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# Effect of 20-Hydroxyecdysone on Proteolytic Regulation in Skeletal Muscle Atrophy

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**Abstract.** *Background/Aim: 20-Hydroxyecdystone (20E) is an ecdysteroid hormone which controls molting and reproduction in arthropods. 20E also produces a variety of effects in vertebrates, including enhancing protein synthesis and skeletal muscle regeneration. The effect of 20E on disuse muscle atrophy has not been reported to date. This study examined the proteolytic regulation of 20E in tenotomized rat slow soleus and fast plantaris muscles. Materials and Methods: Male Wistar rats were randomly divided into three groups: sedentary control (CON), tenotomy without 20E treatment (TEN), and tenotomy with treatment of 5 mg/kg BW of 20E (TEN+20E). The TEN+20E group was administered 20E via subcutaneous injection to the right thigh for 7 days after tenotomy. Results: 20E treatment tended to attenuate disuse muscle atrophy and reduced ubiquitination only in soleus muscle. Conclusion: 20E treatment alleviates skeletal muscle atrophy partially mediated by ubiquitinate pathway, dependent on the muscle phenotype.*

Skeletal muscle atrophy is a debilitating condition associated with a variety of pathological conditions (*e.g.* diabetes mellitus, cancer, chronic obstructive pulmonary disease, and chronic heart failure), physiological consequence of aging (sarcopenia), and disuse (*e.g.* casting-immobilization, microgravity, or tendon injury), subsequently leading to a decrease in mobility and reduced quality of life (1). Muscle atrophy is characterized by a decrease of protein content leading to a reduction in cross-sectional area (CSA) of muscle fibers and impairment of muscle force generation and fatigue resistance (2, 3). Skeletal muscle mass is determined by the balance of protein synthesis and protein breakdown. However, previous compelling evidence has indicated that disuse muscle atrophy is primarily caused by the increase in the rate of protein degradation rather than a decrease in the rate of protein synthesis (4). Although several proteolytic systems are involved in protein turnover in skeletal muscle, recent studies suggested that the ubiquitin-proteasome system (UPS) represents the major intracellular proteolysis machinery responsible for the degradation of major contractile proteins, and contributes significantly to muscle atrophy in both animal and human models (1, 5).

To date, in spite of many attempts, there is no single effective method to completely cure muscle atrophy. Although several pharmacological agents such as β2-adrenergic agonist or proteasome inhibitors have been prescribed to combat muscle atrophy (5), these drugs do not come without side-effects. Therefore, a search for a non-pharmacological therapeutic strategy should be considered as an alternative approach to tackle these clinical problems. There is evidence that ecdysteroids, insect hormones, have an anabolic effect on skeletal muscle (6) with no side-effects in mammals, including humans (7).

Ecdysteroids act as molting hormones in insects and also play a defensive role against insect herbivory in plants (8). There are a large number of structurally related ecdysteriods in plants (phytoecdysteriods). Of these, 20-hydroxyecdysone (20E) is the most commonly found and extensively studied. This compound is present at high concentration in the bark of blackberry tree (*Vitex glabrata*). 20E has low toxicity in mammals: the lethal dose 50 ($LD_{50}$) is 6.4 g/kg in mice for intraperitoneal injection and more than 9 g/kg when given orally (9). The pharmacokinetics of 20E depend on the mode of administration, for example, the half-time of elimination of this substance in lambs when administered *via* oral, intravenous, and intramuscular routes were 0.2, 0.4, and 2 h, respectively (10). It has been reported that the half-life of

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20E was shorter in smaller animals *e.g.*, 8 minutes 15 seconds in mice (11). 20E was taken up by the liver, excreted into the gut *via* bile, and then eliminated into urine and faces in mice (12).

