross-links could be regulated by the type and extent of glycosylation of the helical cross-linking Hyl residues.6 The specific cross-linking pattern appears to be critically important for bone mineralization as indicated by in vitro studies17 and its association with Bruck syndrome, a rare form of osteogenesis imperfecta with congenital contractures.8

1α,25(OH)2D3, the biologically most active hormonal form of vitamin D3, plays critical roles in many physiological processes including calcium homeostasis, bone metabolism, normal function of immune system, and cancer prevention.9 The overall positive effect of vitamin D3 on bone physiology has been known for a century, since it prevents and cures rickets and osteomalacia.10 Alfacalcidol (ALF), 1α(OH)D3, is a well established and effective prodrug of 1α,25(OH)2D3.11–16 Since it is readily converted to the latter without the tightly regulated activation process in the kidney,17,18 it has been widely used to treat various metabolic bone diseases.10,19,20 Though type I collagen gene is considered as one of the 1α,25(OH)2D3 responsive genes in osteoblasts derived from various species,21–24 it also regulates the post-translational modifications of collagen in an osteoblastic cell culture system.25 However, the direct role of vitamin D3 on bone collagen post-translational modifications and collagen maturity in vivo is still not well understood. The objective of this study was to investigate the effects of various doses of ALF on the tibiae of ovariectomized rats, focusing on the quality and quantity of bone collagen.

MATERIALS AND METHODS

Animal Experiments

The protocol for the experiment was approved by the Institutional Animal Care and Use of Teijin Pharma Ltd., and conducted in accordance with accepted standards of humane animal care. Thirty-five 10-week-old female Sprague-Dawley rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were housed in individual cages in a thermo neutral environment (24 ± 2°C, humidity 55 ± 15%) maintained on a 12-h light-dark cycle throughout the study (light on at 6 a.m. and off at 6 p.m.). All animals received ad libitum access to water and standard laboratory chow (1.25% calcium, 1.06% phosphate, CE-2, Clea Japan, Inc. Tokyo, Japan) for the first 2 weeks to allow acclimation to the new environment. Rats

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Grant sponsor: NIH-NIDCR/NIAMS (NIH-R21); Grant number: DE020909, AR060978.

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undertook either ovariectomy or sham-operation under anesthesia with sodium pentobarbital (Kyoritsu Seiyaku, Co. Ltd., Tokyo, Japan) at 12 weeks of age. At 4 weeks post-surgery, rats were divided into the following five groups:

1. **SHAM** (*n* = 7): sham-operated rats treated with Vehicle (Saline/0.2% Triton X-100)
2. **OVX** (*n* = 7): ovariectomized rats treated with Vehicle
3. 0.022 g ALF (*n* = 7): ovariectomized rats treated with 0.022 mg/kg/day
4. 0.067 g ALF (*n* = 7): ovariectomized rats treated with 0.067 mg/kg/day
5. 0.2 g ALF (*n* = 7): ovariectomized rats treated with 0.200 mg/kg/day

Each drug was administered orally five times a week for 12 weeks and the animals were sacrificed on the day after finishing drug administration (28 weeks of age).

**Serum Biochemistry and Bone Mineral Density (BMD) of Lumbar Vertebrae**

Upon sacrifice, blood (*n* = 7) was collected via the abdominal aorta to measure serum calcium (Ca, mg/dL) concentrations with a biochemistry automatic analyzer (Clinical analyzer 7180; HITACHI, Ltd., Tokyo, Japan). At 12 weeks after drug administration, the BMD (g/cm²) of the fourth and fifth lumbar vertebrae (L4, L5) (*n* = 7) were measured by dual-energy X-ray absorptiometry (QDR-2000, Hologic, Inc., Bedford, MA) under inhalation anesthesia with fluothane (Takeda Pharmaceuticals, Co. Ltd., Osaka, Japan).

**Picrosirius-Red (PSR) Staining**

To investigate the organization and maturation of bone collagen matrices in the SHAM, OVX, and ALF-treated groups, the tissue was collected and demineralized with 0.5 M of ethylenediamine-tetraacetic acid (EDTA) (pH 7.4) for 2 weeks, embedded in paraffin and sectioned into 5 µm thick slices. After hydration, the slides were stained with 0.1% solution of Sirius Red in saturated aqueous picric acid (Electron Microscopy Sciences, Hatfield, PA) for 30 min, washed with 0.01 N HCl, dehydrated and mounted. The proximal metaphyses of tissue were observed under a polarized light microscopy (BX40 microscope, Olympus Co., Center Valley, PA) and photographed as previously reported.

