J Bone Miner Metab. **2016 ; 34(2): 171-178. DOI: 10.1007/s00774-015-0664-4.**

Effects of eldecalcitol on bone and skeletal muscles in glucocorticoid treated rats

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Key words: Muscle strength, muscle fatigue, muscle-related gene, bone, eldecalcitol

Abstract

Glucocorticoid causes secondary osteoporosis and myopathy, characterized by type II muscle fiber atrophy. We examined whether a new vitamin D_3 analog, eldecalcitol, could inhibit glucocorticoid-induced osteopenia or myopathy in rats and determined the effects of prednisolone (PSL) and/or eldecalcitol on muscle-related gene expression. Six-month-old female Wistar rats were randomized into four groups: PSL group (10 mg/kg PSL); E group (0.05 μg/kg eldecalcitol); PSL + E group; and control group. PSL, eldecalcitol, and vehicles were administered daily for 2 or 4 weeks. Right calf muscle strength, muscle fatigue, cross-sectional areas (CSAs) of left tibialis anterior muscle fibers, and bone mineral density (BMD) were measured following administration. *Pax7*, *MyoD*, and *myogenin* mRNA levels in gastrocnemius muscles were also determined. The PSL + E group muscle strength was significantly higher than that of the PSL group ($p < 0.05$) after 4 but not 2 weeks. There was no significant difference in muscle fatigue between the groups at 2 or 4 weeks. The CSAs of type II muscle fibers were significantly larger in the E group and PSL + E group than the PSL group at 4 weeks ($p = 0.0093$, $p = 0.0443$, respectively). Eldecalcitol treatment for 4 weeks maintained the same BMD as the PSL + E group. After 2 but not 4 weeks, eldecalcitol treatment significantly increased *Pax7* and *myogenin* mRNA expression in gastrocnemius muscle, while PSL also stimulated *myogenin* expression. Eldecalcitol appears to increase muscle volume and protect against femur BMD loss in PSL-administered rats. It may also stimulate myoblast differentiation into early myotubes.

Introduction

Osteoporotic fractures lead to a decrease in patient quality of life and increase in mortality [1, 2], so their prevention is an important clinical issue in an aging society. Falls are one of the main causes of osteoporotic fractures in addition to low bone mineral density (BMD) and an impaired bone quality. Risk factors for falls include age, a past history of falls, muscle weakness, and an increased intake of medication [3–5], the latter two of which are particularly prevalent in older people [6, 7].

Glucocorticoid is commonly used to treat inflammatory diseases, although it also induces skeletal muscle atrophy [8], known as steroid myopathy, and osteoporosis. Additionally, glucocorticoid causes type II muscle fiber atrophy, which is also observed in sarcopenia. Larsson et al. previously suggested that this might result from a decreased production of insulin-like growth factor-1 (IGF-I), which stimulates the development of muscle mass, and an increased production of myostatin, which inhibits muscle mass development [9, 10]. Type II muscle fibers are fast muscle fibers important in preventing falls [11], indicating that treatment for myopathy should be taken to prevent osteoporotic fractures, especially in older patients receiving glucocorticoid treatment.

In recent years, vitamin D_3 has been shown to prevent osteoporosis and reduce the risk of falls. Activated vitamin D_3, calcitriol, also known as 1, 25-dihydroxyvitamin D_3, has been shown to maintain BMD and to prevent osteoporotic fractures as a treatment for osteoporosis [12]. Additionally, several recent meta-analyses demonstrated that native or activated vitamin D_3 offer preventive effects against falls [13, 14]. Alfacalcidol, a type of activated vitamin D_3, also increased muscle strength in ovariectomized rats [15], and increased muscle strength and prevented muscle fatigue in glucocorticoid-treated rats [16]. However, it had little effect on BMD [17, 18].