Ecdysteroids have a variety of physiological effects in mammals, including hepatoprotective, immune modulatory, as well as hypoglycemic action. Notably, 20E affects certain major metabolic pathways such as protein synthesis, and lipid and carbohydrate metabolism (13). One of the most interesting properties of ecdysteroids in mammals is their anabolic effect, similar to anabolic steroids, but without the androgenic effect (14). Various preparations (*e.g.* Ekdisten® or Retibol®), containing a small amount (about 5 mg/tablet) of ecdysteroid have been used among body builders and sportsman as an ergogenic aid to enhance physical performance (12). Anabolic action of 20E includes increased fiber size in a muscle-specific manner, as well as increasing the myonuclear number in both of normal and regenerating muscles in rat (15). Furthermore, it was reported to enhance physical performance, as measured by a forced swimming test, without training in rats (16). 20E stimulates in dose-dependent manner [$^3$H] leucine incorporation into muscle proteins of C2C12 and isolated human skeletal cells mediating protein kinase B (AKT) activation (17). Although 20E has anabolic action, the response of the muscle proteolytic pathway to this compound is still unknown. Therefore, the primary goal of this study was to evaluate the effect of 20E on the activation of the UPS in rat skeletal muscle atrophy induced by Achilles tendon transection. The reason behind our selecting this animal model is that this type of atrophy is very commonly encountered by clinicians following orthopedic surgery or degenerative musculoskeletal diseases (18); moreover, tenotomy is reproducible in inducing muscle atrophy. Additionally, there is accumulating evidence that slow-twitch fibers are more sensitive to inactivity or microgravity than fast-twitch fibers (19, 20), and 20E enhances muscle hypertrophy of different fiber types in a muscle-specific fashion (15). Thus, the second goal of this study was to evaluate whether 20E induces a fiber type-specific response of proteolytic regulation of tenotomy-induced muscle atrophy in slow-dominant *soleus* and fast-dominant *plantaris* muscles.

## Materials and Methods

*20-Hydroxyecdysteroid*. 20E was isolated from the bark of *Vitex glabrata* using the literature-described procedure (21). The physical and spectroscopic (nuclear magnetic resonance and mass spectra) data of the isolated compound were found to be in accordance with the literature values (22, 23).

*Animals and treatment*. Adult male Wistar rats (10 weeks old), weighing approximately 290-310 g, were obtained from the National Laboratory Animal Centre, Salaya, Nakhon Pathom, Thailand. All animal procedures and experiments were conducted with the approval of the animal use and care guidelines established by the Ethics Committee on the Use of Experimental Animals, Faculty of Science, Mahidol University (protocol no. MUSC54-034-244). Rats were randomly assigned to three groups (n=8 in each group): controls (CON) received vehicle [olive oil plus dimethyl sulfoxide (DMSO; not exceeding 10% of volume injected)]; tenotomy without 20E treatment (TEN), which received vehicle; and tenotomy with 20E treatment (TEN+20E) received 20E at a dose of 5 mg/kg BW diluted in vehicle. Vehicle or 20E treatment were applied to the animal *via* subcutaneous injection into the right thigh for seven consecutive days after tenotomy. All rats were housed at 21˚C and maintained on a 12:12 h light-dark cycle. In order to eliminate confounding factors of food intake between groups, animals were pair-fed with standard food and water. Rats were sacrificed 24 h after the last vehicle or 20E treatment.

*Tenotomy*. Rats were anesthetized with a cocktail of tiletamine and zolazepam (Zoletil®; 25 mg/kg, *i.p.*) and xylazine (8 mg/kg, *i.p.*). All surgical procedures were performed under aseptic technique. The right hindlimb was cleaned with betadine solution to prevent infection, then the skin of the calf down to the Achilles tendon and calcaneous bone was incised. The Achilles tendon was carefully excised from the distal part attached with calcaneous bone to the proximal part for 3 mm in length without disturbing the nerve and blood supply. Thereafter, the skin was sutured with a surgical thread and rats were allowed to recover before returning to their cages.

*Sample collection*. At the end of the experiment, rats were anesthetized with pentobarbital sodium (75 mg/kg, *i.p.*), *soleus* and *plantaris* muscles were rapidly removed, and the rats were euthanized by cardiac puncture. Muscle samples were trimmed of excess connective tissue, weighed, and the entire fiber was horizontally halved. The upper half was immediately frozen in liquid nitrogen and stored at −80˚C for biochemical analysis. The lower half was mounted in Optimal Cutting Temperature Tissue Tek (Electron Microscopy Sciences, Hatfield, PA, USA) and immersed in isopentane (Sigma, St. Louis, MO, USA) pre-cooled by liquid nitrogen and stored at −80˚C for further analysis.

*Western blot analysis*. Approximately 50 mg of muscle sample was homogenized using a glass homogenizer (Glas-Col; Terre Haute, IN, USA) with an ice-cold homogenizing buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM phenylmethane sulfonyl fluoride, and a protease inhibitor cocktail (Sigma) at a mass-to-buffer ratio of 20:1. The homogenates were centrifuged at 1,500 × *g* for 10 min (4˚C), and both supernatant (cytosolic fraction) and pellet (myofibrillar fraction) were collected. The cytosolic fractions were further centrifuged at 10,000 × *g* for 10 min (4˚C) and the supernatant was collected. To remove debris from the myofibrillar fraction, pellets were suspended in ice-cold homogenizing buffer plus 1% Triton X-100 and centrifuged at 1,500 × *g* for 10 min (4˚C) three times. The final pellets were re-suspended with 8 M urea in 50 mM Tris-HCl (pH 7.5). The cytosolic and myofibrillar fractions were aliquoted to determine the protein concentration in triplicate using bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin (Sigma) as a standard.