**Preparation of Bone Sample for Biochemical Analyses**

Approximately one third of the proximal metaphyses of tibia (*n* = 5) was dissected and soft tissues including periosteum and bone marrow were removed. The samples were pulverized to a fine powder under liquid N2 using a Spex Freezer Mill (Spex, Inc., Metuchen, NJ). Pulverized samples were washed with cold phosphate buffered saline (PBS) (pH 7.4), then with cold double distilled water several times and lyophilized. Aliquot of pulverized bone sample was demineralized with 0.5 M of EDTA for 2 weeks with several changes of EDTA, washed with cold distilled water exhaustively and lyophilized.

**Amino Acid and Cross-Link Analyses**

Two to three milligrams of demineralized collagen were reduced with standardized NaB₃H₄, hydrolyzed with 6 N HCl in vacuo, after flushing with N₂ gas, for 22 h at 105°C. An aliquot of each hydrolysate was subjected to amino acid analysis on a high-performance liquid chromatography (HPLC) system (Prostar 240/310, Varian, Walnut Creek, CA) with a strong cation exchange column (AA-911, Tranagenomic, San Jose, CA). The amount of collagen relative to total proteins was calculated as residues of hydroxyproline (Hyp) per 1,000 amino acids. The extent of Lys hydroxylation of collagen was determined using analysis of variance (ANOVA) and Fisher’s PLSD. A p value of less than 0.05 was considered significant.
RESULTS

Serum Calcium Concentrations and BMD of Lumbar Vertebra

ALF treatments raised the serum Ca levels in a dose-dependent manner. Serum Ca level in OVX (9.63 ± 0.24 mg/dL) was significantly decreased compared with other groups (*p < 0.01). With the treatment of ALF at 0.067 μg/kg/day, the serum Ca level (10.18 ± 0.32 mg/dL) was essentially the same as SHAM (10.44 ± 0.28 mg/dL), and the serum Ca level in the 0.2 μg ALF group (11.05 ± 0.31 mg/dL) was significantly higher than that in SHAM (*p < 0.05) (Table 1).

In OVX, the BMD mean values of the lumbar vertebrae (L4, L5) (0.200 ± 0.014 g/cm²) were significantly decreased compared with those in SHAM (0.249 ± 0.017 g/cm²) at 16 weeks after surgery (*p < 0.001). In the ALF-treated groups, the BMD values increased in a dose-dependent manner, and in the 0.067 μg and 0.2 μg ALF groups, the BMD values (0.234 ± 0.017 g/cm² and 0.248 ± 0.022 g/cm², respectively) were significantly higher than those of OVX (*p < 0.01) and comparable to those of SHAM (Table 1).

Table 1. Effect of ALF on Serum Ca and BMD at the Lumbar Vertebra (L4, L5)

<table>
<thead>
<tr>
<th></th>
<th>Serum Ca (mg/dL)</th>
<th>BMD (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>10.44 (0.28)</td>
<td>0.249 (0.017)</td>
</tr>
<tr>
<td>OVX</td>
<td>9.63 (0.24)</td>
<td>0.200 (0.014)</td>
</tr>
<tr>
<td>0.022 μg ALF</td>
<td>10.09 (0.29)</td>
<td>0.215 (0.010)</td>
</tr>
<tr>
<td>0.067 μg ALF</td>
<td>10.18 (0.32)</td>
<td>0.234 (0.017)</td>
</tr>
<tr>
<td>0.2 μg ALF</td>
<td>11.05 (0.31)</td>
<td>0.248 (0.022)</td>
</tr>
</tbody>
</table>

The average values (n=7) and SDs are shown. BMD, bone mineral density. *p < 0.05, **p < 0.001, different from the value of SHAM. *p < 0.01, different from the value of OVX. #p < 0.01, different from the value of 0.2 μg ALF.

Histological Evaluation by PSR Staining

Figure 1 shows the representative images of the proximal metaphysis of tibia stained with PSR observed under polarized light. In SHAM (Fig. 1A), bone collagen matrix stained yellow to red while that of OVX (Fig. 1B) stained green to yellow. In the ALF-treated groups (Fig. 1C–E), the color changed from green to yellow and red in a dose dependent-manner. The collagen matrices at the 0.2 μg ALF group (Fig. 1E) appeared to be well organized a finding similar to SHAM.