Recently, the activated vitamin D_3 analog eldecalcitol [2b-(3-hydroxypropyloxy)-1,25-dihydroxyvitamin D_3] [19] has been authorized for clinical application in Japan. Studies show that eldecalcitol significantly increases BMD and reduces the risk of new vertebral fractures more than alfacalcidol [17, 20]. Ito et al. reported that eldecalcitol increased cortical cross-sectional areas (CSAs), volumetric BMD, and bone mass of the femoral neck, as well as maintaining cortical thickness [21], while Matsumoto et al. reported that wrist fractures were prevented by eldecalcitol administration [17]. These results indicate that eldecalcitol may prevent falls, similar to conventional activated vitamin D_3, and/or maintain the cortical thickness of the radius, although its effects on skeletal muscle strength, fatigue, skeletal muscle tissue, and muscle gene expression remain unclear. This study therefore evaluated the effects of eldecalcitol on the bone and skeletal muscle of PSL-administrated rats to elucidate its actions in muscle differentiation.

Materials and Methods

Animals and experimental protocol

Six-month-old female Wistar rats (Japan SLC Inc., Shizuoka, Japan) were housed in a controlled environment (temperature, 23 ± 2°C; humidity, 40 ± 20%) with a 12-h light–dark cycle. Rats were allowed *ad libitum* access to tap water and commercial standard rodent chow (CE-7; Clea Japan Inc., Tokyo, Japan), and were randomized into the following four groups (n = 5–9 each): 1) PSL group, administered prednisolone and eldecalcitol vehicle; 2) E group, administered eldecalcitol and prednisolone vehicle; 3) PSL + E group, administered both prednisolone and eldecalcitol; and 4) control group, administered eldecalcitol and prednisolone vehicles. Prednisolone (PSL; Predonine®;

Shionogi Pharmaceutical, Osaka, Japan) was dissolved in 0.9% saline and 10 mg/kg/day was injected subcutaneously every day for 2 or 4 weeks. This dose was determined previously to cause steroid myopathy [16]. Eldecalcitol (Chugai Pharmaceutical, Tokyo, Japan) was dissolved in medium-chain triglyceride (MCT) and a dose of 0.05 μg/kg/day or MCT alone was administered orally every day for 2 or 4 weeks. This dose was based on a previous study [22] that elevated rat lumbar spine BMD.

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

Body weight

Body weight was measured at the beginning and end of the experiment (Keimaiko®, Yamato-scale Co., Ltd., Hyogo, Japan). Weight loss during the experiment was compared between the four groups at 2 or 4 weeks.

Muscle strength and fatigability

Calf muscle strength and fatigue were measured the day after the final administration. General anesthesia was induced by intraperitoneal injection of xylazine hydrochloride (Sederac®; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) and ketamine hydrochloride (Ketalar®; Daiichi Sankyo Propharma, Tokyo, Japan). After opening the posterior surface of the right leg to expose the sciatic nerve in the gluteal region, a bipolar cuff electrode (inter-electrode distance, 5 mm; MT Giken, Tokyo, Japan) was attached to the sciatic nerve (Fig. 1). The rat was immobilized on a small platform with the knee in a fully extended position and fixed with Kirschner wire. The

distal end of the Achilles tendon was exposed and cut at the insertion to the calcaneus. A transducer (ZPS-DPU®; Imada, Aichi, Japan) was attached and fixed next to the stump with a load of 0.3 N [16]. Signals transmitted from the force transducer during isometric muscular contraction were digitally recorded on a force-time curve (ZP-Recorder®; Imada).

To prevent muscle desiccation during the study, the exposed area was covered with gauze moistened with saline. The study was conducted at a constant temperature of 25–27°C. To obtain a tetanic contraction, muscle contraction was induced by a monophasic rectangular pulse with a frequency of 40 Hz, a pulse width of 0.2 ms, and a stimulation intensity of 4 V. Each simulation lasted 180 s. We have described stimulation conditions previously [23]. We recorded muscle strength every 10 s after the start of stimulation, and this was defined as the maximum isometric contraction tension of the calf muscles normalized to the body weight [23]. A strength decrement index (SDI) was used to assess muscle fatigue [24], calculated as follows: SDI (%) = [initial contractile tension in the period of stimulation (Ti) − contractile tension at each second from initial stimulation (Tt)] × 100/Ti. This formula provides the attenuation of torque from the beginning of stimulation. A high SDI indicates greater muscle fatigue.

