Alfacalcidol Enhances Collagen Quality in Ovariectomized Rat Bones

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ABSTRACT: The aim of this study was to investigate the effects of alfacalcidol ($1\alpha(OH)D_3$: 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.

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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)/hydroxylysine (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 studies[1,7] and its association with Bruck syndrome, a rare form of osteogenesis imperfecta with congenital contractures.[8]

$1\alpha,25(OH)_2D_3$, the biologically most active hormonal form of vitamin D_3, 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 D_3 on bone physiology has been known for a century, since it prevents and cures rickets and osteomalasia.[10] Alfacalcidol (ALF), $1\alpha(OH)D_3$, is a well established and effective prodrug of $1\alpha,25(OH)_2D_3$.[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 $1\alpha,25(OH)_2D_3$ 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 D_3 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 ($24 \pm 2°C$, humidity $55 \pm 15\%$) 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

underwent 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 µg ALF ($n = 7$): ovariectomized rats treated with 0.022 µg/kg/day
4. 0.067 µg ALF ($n = 7$): ovariectomized rats treated with 0.067 µg/kg/day
5. 0.2 µg ALF ($n = 7$): ovariectomized rats treated with 0.200 µg/kg/day

Each drug was administrated 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/cm^2) 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 tibiae were 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 tibiae were observed under a polarized light microscopy (BX40 microscope, Olympus Co., Center Valley, PA) and photographed as previously reported.[26]

Preparation of Bone Sample for Biochemical Analyses

Approximately one third of the proximal metaphyses of tibia ($n = 5$) was dissected[27] and soft tissues including periosteum and bone marrow were removed. The samples were pulverized to a fine powder under liquid N_2 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.[28]

Amino Acid and Cross-Link Analyses

Two to three milligrams of demineralized collagen were reduced with standardized NaB^3H_4, hydrolyzed with 6 N HCl in vacuo, after flushing with N_2 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, Transgenomic, San Jose, CA).[28] 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 calculated as moles of Hyl/mole of collagen based on a value of 300 residues of Hyp per collagen molecule. The hydrolysates with known amounts of Hyp were analyzed for cross-links on the HPLC system with an AA-911 column (see above) linked to a fluorescence detector (FP1520, Jasco Spectroscopic, Tokyo, Japan) and a liquid scintillation analyzer (500TR series, Packard Instrument, Meriden, CT).[28] The cross-link precursor aldehyde, that is, Hyl aldehyde (Hyl^{ald}), the major reducible cross-links, that is, dehydrodihydroxylysinonorleucine (deH-DHLNL)/its keto amine, and dehydrohydroxylysinonorleucine (deH-HLNL)/its keto amine, were analyzed as their reduced forms, that is, dihydroxynorleucine (DHNL), dihydroxylysinonorleucine (DHLNL), and hydroxylysinonorleucine (HLNL), respectively. The non-reducible, mature cross-links, that is, pyridinoline (Pyr) and deoxypyridinoline (d-Pyr), were also analyzed simultaneously.[28] All cross-links and aldehydes were quantified as moles/mole of collagen.[28] A total number of aldehyde was calculated as a sum of DHNL, DHLNL, HLNL, and $2 \times$ (Pyr+d-Pyr). The analyses were done in five independent experiments.

Immunohistochemical Staining

To determine the relative distribution of LOX, immunohistochemical analyses were performed within the tibia paraffin sections of SHAM, OVX, and ALF-treated groups (see PSR staining, above). The endogenous peroxidase activity was quenched by incubating the slides in 0.3% H_2O_2 in methanol for 30 min. The nonspecific binding was blocked using normal goat serum for 20 min. The sections were incubated overnight at 4°C with primary antibody against LOX (IMGENEX, San Diego, CA), which was diluted 1:200 in PBS. The specificity of immunoreactivity was confirmed by using normal rabbit serum as negative controls. The sections were incubated with biotinylated rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) and then with avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Inc.). After several washes with PBS, the sections were incubated with 3, 3′-diaminobenzidine substrate (Vector Laboratories, Inc.) to visualize the immunoreactivity. The sections were observed under light microscopy (BX40 microscope, Olympus Co.). The area observed was 1.0–2.0 mm below the tibial growth plate and 200 µm thickness from the bone marrow side of cortical bone. LOX expression was evaluated by counting the number of immunopositive osteocytes/area. Data were collected from randomly selected four sections per tibial bone from two animals in each group.[29]