Collagen Content and the Extent of Lys Hydroxylation

In OVX, the collagen content per protein (residue of Hyp/1,000 total amino acids) was significantly lower than those of SHAM and any of the ALF-treated groups (*p < 0.001). The Hyp contents of the ALF-treated groups, even those treated with the lowest dose of ALF (0.022 μg/kg/day), were all comparable to those of SHAM showing no significant difference among the groups (Table 2). The extent of Lys hydroxylation in collagen (Hyl residues/300 residues of Hyp) in OVX was higher than any other group (*p < 0.05), and those of the 0.067 and 0.2 μg ALF groups were comparable with SHAM (Table 2).

Collagen Cross-Linking

Three reducible compounds associated with collagen cross-links (DHNL: Reduced Hylald, DHLNL: Hylald X Hyl and HLNL: Hylald X Lys or Lysald X Hyl), and two non-reducible mature cross-links (Pyr: Hylald X Hylald X Hyl and d-Pyr: Hylald X Hylald X Lys) were identified in all samples. The results of collagen cross-link analyses are summarized in Table 3. DHNL in the 0.2 μg ALF group was significantly increased compared with DHNL in SHAM, OVX and 0.022 μg ALF groups.
The average values ($n=5$) and SDs in Hyp/1,000 are shown as relative amounts in 1,000 total residues. The average values ($n=5$) and SDs in Hyl/collagen are shown as moles/mole of collagen. Hyp, hydroxyproline; Hyl, hydroxylysine. $p<0.05$, $^{**}p<0.001$, different from the value of SHAM. $p<0.05$, $^{*}p<0.01$, and $p<0.005$, different from the value of OVX.

LOX Expression in Tibia

Figure 2A–E shows representative images of immunostaining for LOX in SHAM, OVX, and ALF-treated groups. No immunoreactivity was observed in the respective negative controls (data not shown). The results demonstrated that the levels of LOX protein in tibiae in the ALF-treated groups were increased when compared with those in OVX, and this was confirmed by quantitative analysis of the intracellular expression of LOX in osteocytes ($p<0.05$) (Fig. 2F). The intensity of extracellular LOX staining also appeared to be significantly greater in the ALF-treated groups (Fig. 2C–E) when compared with that in OVX (Fig. 2B).

DISCUSSION

ALF has been widely used for the treatment of osteoporosis in certain countries. The effect of this compound on bone formation has also been investigated in osteoporotic and bone fracture animal models. In our previous study using an osteoblastic cell culture system, we examined the effects of 1α,25(OH)2D3 on collagen cross-linking and related enzymes, that is, LH1-3 and LOX, LOXL1-4. Those results revealed that 1α,25(OH)2D3 directly regulated collagen cross-linking in vitro by upregulating gene expression of specific LHS and LOXs. Thus, we conducted the present study to determine the dose-effect of ALF on collagen matrix in osteoporotic bone, specifically on collagen content, collagen cross-links and collagen organization, by using an ovariectomized rat model.

The results indicated that both BMD (Table 1) and bone volume (Fig. 1) significantly diminished in OVX but gradually increased with ALF treatment in a dose-dependent manner. These are consistent with previous reports. As for the bone matrix composition, the biochemical analyses showed that bone matrix is significantly less collagenous in OVX when compared with control, indicating defective bone matrix composition in ovariectomized rats. However, with ALF treatment including the one with the lowest dose of ALF, that is, 0.022 μg/kg/day, the level of collagen composition in bone matrix became comparable to control (Table 2). This suggests that, even with a low dose of ALF treatment, the composition of newly synthesized bone matrix in OVX bone becomes comparable to normal bone in terms of the collagen content.

Then, we examined the effect of ALF on the quality of bone collagen in ovariectomized rats by histological, immunohistochemical, and biochemical analyses. The

Table 2. Collagen Amount and the Extent of Lysine Hydroxylation

<table>
<thead>
<tr>
<th></th>
<th>Hyp/1,000</th>
<th>Hyl/Collagen</th>
</tr>
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<tbody>
<tr>
<td>SHAM</td>
<td>83.5 (4.1)</td>
<td>41.5 (3.0)</td>
</tr>
<tr>
<td>OVX</td>
<td>72.2 (3.4)*</td>
<td>43.8 (3.5)*</td>
</tr>
<tr>
<td>0.022 μg ALF</td>
<td>81.9 (2.4)*</td>
<td>39.5 (2.2)*</td>
</tr>
<tr>
<td>0.067 μg ALF</td>
<td>82.2 (1.3)*</td>
<td>40.9 (1.8)*</td>
</tr>
<tr>
<td>0.2 μg ALF</td>
<td>80.5 (1.4)*</td>
<td>41.3 (2.0)*</td>
</tr>
</tbody>
</table>

The average values ($n=5$) and SDs are shown. The amounts of cross-links are expressed as moles/mole of collagen. DHNL, dihydroxynorleucine; DHLNL, dihydroxysinosorleucine; HLNL, hydroxylysinonorleucine; Pyr, pyridinoline; d-Pyr, deoxypyridinoline.