Histological analysis of muscle

Rats were euthanized by an injection of sodium pentobarbital (150 mg/kg body weight). The left tibialis anterior (TA) muscle was analyzed histologically after being rapidly frozen in liquid nitrogen, then stored at −80°C. 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 (adenosine triphosphatase

method) with a 15 min preincubation at room temperature in 0.1 M sodium barbital buffer (pH 8.6) containing 0.18 M $CaCl_2$ and distilled water. To measure CSAs of muscle fibers, microscopic images with a magnification of × 200 were captured digitally (BX-50; Olympus, Tokyo, Japan), and individual muscle fibers were traced on-screen using Image J image analysis software (National Institutes of Health, Bethesda, MD, USA). Areas were calculated using Image J software, based on a calibrated pixel-to-actual size (μm) ratio. 50 fibers per muscle were randomly chosen, measured muscle fiber CSA in both type I and type II muscle, and calculated the average of one muscle fiber CSA.

Intraobserver variations, as assessed by the coefficient of variation for three corresponding measurements in 50 randomly selected fibers, ranged from 0.1% to 1.5%. Interobserver variations by three investigators, as assessed by the coefficient of variation of measurements in 50 randomly selected images, ranged from 4.4% to 8.3%.

BMD measurement

Following euthanasia, the right femur was harvested for BMD measurements. The BMD of the total, proximal, middle, and distal femur was measured using dual-energy X-ray absorptiometry (QDR-4500 Delphi; Hologic, Bedford, MA, USA).

Gene expression analysis of skeletal muscle

Gastrocnemius muscles were isolated from the tibias of three rats per group, stored in RNAlater® solution (Qiagen, Hilden, Germany) at −80°C and crushed with a homogenizer (MS-100R; Tomy, Tokyo, Japan). Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Gaithersburg, MD) and reverse transcribed

using an Omniscript® Reverse Transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was carried out by an ABI-PRISM system according to the manufacturer's protocol (PRISM7500; ABI, Foster City, CA, USA) with TaqMan probes specific for rat MyoD (TaqMan probe ID: Rn01457527_g1), myogenin (TaqMan probe ID: Rn01490689_g1), and Pax7 (TaqMan probe ID: Rn00834076_m1). Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for sample normalization (TaqMan probe ID: 4352338E). Data are represented relative to those of a 2-week control rat.

Statistical analyses

All data are expressed as mean ± standard deviation (SD). Differences between groups at each time point were evaluated using one-way analysis of variance (ANOVA). Multiple comparisons were made using Scheffe's and Dunn's post hoc tests, as appropriate. Nonparametric data, including weight loss, 4-week treated muscle strength, SDI, 2-week treated CSA, and BMD of the middle femur, were analyzed by the Dunn's method. Parametric data were analyzed by Scheffe's test. Skeletal muscle gene expression was analyzed by the Wilcoxon rank sum test based on the data of the 2-week control. All statistical analyses were performed using Statistical Package for the Biosciences software (SPBS v9.6) [25]. Values of $p < 0.05$ were considered significant.

Results

There was no significant difference in weight loss between any of the groups after the

2-week administration period. However, after 4 weeks of administration, weight loss in the PSL + E group was significantly higher than that of the control group ($p < 0.01$; Table 1).

Post hoc tests revealed no significant difference in muscle strength between the 2-week groups, but muscle strength of the PSL + E group was significantly higher than that of the PSL group at 4 weeks ($p < 0.05$; Table 2).

