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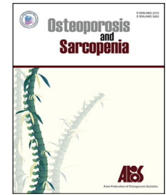

Original article

Effects of eldecalcitol and ibandronate on secondary osteoporosis and muscle wasting in rats with adjuvant-induced arthritis

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ABSTRACT

Objectives: Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovium, progressive erosion of the articular cartilage, and joint destruction. RA also causes secondary osteoporosis and muscle wasting. We investigated the effects of ibandronate (IBN), a bisphosphonate; eldecalcitol (ELD), an active vitamin D3 derivative; and combination treatment with both agents on secondary osteoporosis and muscle wasting using adjuvant-induced arthritis rats.
Methods: Arthritis was induced in 8-week-old male Lewis rats. Rats were randomized into 4 treatment groups and an untreated normal control group: IBN (subcutaneously, once every 2 weeks, 10 μg/kg), ELD (orally, once daily, 30 ng/kg/day), IBN + ELD, vehicle, and control. Paw thickness measurements were performed for evaluation of arthritis. The femur was scanned using dual-energy X-ray absorptiometry. Cross-sectional areas of left tibialis and anterior muscle fibers and the expression of MuRF1, atrogin-1, MyoD, and myogenin in the gastrocnemius muscle were measured to evaluate muscle wasting.
Results: IBN and/or ELD increased bone mineral density (BMD) in the femur. In addition, there was an additive effect of combination treatment compared with single treatments for BMD. However, IBN and/or ELD did not inhibit muscle wasting in adjuvant-induced arthritis rats.
Conclusions: Combination treatment with IBN and ELD may be effective for secondary osteoporosis associated with RA. Other treatments are necessary for muscle wasting associated with RA. Studies in humans are needed to confirm these findings.
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1. Introduction

Rheumatoid arthritis (RA) is a chronic and progressive autoimmune disease characterized by proliferative and invasive synovitis; it results in destruction of joints and erosion of cartilage and bone [1]. In addition, it has been reported that RA is often associated with periarticular or generalized osteoporosis [2,3], decreased muscle function, and low muscle mass [4,5]. Patients with RA frequently have pain and impaired muscle function, which limits physical activity and decreases quality of life [6–8]. Therefore, it is important to prevent physical destruction of joints, bone, and muscle in patients with RA.

Biologics are commonly used to treat RA. However, the effects of biologics on bone mineral density (BMD) and muscle atrophy in patients with RA are still unclear. Additional treatments for preventing or recovering bone or muscle in patients with RA are required. Bisphosphonates and active vitamin D are widely used in the treatment of secondary osteoporosis associated with RA [9]. Bisphosphonates have a high affinity for bone tissue and are incorporated into osteoclasts during bone resorption [10]; they also induce apoptosis [11]. Ibandronate (IBN), a nitrogen-containing bisphosphonate, is effective in preventing bone fragility fractures in patients with osteoporosis [12]. IBN was shown to increase lumbar and total body BMD to a greater extent than did alendronate or risedronate, which are widely used in the treatment of osteoporosis [13]. Although it has been reported that IBN suppresses muscle atrophy in animal experiments of RA [14], its effects on bone and muscle have not been completely clarified.

Vitamin D is necessary for bone health because of its role in the regulation of calcium homeostasis, and vitamin D serum levels influence an adequate response to bisphosphonate treatment [15].

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In addition, it was reported that vitamin D deficiency was common in RA patients [16]. Thus, vitamin D is important in treating secondary osteoporosis with RA. Eldecalcitol (ELD) is an active vitamin D3 derivative used for the treatment of osteoporosis in Japan [17]. It is frequently used in combination with other antiosteoporotic drugs. Sakai et al. [18] reported that treatment with the combination of IBN plus ELD was beneficial in the treatment of osteoporosis in aged postmenopausal osteopenia model rats. There are several reports of the effect of vitamin D on muscle strength and mobility in humans [19,20]. Similarly, ELD has been shown to have a positive effect on skeletal muscle in both animal and human studies [21,22]. However, the effect of ELD used as a single agent or combined with a bisphosphonate for secondary osteoporosis and muscle atrophy associated with RA is unknown.