The accumulation of ubiquitin conjugates and muscle RING-finger-1 (MURF1) protein expression were measured using myofibrillar and cytosolic protein fractions, respectively. Protein in

sample buffer solution (1 mg/ml) was denatured by heating at 60˚C for 10 min and was then loaded into SDS-polyacrylamide gel. The myofibrillar protein was separated on a 5% stacking gel at 60 V for 30 min and on an 8% polyacrylamide gel at 90 V for 360 min at room temperature (RT). However, the cytosolic protein fraction was electrophoresis at 60 V for 30 min on a 5% stacking gel and 110 V for 90 min on a 10% separating gel. Proteins were then transferred for 90 min at 100 V onto nitrocellulose blotting membrane (Pall Corporation® Life science, Port Washington, NY, USA). Non-specific binding was blocked with 5% non-fat milk in Tris-buffered saline (TBS) plus Tween-20 (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 90 min at room temperature. The membrane was incubated with primary antibodies in blocking buffer overnight at 4˚C. The following primary antibodies were used: 1:200 anti-ubiquitin (sc8017; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 1:1,000 anti-MURF1 (AF5366; R&D System, Minneapolis, MN, USA). Following a series of extensive washes with TBS plus Tween-20 buffer, the membrane was incubated for 90 min with 1:10,000 goat anti-mouse IgG peroxidase conjugated (31430; Thermo Scientific) or 1:1,000 rabbit anti-goat IgG conjugated horseradish peroxidase (HAF017; R&D System) secondary antibodies. Protein bands were visualized by enhanced chemiluminescence (ECL) (Thermo Scientific) and exposed to CL-XPosure film (Thermo Scientific). Band intensity was measured using Image J software version 1.44o (NIH; National Institutes of Health, Bethesda, MD, USA).

*Histological analysis*. Embedded muscles were placed at −20˚C for 30 min before sectioning at 10 μm thickness using a cryostat (Lelica, Wetzlar, Germany). Muscle sections were stained with hematoxylin and eosin (H&E) and mounted with Permount (Fisher Scientific, Loughborough, Leicestershire, UK) to visualize muscle morphology and determine the fiber cross-sectional area (CSA). The image acquisitions were taken under a light microscope (Olympus Tokyo, Japan) with digital camera (Olympus) at ×200 magnification. Four images per muscle section were captured to determine the CSA with cell Sens Dimension 1.8.1 software (Olympus). The muscle fiber CSA was quantified by using ImageJ software version 1.44o (NIH).

*Statistical analysis*. Data are presented as means±SEM. Normal distribution and homogeneity of variance were determined using Shapiro–Wilk test and Levene's test, respectively. One-way ANOVA with Student-Newman-Keuls *post hoc* test was used to determine differences between treatment conditions. When homogeneity of variance was not assumed, data were analyzed by the Kruskal–Wallis test. If statistical significance was detected, Dunn's test was used to indicate significant differences with the level of acceptance at $p<0.05$. Statistical analysis was performed using SPSS version 17.0 (Chicago, IL, USA).

## Results

*Effect of 20E on body weight, muscle wet weight and myofibrillar protein content after tenotomy*. Eight days after tenotomy, the body weight of TEN and TEN+20E groups were significantly ($p<0.05$) lower than that of the CON group (Figure 1A). There were significant ($p<0.01$) decreases in the absolute and relative wet weight of *soleus* muscles (by 58.97% and 54.4%, respectively) 8 days after Achilles tendon transection compared to the CON group. Additionally in the tenotomized groups, the absolute and relative *plantaris* wet weights decreased by 18.85% and 11.25%, respectively, as compared with the CON group. Interestingly, 20E treatment attenuated tenotomy-induced muscle loss in *soleus* muscle, the reduction of muscle wet weight was less in the TEN+20E group when compared with the TEN group (49.13% *vs*. 58.97%) (Figure 1C and D). Corresponding with the muscle wet weight, the myofibrillar protein content was significantly reduced in both *soleus* and *plantaris* muscles ($p<0.01$ and $p<0.05$, respectively) in TEN compared with CON groups. However, 20E treatment had no additional effect on tenotomized muscle.