Muscle fatigue, as evaluated by SDI, was not found to differ significantly between the 2- or 4-week administrated groups at 30, 60, 90, and 180 s after stimulation (Table 2). Similarly, no significant differences in the CSA of both type I and type II fibers were seen between the 2-week administrated groups. The type II fiber CSAs of the E group and PSL + E group were significantly greater than those of the PSL group at 4 weeks ($p = 0.0093$ and $p = 0.0443$, respectively). However, there was no significant difference about the CSA of type I fibers between the 4-week administrated groups.

The distal femoral BMD was significantly different between the PSL and E groups after 2 weeks of treatment ($p = 0.0261$; Table 3). After 4 weeks, the total, proximal, and distal BMD of the femur in the PSL group were significantly lower than those of the control group, E group and PSL + E group ($p < 0.05$–0.001) (Table 3). Similarly, the middle femoral BMD was significantly increased in the E-treated compared with the PSL-treated group at 4 weeks ($p = 0.0314$).

In 2-week treated gastrocnemius muscle, *Pax7* and *myogenin* mRNA expression levels were significantly higher in the PSL group and E group compared with the control [$p = 0.0495$ and $p = 0.0495$, respectively (*Pax7*); $p = 0.0495$ and $p = 0.0495$, respectively (*myogenin*)]. There was no significant difference in *MyoD* expression between the groups, nor for any of the three mRNA levels in the 4-week treated groups.

Discussion

Glucocorticoid-induced osteoporosis (GC-OP) is a form of osteoporosis that occurs as a result of osteocyte and osteoblast apoptosis [26] and myopathy characterized by type II muscle atrophy and weakness. In this study, glucocorticoid (PSL) administration caused osteopenia in rats. The new activated vitamin D_3 analog eldecalcitol was found to increase the CSA of type II muscle fibers in PSL-treated rats and to prevent PSL-induced bone loss. Together with PSL, it was also shown to elevate the gene expression of *Pax7* and *myogenin*. These results indicate that eldecalcitol could increase muscle volume and have preventive effects on osteoporosis in glucocorticoid-treated patients.

Saito et al. recently reported that eldecalcitol dose-dependently increased the expression of *IGF-I* in C2C12 cells [27], while it also induced the expression of *MyoD* and *myogenin* in the same cells when combined with 1α,25-dihydroxyvitamin D_3 [28]. We speculated that eldecalcitol would also increase muscle strength via a genomic action, so to elucidate its mechanisms of action we analyzed the expression of muscle-related genes in the skeletal muscle of rats treated with PSL and/or eldecalcitol. Eldecalcitol increased the expression of *Pax7* and *myogenin* during 2 weeks of treatment in the gastrocnemius muscle of normal rats in this study (Fig. 2). However, there was no significant difference in the expression of *MyoD*.

Pax7 is considered to be a specific marker of satellite cells or myoblasts [29], while myogenin stimulates the differentiation of myoblasts into early myotubes [30]. Following muscle damage, MyoD stimulates the differentiation of satellite cells in MyoD-positive myoblasts that migrate to the area of damage and form myotubes [31].

To prevent the exhaustion of satellite cells, some myoblasts express Pax7 without MyoD and return to their quiescent satellite cell state. This self-renewal pathway is strictly controlled [32]. Based on these findings, it is conceivable that eldecalcitol contributes to the differentiation of skeletal muscle. This would have the effect of increasing muscle strength.

The observed similar changes in prednisolone- and eldecalcitol-induced muscular gene expression, such as *Pax7* and *myogenin*, nevertheless led to different effects of the treatment in gastrocnemius muscle. We do not fully understand these findings, but speculate that the temporary increase in *Pax 7* and *myogenin* caused muscle damage to be repaired by PSL following 2 weeks of treatment.