Adjuvant-induced arthritis (AIA) is a widely used experimental animal model because it shows similarities with human RA [23,24]. After immunization, rats develop inflammation and polyarthritis that lead to decreased body weight and loss of skeletal muscle mass [25]. Muscle wasting in arthritic rats is associated with an increase in the activity of the ubiquitin-proteasome pathway, and the key enzymes in this process are two E3 ubiquitin ligases, *muscle ring finger protein-1* (*MuRF1*) and *atrogin-1* [26].

In this study, we investigated the effects of IBN, ELD, and a combination of IBN + ELD on secondary osteoporosis and muscle wasting using AIA rats.

2. Methods

2.1. Animals and experimental protocol

Eight-week-old male Lewis rats (Japan SLC, Shizuoka, Japan) were used in this study. Adjuvant arthritis was induced by subcutaneous injection of a prepared suspension of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI, USA) in paraffin oil (5 mg/mL) into the footpad of the right hind paw (50 μL). Normal untreated rats were used as a control (CON) group. From day 21 after adjuvant injection, the CON and AIA rats were divided into 5 groups (n = 10 in each group): (1) CON group, normal control rats for the AIA group; (2) Vehicle group, AIA control rats treated with isotonic sodium chloride solution (Otsuka Pharmaceutical Factory, Tokushima, Japan) for vehicle of IBN and medium-chain triglyceride (The Nisshin Oillio Group, Tokyo, Japan) for vehicle of ELD; (3) IBN group, AIA rats treated with IBN (subcutaneously, once every 2 weeks, 10 μg/kg) (Bonviva, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan); (4) ELD group, AIA rats treated with ELD (orally, once daily, 30 ng/kg/day) (Chugai Pharmaceutical Co., Ltd.); and (5) IBN + ELD group, AIA rats treated with a combination of IBN and ELD. The dose of IBN or ELD was based on previous studies [18,27], in which IBN and ELD elevated femoral and lumbar spinal BMD in ovariectomized rats. Rats were allowed ad libitum access to tap water and commercial standard rodent chow (CE-7; Clea Japan, Tokyo, Japan), and housed in a controlled environment (temperature 23 ± 2 °C, humidity 40 ± 20%) with a 12-h light-dark cycle. After 2 or 4 weeks treatment, rats were euthanized by an injection of sodium pentobarbital (150 mg/kg body weight, intraperitoneally) (Nembutal, Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan).

2.2. Tissue preparation

Left femurs were harvested and used for measurement of BMD by dual-energy X-ray absorptiometry (DXA). Left tibialis anterior (TA) muscles were harvested and stored in liquid nitrogen for measurement of cross-sectional areas (CSAs), and left gastrocnemius muscles were harvested and stored in RNAlater solution (Qiagen, Hilden, Germany) at −80 °C for real-time polymerase chain reaction (PCR).

The Animal Research Committee of our institute approved the protocol for all animal experiments, and all animal experiments adhered to the "Guidelines for Animal Experimentation" of the university.

2.3. Body weight

Body weight was measured at the beginning and end of the experiment (Keimaiko; Yamato-scale, Hyogo, Japan).

2.4. Clinical measurements of arthritis by paw thickness

Clinical signs of arthritis in each hind paw were assessed once weekly by investigators blind to the treatment group, as previously described [28,29]. Hind footpad width was also measured once weekly with calipers (Dial Thickness Gauge; Ozaki Mfg. Co., Ltd., Tokyo, Japan) [30].

2.5. BMD measurement

BMD of the total femur was measured using DXA (QDR-4500 Delphi; Hologic, Bedford, MA, USA). Results were used to assess secondary osteoporosis.