Table 3. Comparison of the Collagen Cross-Links

<table>
<thead>
<tr>
<th></th>
<th>DHNL</th>
<th>DHLNL</th>
<th>HLNL</th>
<th>Pyr</th>
<th>d-Pyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>0.088 (0.010)</td>
<td>0.863 (0.097)</td>
<td>0.297 (0.055)</td>
<td>0.207 (0.026)</td>
<td>0.155 (0.027)</td>
</tr>
<tr>
<td>OVX</td>
<td>0.090 (0.006)</td>
<td>0.890 (0.113)</td>
<td>0.301 (0.036)</td>
<td>0.175 (0.018)*</td>
<td>0.142 (0.027)</td>
</tr>
<tr>
<td>0.022 μg ALK</td>
<td>0.085 (0.018)*</td>
<td>0.836 (0.119)</td>
<td>0.306 (0.056)*</td>
<td>0.214 (0.022)*</td>
<td>0.169 (0.042)</td>
</tr>
<tr>
<td>0.067 μg ALF</td>
<td>0.099 (0.009)</td>
<td>0.878 (0.097)</td>
<td>0.305 (0.037)*</td>
<td>0.238 (0.043)*</td>
<td>0.155 (0.027)</td>
</tr>
<tr>
<td>0.2 μg ALK</td>
<td>0.105 (0.016)*</td>
<td>0.998 (0.217)</td>
<td>0.373 (0.051)*</td>
<td>0.276 (0.044)*</td>
<td>0.178 (0.030)</td>
</tr>
</tbody>
</table>

The average values ($n=5$) and SDs are shown. The amounts of cross-links are expressed as moles/mole of collagen. DHNL, dihydroxynorleucine; DHLNL, dihydroxysinosorleucine; HLNL, hydroxylysinonorleucine; Pyr, pyridinoline; d-Pyr, deoxypyridinoline. $p<0.05$, $^{*}p<0.01$, different from the value of SHAM. $p<0.05$, $^{*}p<0.01$, and $p<0.001$, different from the value of OVX. $p<0.05$, different from the value of 0.2 μg ALF.
result of PSR staining indicated that immature, poorly
organized collagen matrix seen in OVX became more
mature and organized with increasing ALF doses. This
suggests that higher doses of ALF better facilitate the
process of collagen maturation and organization in
ovariectomy-induced osteoporotic bones. The increased
levels of LOX expression in the ALF-treated groups also
support this notion, as LOX catalyzes the reaction that
initiates the process of collagen cross-linking. Since
collagen cross-linking plays crucial roles in bone forma-
tion, mineralization and maturation,1,33–35 we then ana-
alyzed collagen cross-links by biochemical means. Cross-
link analysis identified DHNL, DHLNL, HLNL, Pyr,
and d-Pyr in all groups indicating the major cross-
linking pathway to be the Hylαδ-derived, and this did
not change by ALF treatment. In OVX, the amounts of a
cross-link precursor aldehyde (DHNL) and immature
cross-links (DHLNL and HLNL) tended to increase
compared with those of SHAM though not statistically
significant. However, with the treatment of ALF at
0.2 μg/kg/day, DHNL and HLNL were higher than those
of OVX and SHAM. Recently, Saito et al. reported that
DHNLN in the femoral bone of ovariectomized rats was
significantly lower than that in sham-operated rats.31

The average values (n = 5) and SDs are shown. Total aldehyde: sum of DHNL, DHLNL, HLNL, and 2 × (Pyr+d-Pyr). Pyr, pyridinoline;
d-Pyr, deoxypyridinoline; DHNL, dihydroxylnorleucine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine. *p < 0.05,
different from the value of SHAM. *p < 0.05, a p < 0.001, different from the value of OVX. #p < 0.05, different from the values of 0.2 μg
ALF.