Statistical Analysis

All statistical evaluations were performed using Stat View software (SAS Institute Inc., Cary, NC). Values were expressed as means standard deviation in the text and the tables, and the difference (in comparisons with SHAM, OVX, 0.022 µg ALF, 0.067 µg ALF, and 0.2 µg ALF) was determined using analysis of variance (ANOVA) and Fisher's PLSD. A p value of less than 0.05 was considered significant.

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

	Serum Ca (mg/dL)	BMD (g/cm^2)
SHAM	10.44 (0.28)^a	0.249 (0.017)
OVX	9.63 (0.24)**	0.200 (0.014)**
0.022 μg ALF	10.09 (0.29)**,$^{a,\#}$	0.215 (0.010)**,$^{\$}$
0.067 μg ALF	10.18 (0.32)$^{a,\#}$	0.234 (0.017)^a
0.2 μg ALF	11.05 (0.31)*,^{a}	0.248 (0.022)^a

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. $^aP < 0.01$, different from the value of OVX. $^{\$}P < 0.05$, $^{\#}p < 0.01$, different from the value of 0.2 μg ALF.

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/cm^2) were significantly decreased compared with those in SHAM (0.249 ± 0.017 g/cm^2) 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/cm^2 and 0.248 ± 0.022 g/cm^2, respectively) were significantly higher than those of OVX ($p < 0.01$) and comparable to those of SHAM (Table 1).

### 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 Hyl^{ald}, DHLNL: Hyl^{ald} X Hyl and HLNL: Hyl^{ald} X Lys or Lys^{ald} X Hyl), and two non-reducible mature cross-links (Pyr: Hyl^{ald} X Hyl^{ald} X Hyl and d-Pyr: Hyl^{ald} X Hy^{lald} 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
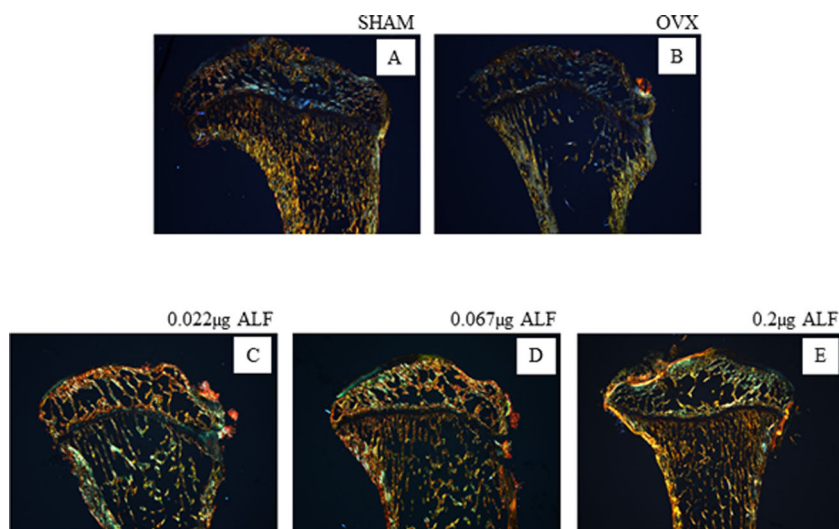
Figure 1. Representative PSR staining of proximal metaphysis of tibiae in SHAM (A), OVX (B), 0.022 μg ALF (C), 0.067 μg ALF (D), and 0.2 μg ALF (E). Bone collagen matrix stained yellow to red in SHAM (Fig. 1A) and green to yellow in OVX (Fig. 1B). In the ALF-treated groups (Fig. 1C–E), the color changed 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.