2.6. Histological analysis of muscle

Left TA muscles in rats in the 5 groups that were treated for 4 weeks (n = 5) were analyzed histologically. Samples were cut into 10-μm thick transverse serial sections at the thickest part of the muscle belly, with the cryostat maintained at −18 °C. Sections were stained histochemically with hematoxylin and eosin. To measure CSAs of muscle fibers, microscopic images at a magnification of 200× were captured digitally (Facescope IIPS20; Alfabio, Gunma, Japan), and individual muscle fibers were traced on-screen using ImageJ image analysis software (National Institutes of Health, Bethesda, MD, USA). Areas were calculated using ImageJ software based on a calibrated pixel-to-actual size (micrometer) ratio. Fifty fibers per muscle were randomly chosen, muscle fiber CSA was measured, and mean CSA for one muscle fiber was calculated.

With this method, intraobserver variation, as assessed by the coefficient of variation for 3 corresponding measurements in 50 randomly selected fibers, ranged from 0.1% to 1.2%. Interobserver variation between the three investigators, as assessed by the coefficient of variation of measurements in 50 randomly selected images, ranged from 3.7% to 8.5%.

2.7. Gene expression analysis of skeletal muscle

Gastrocnemius muscles were isolated from the tibias of rats from all groups treated for 2 and 4 weeks (n = 5). We evaluated the gene expression of *MyoD*, and *myogenin* as muscle anabolic markers and *atrogin-1* and *MuRF1* as muscle catabolic markers. Samples were crushed with a homogenizer (MS-100R; Tomy, Tokyo, Japan). Total RNA was collected from tissue using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The final concentration of RNA was determined with NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA synthesis was performed using the First-Strand cDNA Synthesis Kit (GE Healthcare, Milwaukee, WI, USA). Quantitative reverse-transcription PCR was done using Light Cycler 480 (Roche, West Sussex, United Kingdom) according to the manufacturer's protocol, with TaqMan probes specific for rat *MyoD* (TaqMan probe ID: Rn01457527_g1), *myogenin* (TaqMan probe ID: Rn01490689_g1), *atrogin-1* (TaqMan probe ID: Rn00591730_m1), and *MuRF1*

(TaqMan probe ID: Rn00590197_m1).

Amplification of glyceraldehyde3-phosphate dehydrogenase was used as an internal control for sample normalization (TaqMan probe ID: Rn01775763_g1).

2.8. Statistical analyses

Statistical analyses were performed using the Statistical Package for the Biosciences software ver. 9.6 (Akita University, Akita, Japan) [31]. Continuous variables were expressed as mean ± standard deviation. Differences between groups at each time point were evaluated using one-way analysis of variance (ANOVA). Multiple comparisons were made using Scheffe and Dunn *post hoc* tests, as appropriate. Nonparametric data, including skeletal muscle gene expression, was analyzed by Dunn method. Parametric data were analyzed by Scheffe method. Skeletal muscle gene expression was analyzed by the Wilcoxon rank-sum test based on the data of control rats at 2 weeks. Data were analyzed by 1-way ANOVA followed by a least significant difference test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Body weight and arthritis

The body weight of rats in the CON group was significantly higher than rats in the AIA groups (Vehicle, IBN, ELD, and IBN + ELD group) at the beginning and end of the experiment ($P < 0.05$). However, no significant differences in weight were seen among any of the AIA groups (Table 1).

The paw thickness of rats in the AIA groups was significantly greater than rats in the CON group at the end of 2 and 4 weeks. However, there were no significant differences in the paw thickness of the AIA groups at the 2 and 4 weeks (Fig. 1).

3.2. Bone mineral density

At the end of 2 and 4 weeks, total femoral BMD was significantly decreased in the AIA groups (Vehicle, IBN, ELD, and IBN + ELD groups) compared with the CON group ($P < 0.01$).