It was previously reported that glucocorticoid-induced myopathy occurred through an imbalance of proteosynthesis and proteolysis in skeletal muscle [10]. Atrogin-1, muscle ring finger 1 (MuRF1), and regulated in development and DNA damage responses 1 (REDD1) are proteolysis regulators whose expression is increased in muscle cells *in vitro* and skeletal muscle in mice and rats during steroid-induced myopathy [10]. The expression of REDD1, a repressor of mTOR signaling, was also increased in rats treated with the glucocorticoid dexamethasone [10], and only one report found that vitamin D had no effect on *MuRF1* expression in rats [33]. Thus, one of the mechanisms by which eldecalcitol acts in steroid-treated rats may be exerted via controlling the expression of genes such as *atrogin-1*, *MuRF1*, and *REDD1*. The effect of eldecalcitol on the expression of these genes has not been elucidated, so future work should investigate these effects in glucocorticoid-treated animals.

Our previous studies showed that alfacalcidol increased muscle strength but did not affect muscle fatigue in ovariectomized rats [15], while it preserved not only BMD

but also muscle strength and volume, and prevented muscle fatigue in glucocorticoid-treated rats [16]. Similarly, in the present study, eldecalcitol increased muscle strength and volume, but did not affect muscle fatigue, in glucocorticoid-treated rats. Lieu et al. previously reported that high doses of glucocorticoid treatment induced a reduction in phosphofructokinase (PFK) activity in rats [34]. PFK is a glycolytic enzyme that contributes to anaerobic energy production, so a decrease of its activity could lead to increased muscle fatigue. More recently, Dong et al. found that glucocorticoid stimulated the expression of myostatin in muscle satellite cells derived from mice [35]; however, te Pas et al. reported that glucocorticoid increased the expression of MyoD1, myf-5, and MRF4 in the C2C12 cell line [36]. This result indicates that steroid use may exert anabolic or catabolic effects on muscle, dependent on the experimental dosing regimen. In the present study, the expression of myogenin and Pax7 was increased by steroid treatment in gastrocnemius muscle, which may explain why no muscle weakness or fatigue was observed.

Recently, eldecalcitol has been shown to exert a clinically positive effect both on bone and muscle. For instance, in 1,054 osteoporotic patients, the incidence of wrist fractures caused by falls was significantly lower in the eldecalcitol-treated compared with the alfacalcidol-treated group after 36 months [17]. Eldecalcitol also improved the chair-rising time, which is associated with past falls [37], in postmenopausal osteoporotic women treated with bisphosphonates [38]. Taking these findings and our own into account, eldecalcitol appears to have the potential to prevent osteoporotic fractures by increasing BMD and decreasing the incidence of falls by increasing muscle volume in older patients receiving glucocorticoid treatment.

The present study has two limitations that should be taken into account when

considering the results. First, we administered only a single regimen of eldecalcitol treatment to glucocorticoid-treated rats to evaluate its effects on muscle strength and fatigue. This dose was based on a previous study that reported increased BMD in rats [22]. However, it is possible that higher doses or a longer duration of eldecalcitol treatment would result in significant effects on muscle strength or fatigue. Second, we did not evaluate the expression of other genes such as *atrogin-1*, *MuRF1*, or *REDD1* which may have characterized glucocorticoid-induced muscle atrophy [10].

In conclusion, eldecalcitol appears to increase TA muscle type II fiber CSA, to improve the gastrocnemius muscle strength by inducing the higher expression of *myogenin* and *Pax7*, and to protect against the loss of femur BMD in PSL-administrated rats. Eldecalcitol therefore seems to prevent osteoporosis and increase muscle volume in glucocorticoid-treated rats. Further studies are needed to evaluate other genes like *atrogin-1*, *MuRF1*, or *REDD1* and to compare the effects of eldecalcitol on skeletal muscle with those of conventional activated vitamin D_3 such as alfacalcidol.

Acknowledgment

This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant No. 25462284).