*Effect of 20E on muscle histology and fiber CSA after tenotomy*. Muscle morphology was assessed by H&E staining. As seen in Figure 2A, the normal muscle fibers were polygonal shapes and packed together to form muscle bundles. In contrast, the muscle fibers revealed smaller in size associated with the significant ($p<0.01$) reduction of mean fiber CSA in *soleus* and *plantaris* muscles of tenotomized groups when compared with the CON group. However, 20E treatment did not alleviate the tenotomy-induced reduction of fiber CSA (Figure 2). In parallel, muscle fiber distribution in TEN and TEN+20E groups presented a leftward shift of the CSA curve in both muscles examined (Figure 2B).

*Effect of 20E on components of the UPS after tenotomy*. In order to determine whether tenotomy-induced muscle atrophy involved the activation of the UPS pathway, ubiquitin conjugates were examined in the myofibrillar fraction from both *soleus* and *plantaris* muscles. As expected, tenotomy significant ($p<0.01$) increased high molecular weight (HMW.) ubiquitin conjugates in both *soleus* and *plantaris* muscles (151.1% and 128.3%, respectively) compared to the CON group. Interestingly, the level of HMW–ubiquitin conjugates was attenuated by 63.1% in *soleus* of tenotomized rat treated with 20E, but this effect was not observed in the *plantaris* muscles (Figure 3A and B).

Since E3 ligases are considered to be rate-limiting in the UPS pathway and their mRNA levels were consistently up-regulated in several models of muscle disuse atrophy, MURF1 protein expression was also determined in this study. Surprisingly, MURF1 protein expression in *soleus* and *plantaris* muscles was not significantly affected by tenotomy nor by 20E (Figure 4A and B).

## Discussion

This study aimed to investigate the effect of 20E on skeletal muscle atrophy in both slow- and fast-twitch muscles following tenotomy. The results demonstrated that subcutaneous