IBN and/or ELD treatment for 2 weeks significantly increased the BMD of total femur compared with that of vehicle group ($P < 0.05$). At 4 weeks, an additional increase was observed at the total femoral BMD in the IBN + ELD group, compared with IBN alone or ELD alone groups ($P < 0.05$) (Table 2).

3.3. Cross-sectional area

The CSA of TA muscle fibers of rats in the CON group was significantly greater than rats in the AIA groups at the end of 2 and 4 weeks. However, no significant differences in the CSA of TA muscle fibers were seen among the AIA groups at the both of 2 and 4 weeks (Table 3).

3.4. Gene expression analysis of skeletal muscle

Two weeks of treatment with ELD alone and IBN + ELD showed significantly higher mRNA expression of *MuRF1* ($P < 0.05$) compared with the CON group (Fig. 2B). IBN and/or ELD treatment did not affect mRNA expression of *atrogin-1* at 2 or 4 weeks (Fig. 2A, C).

On the other hand, in regard to the myogenic regulatory factors, AIA rats (Vehicle group) revealed significantly higher mRNA expression levels of *MyoD* at 2 and 4 weeks compared with the CON group ($P < 0.05$) (Fig. 3A, C). Two weeks' treatment with IBN and 4 weeks' treatment with ELD showed significantly higher *MyoD* expression compared with the CON group ($P < 0.05$) (Fig. 3A, C). *Myogenin* mRNA expression levels in the IBN, ELD, and IBN + ELD groups at 2 weeks and IBN group at 4 weeks were significantly higher than levels in the CON group ($P < 0.05$) (Fig. 3B, D).

However, there were no significant differences in the muscle anabolic (*MyoD* and *myogenin*) and catabolic markers (*MuRF1* and *atrogin-1*) among the AIA groups at both 2 and 4 weeks (Figs. 2 and 3).

4. Discussion

In the present study, we demonstrated that IBN and/or ELD increased BMD in the femur, whereas IBN and/or ELD did not inhibit muscle wasting in AIA rats.

There are several reports on combination therapy with IBN and ELD. Sakai et al. [18] reported that treatment with IBN + ELD had a synergistic effect on inhibition of bone resorption without suppressing bone formation in ovariectomized rats. Takada et al. [32] reported that IBN + ELD for 6 months significantly improved bone strength of the proximal femur in postmenopausal women with osteoporosis. Bisphosphonates augment BMD through the inhibition of osteoclast activity [33]. Third-generation nitrogen-containing bisphosphonates, such as IBN, inhibit farnesyl pyrophosphate synthetase in the mevalonate pathway in osteoclasts and induce apoptosis of osteoclasts, thereby inhibiting osteoclastic activity [11]. On the other hand, it was reported that ELD increased BMD by suppressing the expression of receptor activator of nuclear factor-kappa B ligand in bone tissue, a stimulator of osteoclast differentiation and bone resorption [34]. This difference in mechanism of action on osteoclasts might be the reason for the additive effect of ELD + IBN on the increase in BMD.

AIA rats revealed significantly higher mRNA expression levels of *MyoD* at 2 and 4 weeks compared with the CON group. Impaired skeletal muscle regeneration affects muscle atrophy, and *MyoD* and *myogenin* are important factors in muscle regeneration. Following damage in adult skeletal muscle, satellite cells start to proliferate and differentiate, and expression of *MyoD* and *myogenin* increases [35]. Expression of *MyoD* is related to activation and proliferation of satellite cells, whereas *myogenin* reflects terminal myoblast differentiation [36]. Castillero et al. [37] reported that AIA increases the expression of *MyoD* and *myogenin* in rats. The increased expression of *MyoD* was considered to reflect stimulated muscle regeneration in AIA rats.