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Figure legends

Fig. 1. Schematic illustration of the rat posterior right hindlimb, indicating the procedures used for the measurement of muscle strength and fatigue. The lower extremity was immobilized with the knee in a fully extended position and fixed with Kirschner wire. After exposing the sciatic nerve in the gluteal region, the stump of the Achilles tendon was attached to a bipolar cuff electrode. Signals transmitted from the force transducer during isometric muscular contraction were recorded.

Fig. 2. Measurement of *MyoD*, *myogenin*, and *Pax7* expression in gastrocnemius muscle at 2 or 4 weeks using RT-PCR. Values represent means + SD (n = 3 per group). Differences were classed as meaningful if the gene expression ratio to 2-week treated control and other groups was more than 2-fold as analyzed by the Wilcoxon rank sum test. Asterisk represents a significant difference compared with the 2-week treated control. *Pax7 e*xpression of the PSL group and E group was significantly higher than that of the control at 2 weeks (A). There was no significant difference in *MyoD* expression between groups at 2 weeks (B). *Myogenin* expression of the PSL group and E group was higher than that of the control group at 2 weeks (C). In the 4-week treated groups, no significant difference in *Pax7*, *MyoD*, or *myogenin* expression of gastrocnemius muscle was observed (D, E, and F).

TABLE 1. Body weight and weight loss of experimental groups

	Control group		PSL group		E group		PSL + E group		ANOVA
	Start	End	Start	End	Start	End	Start	End	
2W									
Body weight (g)	227 ± 6	217 ± 10	247 ± 5	227 ± 7	244 ± 20	234 ± 17	262 ± 17	242 ± 15	
Weight loss (g)	10 ± 10		20 ± 4		10 ± 6		20 ± 14		0.0401
4W									
Body weight (g)	259 ± 8	249 ± 8	238 ± 14	214 ± 14	242 ± 15	23 ± 15	240 ± 10	202 ± 14	
Weight loss (g)	10 ± 9		24 ± 6		19 ± 12		38 ± 19[a]		0.0007

n = 6–9 per group; values are mean ± SD

[a] $p < 0.01$ vs. control group by Dunn's method

2W, 2-week treatment; 4W, 4-week treatment; Start, at the beginning of the experiment; End, at sacrifice; ANOVA, one-way analysis of variance.

TABLE 2. Muscle strength, SDI, and CSA

		Control group	PSL group	E group	PSL + E group	ANOVA
Muscle strength (N/kg)						
2W		16.73 ± 1.55	15.20 ± 2.46	17.23 ± 3.37	12.91 ± 3.76	0.0452
4W		14.70 ± 1.95	14.31±1.98	18.16 ± 4.49	17.97 ± 2.12[a]	0.0090
SDI (%)						
2W	30 s	64.84 ± 5.69	63.58 ± 7.66	66.34 ± 6.75	70.20 ± 11.22	0.4476
	60 s	76.23 ± 5.32	74.50 ± 5.80	80.85 ± 7.50	82.98 ± 11.20	0.1690
	90 s	80.05 ± 6.00	78.59 ± 6.62	88.02 ± 8.30	88.72 ± 11.17	0.0575
	180 s	83.27 ± 4.29	90.64 ± 9.48	95.82 ± 6.91	93.62 ± 8.46	0.0567
4W	30 s	71.89 ± 10.61	69.00 ± 13.82	69.77 ± 6.38	63.95 ± 6.22	0.4313
	60 s	86.05 ± 9.31	79.74 ± 11.32	80.74 ± 4.41	78.61 ± 3.07	0.2389
	90 s	91.84 ± 7.32	84.79 ± 8.95	84.37 ± 3.36	85.20 ± 3.82	0.0663
	180 s	96.15 ± 5.33	92.94 ± 6.70	90.26 ± 4.73	91.53 ± 1.80	0.1131
CSA (μm^2)						
2W	type I	187.0 ± 68.6	197.5 ± 69.4	191.8 ± 73.5	197.4 ± 68.2	0.8535
	type II	206.2 ± 55.3	199.0 ± 52.3	221.1 ± 80.4	208.2 ± 47.7	0.3246
4W	type I	193.3 ± 71.4	196.4 ± 76.1	177.6 ± 66.8	186.2 ± 89.57	0.6183
	Type II	209.3 ± 58.3	183.4 ± 48.4	221.0 ± 56.2 [b]	214.7 ± 55.2 [c]	0.0041