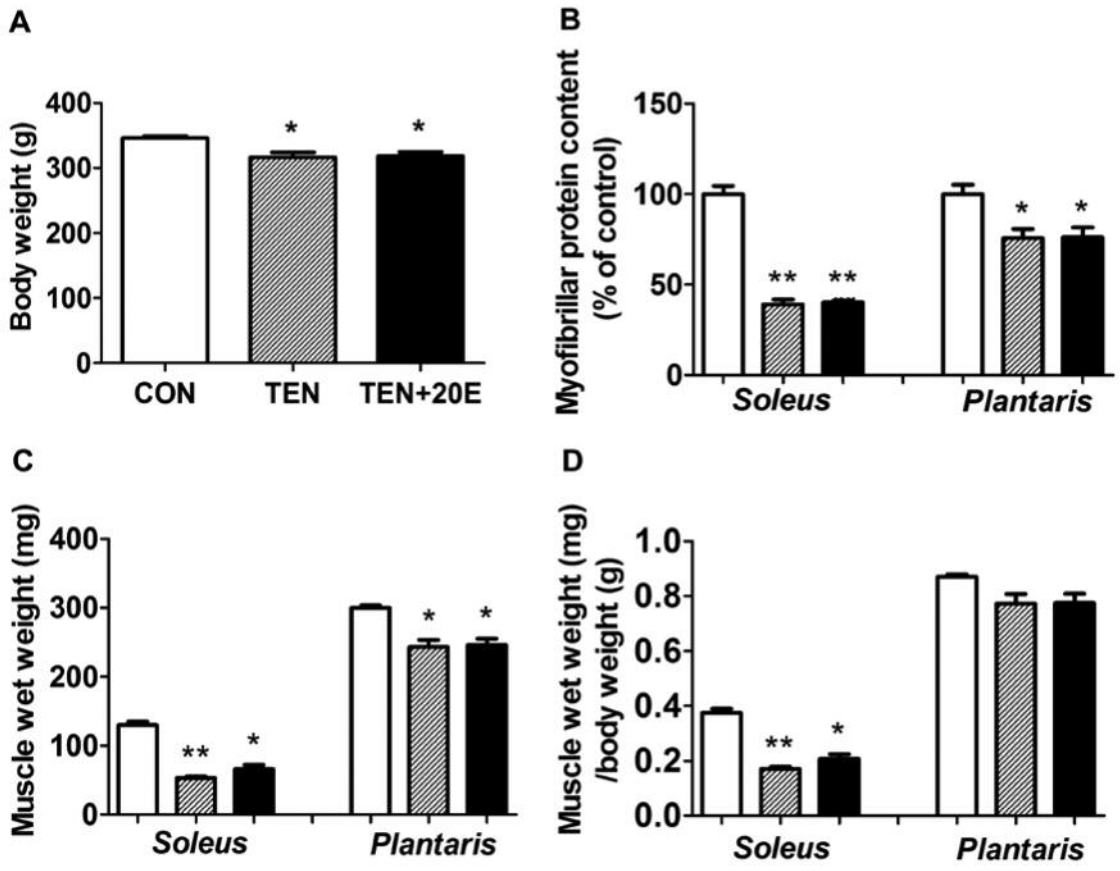


Figure 1. *Effects of 20-hydroxyecdystone (20E) on body weight (A), myofibrillar protein content (B), absolute muscle wet weight (C) and relative muscle wet weight normalized to body weight (D) of soleus and plantaris muscles 8 days after tenotomy (n=8 rats/group). *p<0.05 and **p<0.01, vs. control non-tenotomized (CON) rats for each muscle.*

administration of 5 mg/kg BW of 20E tended to alleviate tenotomy-induced reduction of muscle mass and attenuate tenotomy-induced ubiquitinated protein in *soleus* muscle. Despite this, 20E had no additional effect on body weight, myofibrillar protein content, or fiber CSA in either muscle after tenotomy.

Our findings that transection of the Achilles tendon for 8 days induced muscle atrophy in both *soleus* and *plantaris* muscles and this atrophic effect was greater in the predominantly slow *soleus* muscle than the predominantly fast *plantaris* muscle were in accordance with other literature (20). In the present study, the finding that 20E treatment had a tendency to preserve muscle mass in slow-twitch muscles but had no effect on fast-twitch muscles following tenotomy was in line with previous reports on the effect of anabolic steroid supplements (24, 25). In one study, 2 mg/kg BW of nandrolone decanoate injection for 1 week increased muscle mass and myosin heavy chain type I expression on rat regenerating *soleus* but not on predominantly fast *extensor digitorum longus muscles* (24). Moreover, 1-week administration of 100 mg testosterone enanthate increased muscle fiber diameter 1.5-fold more in slow fiber compared to fast fiber *vastus lateralis* muscle in elderly men (25).

In this study, the dose of 5 mg/kg BW of 20E was applied to the rat, since the dose-dependent effect of this compound on the muscle fiber growth has been shown and this was an effective dose to promote regeneration in rat skeletal muscle (15). There is also evidence that intraperitoneal injection of 5 mg/kg BW of 20E for 7 days enhanced protein synthesis in mouse heart and muscle (26), increased body and muscle weight, as well as liver growth in castrated or ovariectomized rats, but had no any androgenic effect (14). Furthermore, continuous infusion of 20E (5 mg/kg BW) for 5 days using subcutaneously implanted osmotic pump revealed an increase in mass of *triceps brachii* muscle, but there were no differences in the masses of *tibialis anterior*, *biceps femoris*, and *gastrocnemius* muscles, nor any body weight change in mice (27).