In the skeletal muscle, when muscle satellite cells differentiate and proliferate, and damaged muscles are regenerated, several myogenic regulatory factors are involved, such as *MyoD* and *myogenin* [36]. An in vitro study showed that ELD upregulated gene expression of vitamin D receptors, *MyoD*, insulin-like growth factor, and myosin heavy chain subtypes in differentiated C2C12

Table 1
Body weight (g) in each group at 2 and 4 weeks.

	CON	Vehicle	IBN	ELD	IBN + ELD
2 Weeks					
Start	258 ± 61	199 ± 7*	202 ± 12*	199 ± 9*	195 ± 11*
End	291 ± 11	228 ± 12*	223 ± 12*	212 ± 11*	209 ± 12*
4 Weeks					
Start	269 ± 11	193 ± 9*	191 ± 6*	198 ± 12*	203 ± 11*
End	321 ± 16	246 ± 13*	245 ± 11*	235 ± 18*	237 ± 12*

n = 10 per group.
Values are presented as mean ± standard deviation.
CON, control; IBN, ibandronate; ELD, eldecalcitol; Start, beginning of drug administration; End, at sacrifice.
*$P < 0.05$ vs. CON by Scheffe method.

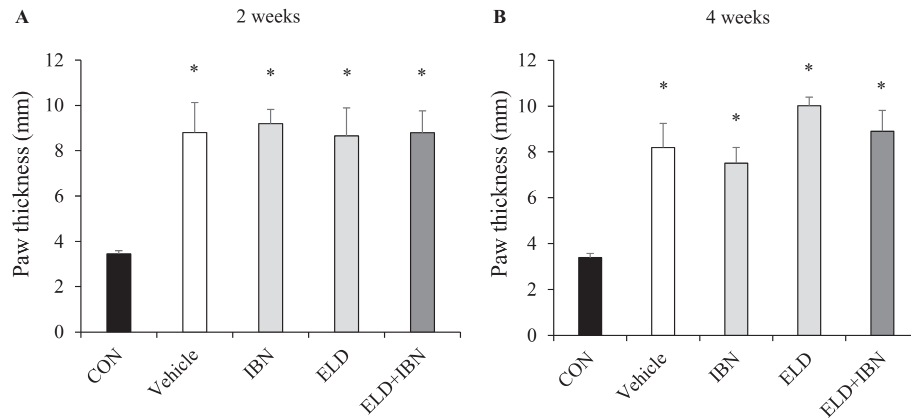

Fig. 1. Paw thickness. Paw thickness at 2 weeks (A) and 4 weeks (B). n = 10 per group. Values are presented as mean ± standard deviation. CON, control; IBN, ibandronate; ELD, eldecalcitol. *P < 0.05 vs. CON by Scheffe method.

Table 2
Bone mineral density (g/cm^2) of total femur in each group at 2 and 4 weeks.

	CON	Vehicle	IBN	ELD	IBN + ELD
2 Weeks	0.230 ± 0.009	0.165 ± 0.013*	0.181 ± 0.007*,†	0.189 ± 0.010*,†	0.197 ± 0.010*,†,‡
4 Weeks	0.246 ± 0.009	0.153 ± 0.010*	0.178 ± 0.006*,†	0.186 ± 0.010*,†	0.202 ± 0.012*,†,‡,¶

n = 10 per group.
Values are presented as mean ± standard deviation.
CON, control; IBN, ibandronate; ELD, eldecalcitol.
*P < 0.05 vs. CON by Scheffe method.
†P < 0.05 vs. Vehicle by Scheffe method.
‡P < 0.05 vs. IBN by Scheffe method.
¶P < 0.05 vs. ELD by Scheffe method.

Table 3
Cross-sectional areas of tibialis anterior muscle fibers (μm^2).