Table 4. The Ratio of Pyr/d-Pyr, Pyr+dPyr/DHLNL+HLNL, and Total Aldehyde

<table>
<thead>
<tr>
<th></th>
<th>Pyr/d-Pyr</th>
<th>Pyr+dPyr DHLNL+HLNL</th>
<th>Total aldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>1.354 (0.162)</td>
<td>0.720 (0.078)</td>
<td>1.972 (0.225)</td>
</tr>
<tr>
<td>OVX</td>
<td>1.252 (0.195)</td>
<td>0.662 (0.081)</td>
<td>1.915 (0.165)</td>
</tr>
<tr>
<td>0.022 μg ALF</td>
<td>1.315 (0.254)*</td>
<td>0.768 (0.084)</td>
<td>1.994 (0.270)*</td>
</tr>
<tr>
<td>0.067 μg ALF</td>
<td>1.546 (0.145)*</td>
<td>0.758 (0.101)</td>
<td>2.068 (0.186)*</td>
</tr>
<tr>
<td>0.2 μg ALF</td>
<td>1.568 (0.251)*</td>
<td>0.859 (0.160)*</td>
<td>2.383 (0.244)*</td>
</tr>
</tbody>
</table>

Figure 2. Immunohistochemical analysis for LOX. Representative distribution of LOX in the cortical bone 1.0–2.0 mm distal below
the tibial growth plate and 200 μm thickness from bone marrow side of SHAM (A), OVX (B), 0.022 μg ALF (C), 0.067 μg ALF (D), and
0.2 μg ALF (E). Arrowheads indicate the areas of strong LOX expression in osteocyte cells. Magnification: 40×. The bar represents
200 μm. (F) Quantitative analysis of LOX expression (*p < 0.05). The levels of LOX expression in tibiae in the ALF-treated groups were
increased when compared with those in OVX. The intensity of extracellular LOX staining also appeared to be significantly higher in
the ALF-treated groups (Fig. 2C–E) when compared with that in OVX (Fig. 2B).
The difference between the results of their study and the current one is not clear, but it could be due to aspects of the experimental protocol such as the timing of sacrifice, the strain of the rats used or the locations of bones analyzed. As for the mature cross-links, Pyr in OVX was significantly decreased compared with that in SHAM. Pyr in 0.067 and 0.2 μg ALF groups were significantly increased compared with that of OVX. The cross-link maturation assessed by Pyr+t-d-Pyr/DHLNL+HLNL in 0.2 μg ALF group was significantly increased compared with that in OVX and SHAM. In addition, Pyr/DHLNL in 0.2 μg ALF group was significantly increased compared with that in OVX, and the total number of aldehyde in 0.2 μg ALF group was significantly higher than in all other groups. These biochemical analyses indicated that ALF treatment increased Hyl ald-derived cross-links and the collagen maturation in ovariectomy-induced osteoporotic bone, which was consistent with our previous in vitro study. This is likely caused by increased expression/activities of LOX and, possibly, LOXL enzymes, resulting in the formation of highly cross-linked and mature collagen fibrils.

We previously reported that, among LOX family members, LOXL2 was most responsive to 1α,25(OH)2D3. It is not clear if this LOX isoform plays a role on collagen cross-linking phenotype seen in the ALF-treated groups in this study. The investigation of the level of mRNA in the tissue could provide further insight into the mechanisms of changing of cross-linking profile in vivo. We have recently reported that the pattern of glycosylation of the helical Hyl may regulate the maturation of collagen cross-links. Since the cross-link maturation is accelerated with a high dose of ALF, ALF may affect collagen glycosylation as well. In this study, we could not analyze another trivalent cross-link, pyrrole/deoxypyrrole, a condensation product of Hyl ald, Lys ald and Hyl/Lys due to its inherent lability in acid hydrolysis. Though the quantitative analysis of this cross-link is still not established, characterization of the pyrrole containing cross-linked peptides by HPLC combined with mass spectrometry could provide more comprehensive information about the effect of ALF on collagen cross-links. Further studies are warranted to address these issues. Shiaishi et al. reported the effects of ALF (0.05, 0.1, and 0.2 μg/kg) on mechanical strength at cancellous bone in lumbar vertebra and cortical bone in femoral midshaft. The results showed that bone strength in ovariectomized rats without ALF treatments decreased slightly compared with that in sham-operated rats without ALF treatments, and ALF treatment increased the bone strength of both cancellous bone in lumbar vertebra and cortical bone in a dose-dependent manner. Interestingly, even with the lowest dose of ALF (0.05 μg/kg), bone strength was significantly increased compared with that in sham-operated rats treated with vehicle. Those results are consistent with those of the current study, which demonstrate dose-dependent increases in collagen cross-links and improved collagen matrix maturation and organization by ALF.

In conclusion, our study revealed that ALF effectively normalizes the content of collagen, Lys hydroxylation of collagen, collagen cross-linking and collagen maturation/organization in bone. These data strongly indicate that ALF enhances not only collagen deposition but also the maturation and stability of collagen in osteoporotic bones, which likely contributes to the improvement of bone quality.

REFERENCES