Table 2. Collagen Amount and the Extent of Lysine Hydroxylation

	Hyp/1,000	Hyl/Collagen
SHAM	83.5 (4.1)	41.5 (3.0)
OVX	72.2 (3.4)**	43.8 (3.5)*
0.022 μg ALF	81.9 (2.4)^c	39.5 (2.2)^a
0.067 μg ALF	82.2 (1.3)^c	40.9 (1.8)^a
0.2 μg ALF	80.5 (1.4)^b	41.3 (2.0)^a

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. $^a p < 0.05$, $^b p < 0.0l$, and $^c p < 0.005$, different from the value of OVX.

($p < 0.05$). DHLNL showed no significant difference among all of the groups examined. HLNL in the 0.2 μg ALF group was significantly increased when compared with HLNL in all other groups examined ($p < 0.05$). The non-reducible fluorescent cross-link, Pyr, in OVX was significantly decreased when compared with that of SHAM ($p < 0.05$), but Pyr in the 0.067 and 0.2 μg ALF groups was significantly higher than it was in OVX ($p < 0.01$, $p < 0.005$, respectively). Pyr in the 0.2 μg ALF group was significantly higher than that in SHAM ($p < 0.01$). d-Pyr showed no significant difference among any of the groups examined, though it tended to be higher in the ALF-treated groups compared with OVX. Table 4 shows the ratios of Pyr/d-Pyr (the extents of Lys hydroxylation of the cross-links), Pyr+d-Pyr/DHLNL+HLNL (the maturity of collagen cross-links) and the total aldehyde (the sum of free aldehyde and aldehyde involved in cross-links) of each group. Pyr/d-Pyr in the 0.067 and 0.2 μg ALF groups was significantly higher than in OVX ($p < 0.05$). Pyr+d-Pyr/DHLNL+HLNL in 0.2 μg ALF group were significantly increased in comparison to that in SHAM and OVX ($p < 0.05$, $p < 0.001$, respectively). In 0.2 μg ALF group, Pyr/DHLNL was also significantly higher than that in OVX ($p < 0.05$) (data not shown). The total aldehyde in 0.2 μg ALF group was significantly higher than in all other groups ($p < 0.05$).

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.[15,30–32] In our previous study using an osteoblastic cell culture system, we examined the effects of 1α,25 $(OH)_2D_3$ on collagen cross-linking and related enzymes, that is, LH1-3 and LOX, LOXL1-4. Those results revealed that 1α,25$(OH)_2D_3$ directly regulated collagen cross-linking in vitro by upregulating gene expression of specific LHs and LOXs.[25] 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.[25,31] 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 3. Comparison of the Collagen Cross-Links

	DHNL	DHLNL	HLNL	Pyr	d-Pyr
SHAM	0.088 (0.010)	0.863 (0.097)	0.297 (0.055)	0.207 (0.026)	0.155 (0.027)
OVX	0.090 (0.006)	0.890 (0.113)	0.301 (0.036)	0.175 (0.018)*	0.142 (0.027)
0.022 μg ALK	0.085 (0.018)$^#$	0.836 (0.119)	0.306 (0.056)$^#$	0.214 (0.022)$^#$	0.169 (0.042)
0.067 μg ALF	0.099 (0.009)	0.878 (0.097)	0.305 (0.037)$^#$	0.238 (0.043)$^{b,#}$	0.155 (0.027)
0.2 μg ALK	0.105 (0.016)^{*,a}	0.998 (0.217)	0.373 (0.051)^{*,a}	0.276 (0.044)^{**,c}	0.178 (0.030)

The average values ($n = 5$) and SDs are shown. The amounts of cross-links are expressed as moles/mole of collagen. DHNL, dihydroxynorleucine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; Pyr, pyridinoline; d-Pyr, deoxypyridinoline. *$p < 0.05$, **$p < 0.01$, different from the value of SHAM. $^a p < 0.05$, $^b p < 0.01$, and $^c p < 0.001$, different from the value of OVX. $^# p < 0.05$, different from the value of 0.2 μg ALF.