	CON	Vehicle	IBN	ELD	IBN + ELD
2 Weeks	3682.1 ± 586.5	1091.7 ± 177.6*	1230.1 ± 175.7*	1304.0 ± 105.1*	1476.7 ± 417.0*
4 Weeks	3655.3 ± 781.4	1192.4 ± 262.9*	1317.8 ± 201.0*	1385.1 ± 466.7*	1336.1 ± 342.6*

n = 5 per group.
Values are presented as mean ± standard deviation.
CON, control; IBN, ibandronate; ELD, eldecalcitol.

myoblasts [38]. Kinoshita et al. [21] reported that ELD treatment increased TA muscle type II fiber CSA and improved muscle strength by inducing higher expression of *myogenin* in the glucocorticoid-induced myopathy rat model. Based on these results, we expected that IBN and/or ELD treatment regenerated muscle atrophy in the AIA rats by stimulating the expression of myogenic regulatory factors such as *myogenin* and *MyoD* at the beginning of the experiment. However, muscle atrophy was not improved by administration of IBN and/or ELD, and there were no significant differences in the expression of the anabolic skeletal muscle genes (*MyoD* and *myogenin*) among the AIA rats. This might be caused by the severe muscle atrophy in AIA rats or a lower dose and shorter duration of IBN and ELD treatment in the present study.

MuRF1 and *atrogin-1* are key enzymes in the ubiquitin-proteasome proteolytic pathway [26,39]. In addition, inflammatory cytokines such as tumor necrosis factor-α and IL-6 promote NFκB signaling and induce muscle protein degradation and apoptosis [40]. Granado et al. reported that increased expression of *MuRF1* and *atrogin-1* was involved in muscle wasting in AIA rats [26], and Watanabe et al. reported that IBN treatment significantly inhibited skeletal muscle atrophy by suppressing the expression of atrogenes (*atrogin-1* and *MuRF1*) in denervation-induced immobilization model mice [14]. Thus, we hypothesized that IBN and/or ELD administration would suppress the expression of atrogenes such as *MuRF-1*. However, instead, ELD and IBN + ELD stimulated the expression of *MuRF-1* in AIA rats. Although there are several reports that administration of active vitamin D decreased food intake and increased expression of *MuRF1* in rats [41,42], further investigation is needed regarding the effects of IBN and/or ELD on atrogenes.

5. Conclusions

In conclusion, IBN and/or ELD treatment increased femoral BMD in AIA rats. However, IBN and/or ELD treatment did not inhibit muscle wasting. These results suggest that combination treatment of IBN and ELD may be effective for secondary osteoporosis associated with RA, but another treatment might be necessary for muscle wasting associated with RA. Studies in humans are needed to confirm these findings.