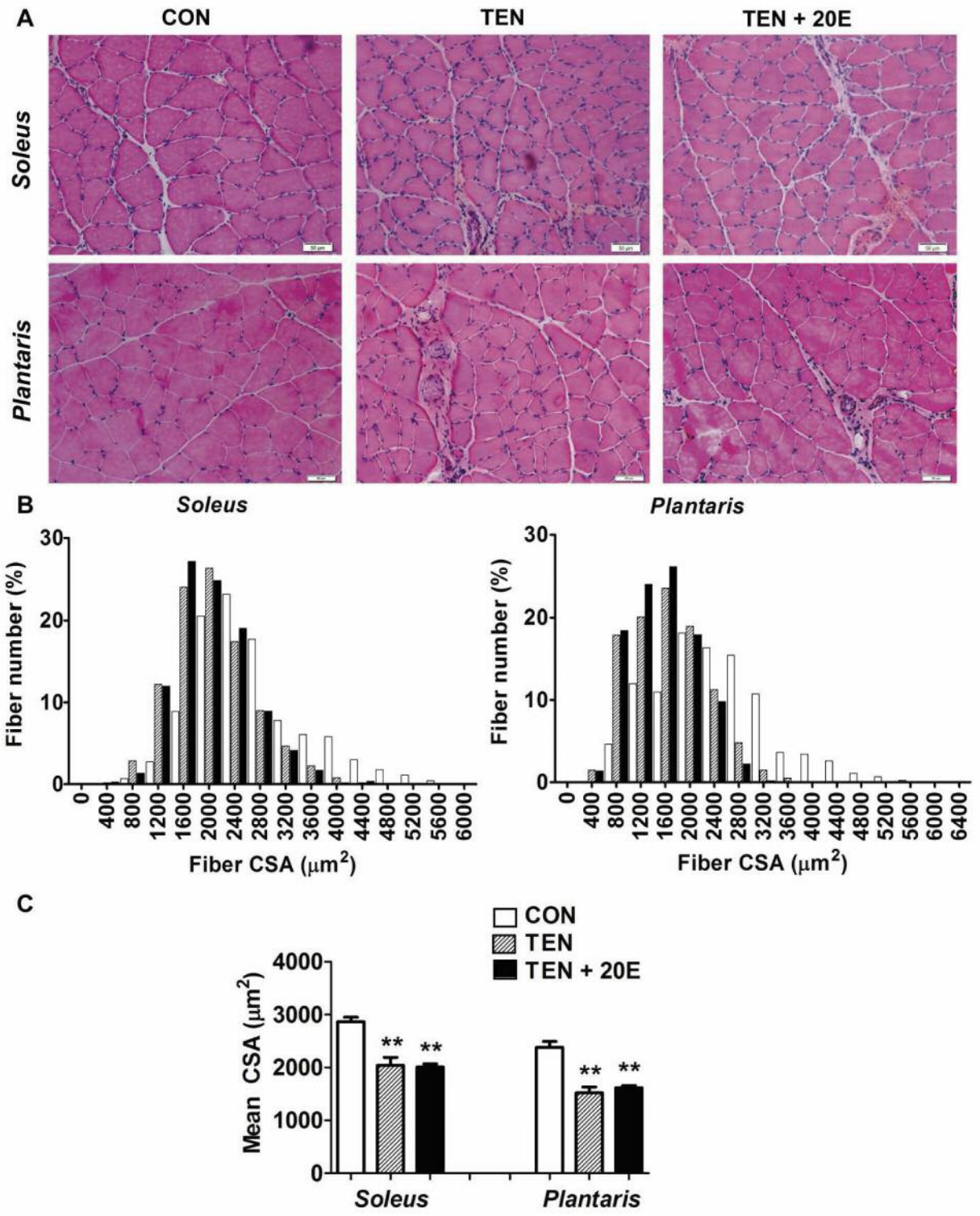


Figure 2. *Effects of 20-hydroxyecdystone (20E) on muscle morphology and fiber cross-sectional area (CSA) in soleus and plantaris muscles 8 days after tenotomy. A: Hematoxylin and eosin staining of soleus and plantaris muscle cross-sections. Left panel is muscle from control non-tenotomized rats (CON); middle panel is muscle from tenotomized rat (TEN); right panel is muscle from tenotomized rat treated with 20E (TEN+20E). Scale bars=50 μm. B: Histogram of fiber CSA distribution (a total of 1168 fibers from CON, 1113 fibers from TEN, and 1109 fibers from TEN+20E of soleus muscle; 1192 fibers from CON, 1225 fibers from TEN, and 1172 fibers from TEN + 20E of plantaris muscle) C: Mean fiber CSA (n=3 rats/group). **p<0.01 vs. CON for each muscle.*