n = 5–9 per group; values are mean ± SD; ANOVA, one-way analysis of variance; SDI, strength decrement index; CSA, cross sectional area; [a] Significantly different from PSL group, $p < 0.05$ by Dunn's method; [b] Significantly different from PSL group, $p < 0.01$

11 by Sheffe's method; [c] Significantly different from PSL group, $p < 0.05$ by Scheffe's method

12

TABLE 3. Bone mineral density of femur (g/cm^2)

	Control group	PSL group	E group	PSL + E group	ANOVA
2W					
Total	0.216 ± 0.004	0.217 ± 0.006	0.225 ± 0.008	0.222 ± 0.007	0.0284
Proximal	0.216 ± 0.004	0.218 ± 0.008	0.225 ± 0.013	0.228 ± 0.010	0.0897
Middle	0.187 ± 0.002	0.186 ± 0.005	0.189 ± 0.009	0.185 ± 0.005	0.5929
Distal	0.241 ± 0.007	0.237 ± 0.008[a]	0.251 ± 0.009	0.248 ± 0.010	0.0138
4W					
Total	0.227 ± 0.008	0.209 ± 0.011[b,c]	0.231 ± 0.007	0.224 ± 0.006	< 0.0001
Proximal	0.226 ± 0.008	0.210 ± 0.009[b,c]	0.232 ± 0.007	0.223 ± 0.007	< 0.0001
Middle	0.195 ± 0.006	0.182 ± 0.012[d]	0.195 ± 0.009	0.188 ± 0.008	0.0060
Distal	0.254 ± 0.009	0.230 ± 0.013[b,e]	0.257 ± 0.013	0.253 ± 0.008	< 0.0001

n = 6–9 per group; values are mean ± SD

2W, 2-week treatment; 4W, 4-week treatment; ANOVA, one-way analysis of variance

[a] Significantly different from E group, $p = 0.0261$ by Scheffe's method

[b] Significantly different from control and E group, $p < 0.001$ by Scheffe's method

[c] Significantly different from PSL + E group, $p < 0.05$ by Scheffe's method

[d] Significantly different from control and E group, $p < 0.05$ by Scheffe's method