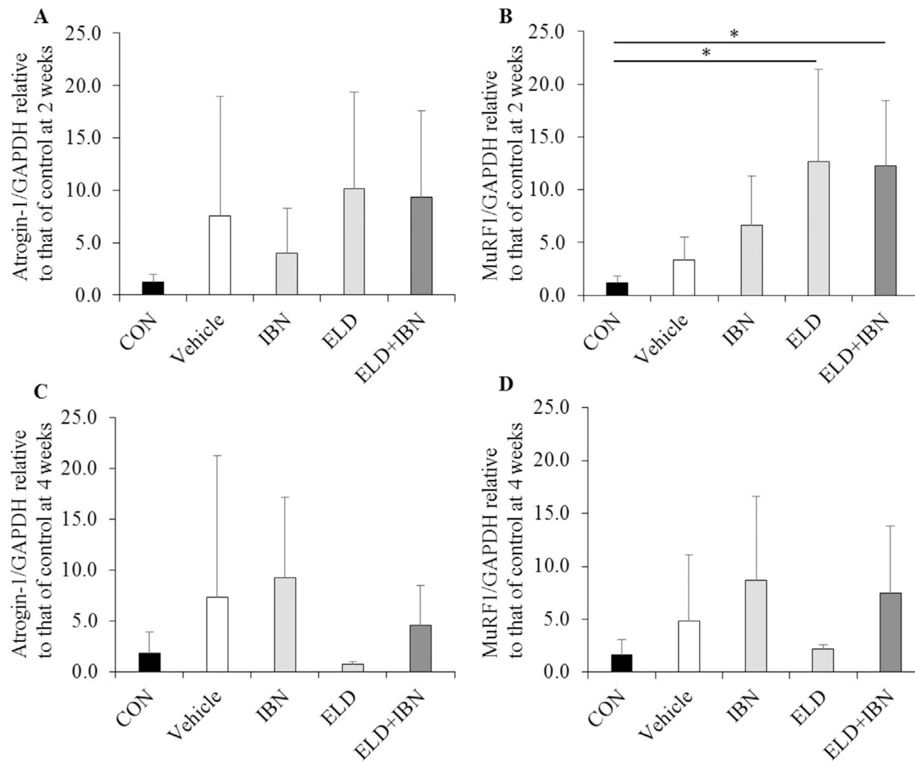

Fig. 2. Muscle catabolic markers. Measurement of Atrogin-1 (A, C) and MuRF1 (B, D) expression in gastrocnemius muscle at 2 and 4 weeks using reverse-transcription polymerase chain reaction. n = 5 per group. Values are presented as mean ± standard deviation. *P < 0.05 by Dunn multiple comparison test. MuRF1, muscle ring finger protein-1, CON, control; IBN, ibandronate; ELD, eldecalcitol.

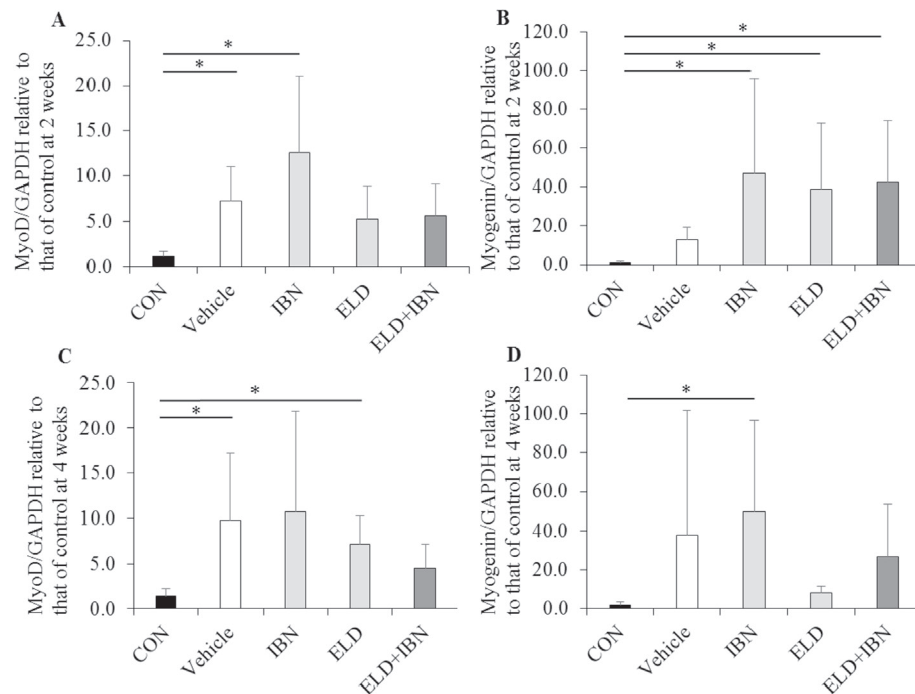

Fig. 3. Muscle anabolic markers. Measurement of MyoD (A, C) and Myogenin (B, D) expression in gastrocnemius muscle at 2 and 4 weeks using reverse-transcription polymerase chain reaction. n = 5 per group. Values are presented as mean ± standard deviation. *P < 0.05 by Dunn multiple comparison test. CON, control; IBN, ibandronate; ELD, eldecalcitol.

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

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