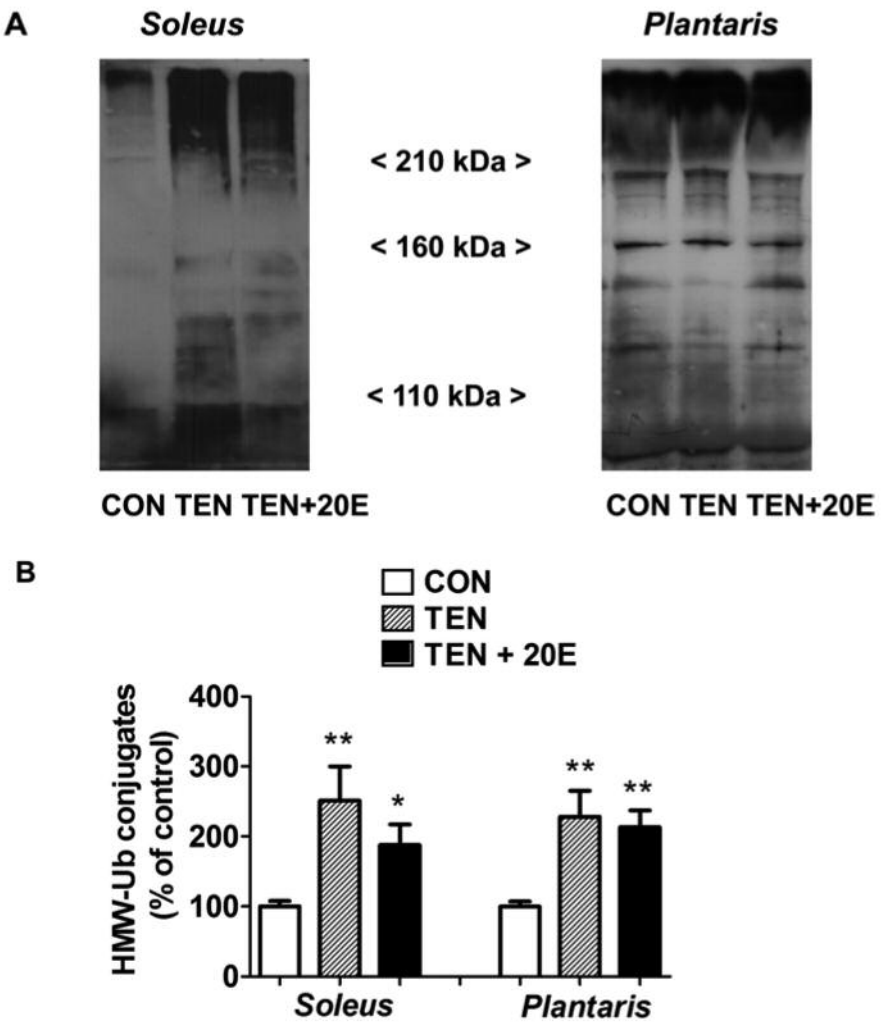


Figure 3. *Effects of 20-hydroxyecdystone (20E) on high molecular weight (HMW)–ubiquitin (Ub) conjugates in soleus and plantaris muscles 8 days after tenotomy. A: Western blot analysis of HMW–Ub conjugates. B: Quantified data of HMW–Ub (n=6-7 rats/group). *p<0.05 and **p<0.01, vs. control non-tenotomized (CON) rats for each muscle.*

Although the anabolic effect of 20E was demonstrated in several studies, its effect on body weight or muscle mass was inconsistent (15, 27). These variations may stem from the distinction in animal strains and the animal models used in those studies, and also the differences in routes and methods of 20E administration. 20E was found to be rapidly eliminated by various routes *e.g.* bile, urinary and feces with low toxicity in mammals (12). Thus, the half-life of this compound in rodents is short *e.g.* 8 min 15 sec in mice (11). This may explain discrepancies in muscle mass adaptation to 20E treatment. Considering the effect of 20E on disuse muscle atrophy, further study is required to investigate the optimal dose and duration of 20E administration, and to determine the plasma concentration of 20E throughout the experiment.

In this study, 20E treatment had no effect on fiber CSA in tenotomy-induced muscle atrophy. This finding was in contrast with that of Toth *et al.*, who reported that 20E increased *soleus* CSA following cardiotoxin-induced muscle injury (15). The distinction may result from the differences in animal models used in the study.

There are three major conditions regulating muscle fiber size: i) the number of myonuclei, ii) the rate of protein synthesis, iii) the rate of protein degradation. A previous study proposed that 20E exerted an anabolic effect (increased fiber CSA) in regenerating *soleus* muscle *via* an increased myonuclear number (15). In response to stimuli such as muscle injury, the resident mesenchymal stem cells, termed satellite cells, become activated, proliferate and express myogenic markers (myoblasts), leading them to fuse together