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

	Pyr/d-Pyr	Pyr+dPyr DHLNL+HLNL	Total aldehyde
SHAM	1.354 (0.162)	0.720 (0.078)	1.972 (0.225)
OVX	1.252 (0.195)	0.662 (0.081)	1.915 (0.165)
0.022 µg ALF	1.315 (0.254)[#]	0.768 (0.084)	1.994 (0.270)[#]
0.067 µg ALF	1.546 (0.145)[a]	0.758 (0.101)	2.068 (0.186)[#]
0.2 µg ALF	1.568 (0.251)[a]	0.859 (0.160)[*,b]	2.383 (0.244)[*,a]

The average values ($n = 5$) and SDs are shown. Total aldehyde: sum of DHNL, DHLNL, HLNL, and $2 \times$ (Pyr+d-Pyr). Pyr, pyridinoline; d-Pyr, deoxypyridinoline; DHNL, dihydroxynorleucine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine. [*]$p < 0.05$, different from the value of SHAM. [a]$p < 0.05$, [b]$p < 0.001$, different from the value of OVX. [#]$p < 0.05$, different from the values 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 formation, mineralization and maturation,[1,33–35] we then analyzed 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 Hyl[ald]-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 DHLNL in the femoral bone of ovariectomized rats was significantly lower than that in sham-operated rats.[31]
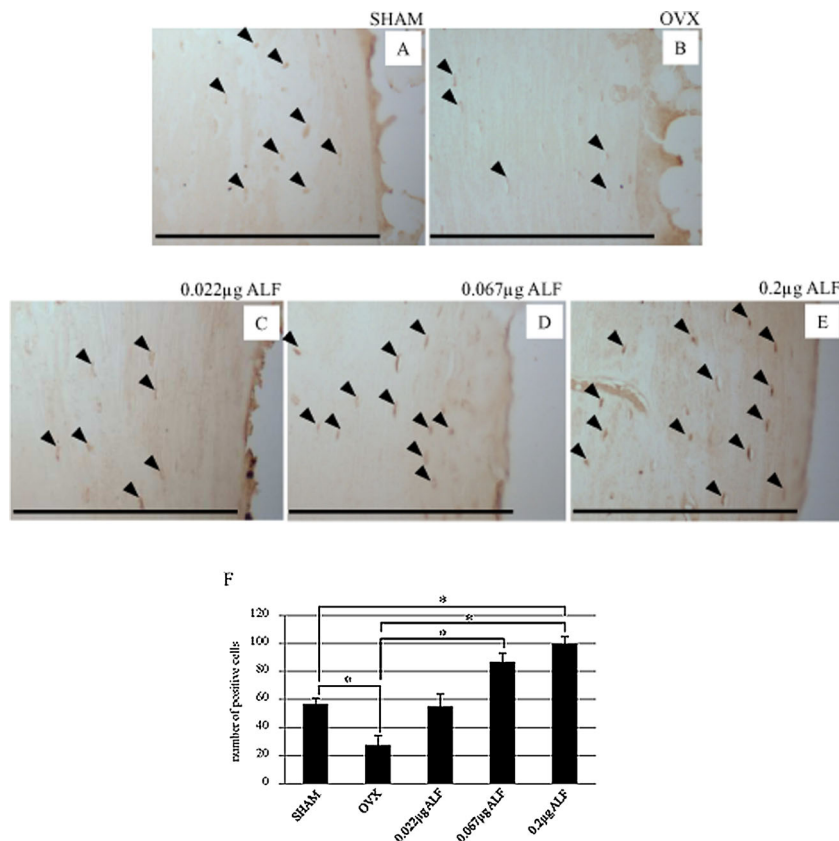
Figure 2. Immunohistochemical analysis for LOX. Representative distribution of LOX in the cortical bone 1.0–2.0 mm distal below the tibial growth plate and 200 µm thickness from bone marrow side of SHAM (A), OVX (B), 0.022 µg ALF (C), 0.067 µg ALF (D), and 0.2 µg ALF (E). Arrowheads indicate the areas 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+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.[25] 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 $1\alpha,25$ $(OH)_2D_3$.[25] 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.[6] 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[36] 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.[15] reported the effects of ALF (0.05, 0.1, and 0.2 µg/kg) on mechanical strength at cancellous bone in lumber 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 lumber 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 im-proved collagen matrix maturaedion 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.

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