[e] Significantly different from PSL + E group, $p < 0.001$ by Scheffe's method

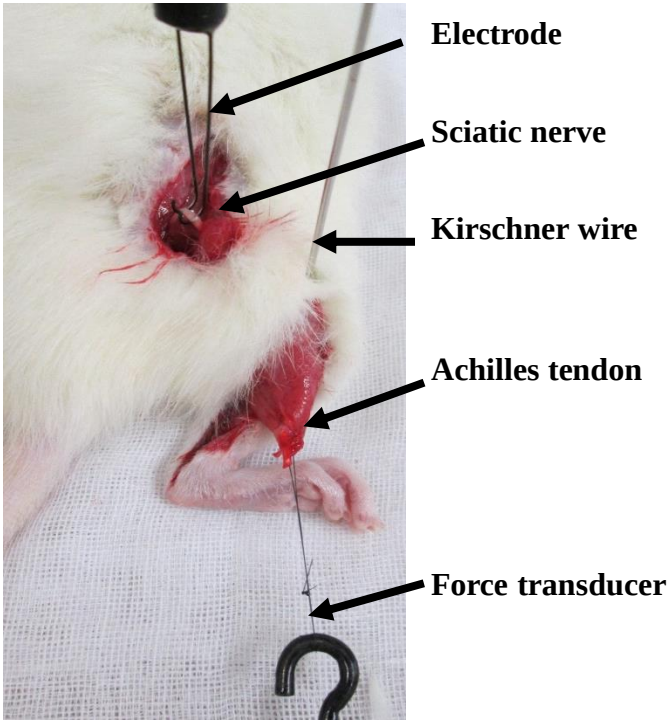
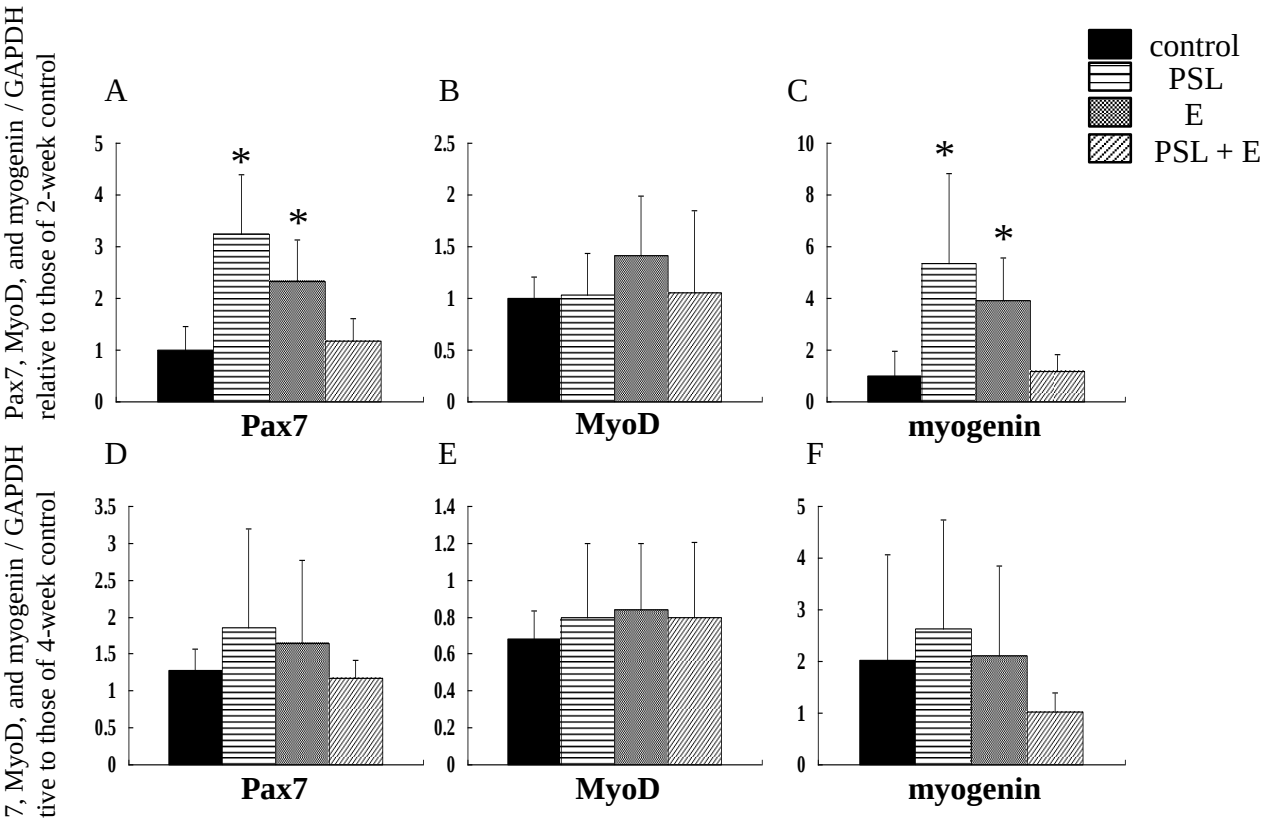

Electrode
Sciatic nerve
Kirschner wire
Achilles tendon
Force transducer

Figure 1



* $p = 0.0495$ vs 2-week control (Wilcoxon rank sum test)

Figure 2