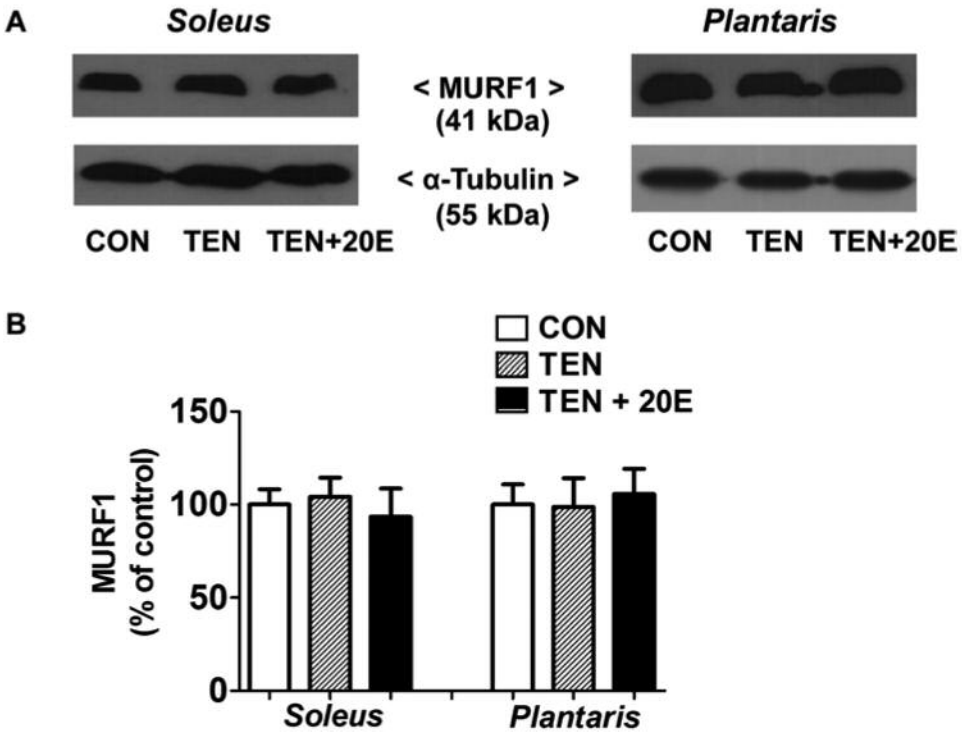


Figure 4. *Effects of 20-hydroxyecdystone (20E) on muscle RING-finger-1 (MURF1) protein expression in soleus and plantaris muscles 8 days after tenotomy. A: Western blot analysis of MURF1 protein expression. B: Quantified data of MURF1 expression. MURF1 band density was normalized using α-tubulin (n=5 rats/group).*

to form new multinucleated muscle fibers or to repair existing myofibers (28). Nevertheless, many studies demonstrated that myonuclei number did not change during disuse muscle atrophy (29, 30). It is possible that 20E administration had no additional effect on myogenic precursor cell activation in tenotomized muscle. Thus, 5 mg/kg BW of 20E treatment may be an effective dose in regenerating *soleus* fiber CSA but is not sufficient for increasing the CSA in atrophied muscle.

Muscle atrophy occurs when the balance between protein synthesis and protein degradation is in favor of degradation, leading to the loss of myofibrillar protein (31). In this study, tenotomy induced the loss of myofibrillar protein associated with the reduction of fiber CSA in both *soleus* and *plantaris* muscles. Although the precise mechanism leading to skeletal muscle atrophy is not clearly understood, the UPS, which is the major proteolytic system, has been implicated in the pathogenesis of skeletal muscle wasting, including muscle disuse atrophy and cachexia (32). Our finding that tenotomy increased accumulation of ubiquitinated proteins in both muscles is consistent with another study (5). Interestingly, 20E treatment resulted in lower ubiquitinated protein accumulation only in *soleus* muscle, not *plantaris* muscle. Collectively, these data indicate that 20E can alleviate tenotomy-induced atrophy, particularly in slow muscle, at least in part through a muscle-specific inhibition of the ubiquitin-proteasome pathway.

Nevertheless, the finding that tenotomy did not alter protein expression of MURF1, muscle-specific E3 ligase, in both muscles examined was unexpected, given that this protein is mostly up-regulated in various models of muscle atrophy including immobilization, denervation, and hindlimb suspension (33). The reason behind this discrepancy may stem from the use of different animal models of muscle atrophy. Microarray analysis of tenotomized muscle in mice demonstrated increased expression of genes involved in lysosomal protein degradation (*e.g.* cathepsin) (1) and calcium-activated protease (34) in mouse tenotomized muscle. Furthermore, a recent study demonstrated that the *MURF1* mRNA tended to increase to the highest level at after 3 days and then declined at 7 days in the ambulatory muscles following immobilization (35). Thus, in this study, it is possible that the level of MURF1 protein expression might have already returned to the baseline level at 8 days after tenotomy. Therefore, other proteolytic mechanisms and time course need to be further investigated to verify the effect of 20E on tenotomy-induced muscle atrophy.

In conclusion, we demonstrated that 20E attenuated tenotomy-induced muscle atrophy in predominantly slow-twitch muscle, partially mediated by ubiquitin proteasome pathway in rat skeletal muscle. Thus, 20E may be beneficial as a supplement for promoting muscle recovery from disuse muscle atrophy.

## Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

## Acknowledgements

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