

1
2
3
4

TITLE PAGE
- Korean Journal for Food Science of Animal Resources -
Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Title	Effect of deer antler extract on muscle differentiation and AICAR-induced muscle atrophy in C2C12 cells
Running Title (within 10 words)	Effect of deer antler extract on myoblast differentiation and atrophy
Author	Kyungae Jo ¹ , Woo Young Jang ¹ , Beom Sik Yun ² , Jin Soo Kim ² , Hyun-Sun Lee ³ , Yeok Boo Chang ¹ , Hyung Joo Suh ¹
Affiliation	1 Department of Integrated Biomedical and Life Sciences, Graduate School, Korea University, 145 Anam-ro, Sungbuk-gu, Seoul 02841, Republic of Korea 2 R&D center, Kwangdong Pharm Co., Ltd, 271 Digital-ro, Guro-gu, Seoul 08381, Republic of Korea 3 Agency for Korea National Food Cluster, Iksan 54576, Republic of Korea
Special remarks – if authors have additional information to inform the editorial office	
ORCID (All authors must have ORCID) https://orcid.org	Kyungae Jo (https://orcid.org/0000-0003-4056-6706) Woo Young Jang (https://orcid.org/0000-0002-5104-4949) Beom Sik Yun (https://orcid.org/0000-0002-0065-5555) Jin Soo Kim (https://orcid.org/0000-0001-8218-439X) Hyun-Sun Lee (https://orcid.org/0000-0003-0435-0107) Yeok Boo Chang (https://orcid.org/0000-0002-2445-5750) Hyung Joo Suh (https://orcid.org/0000-0001-8869-3929)
Conflicts of interest List any present or potential conflicts of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This research was supported by the Kwang Dong Pharmaceutical Co., Ltd (Q1823491).
Author contributions (This field may be published.)	Conceptualization: Jo K, Suh HJ. Data curation: Jang WY, Chang YB. Formal analysis: Yun BS, Kim JS, Lee HS. Methodology: Jo K, Suh HJ. Software: Jang WY, Chang YB. Validation: Yun BS, Lee HS. Investigation: Kim JS, Lee HS. Writing - original draft: Jo K, Suh HJ. Writing - review & editing: Jo K, Jang WY, Yun BS, Kim JS, Lee HS, Chang YB, Suh HJ.
Ethics approval (IRB/IACUC) (This field may be published.)	This manuscript does not require IRB/IACUC approval because there are no human and animal participants.

5
6

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Hyung Joo Suh
Email address – this is where your proofs will be sent	suh1960@korea.ac.kr
Secondary Email address	foodsuh@naver.com

Postal address	Department of Integrated Biomedical and Life Sciences, Graduate School, Korea University, 145 Anam-ro, Sungbuk-gu, Seoul 02841, Republic of Korea
Cell phone number	+82 10 4214 2853
Office phone number	+82 2 940 2764
Fax number	+82 2 921 7207

7
8

ACCEPTED

9 **Abstract**

10 The effect of deer antler extract on muscle differentiation and muscle atrophy were evaluated to
11 minimize muscle loss following aging. Various deer antler extracts (HWE, hot water extract of deer
12 antler; FE, hot water extract of fermented deer antler; ET, enzyme-assisted extract of deer antler; UE,
13 extract prepared by ultrasonication of deer antler) were evaluated for their effect on muscle
14 differentiation and inhibition of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)-induced
15 muscle atrophy in C2C12 cells. To confirm the morphological changes caused by the effect of deer
16 antler extracts on muscle differentiation, the length and thickness of myotubes were measured by
17 Jenner-Giemsa staining. In addition, the expression levels of genes related to muscle differentiation and
18 atrophy were confirmed through qRT-PCR. In the presence of antler extracts, the length and thickness
19 of myotubes and MyoD1 and Myf5 gene expression were increased compared to those in the control
20 group. Gene expression of AMPK, MyoD1, and myogenin, along with the muscle atrophy factors
21 MuRF-1 and FoxO3a upon addition of deer antler extracts to muscle-atrophied C2C12 cells was
22 determined by qRT-PCR after treatment with AICAR. The expression of MuRF-1 and FoxO3a
23 decreased in the groups treated with antler extracts compared to that in the group treated with AICAR
24 alone. In addition, gene expression of MyoD1 and myogenin in the muscle atrophy cell model was
25 significantly increased compared that into the control group. Therefore, our findings indicate that deer
26 antler extract can increase the expression of MyoD1, Myf5 and myogenin, inhibit muscle atrophy, and
27 promote muscle differentiation.

28

29 **Keywords:** Deer antler; muscle differentiation; muscle atrophy; AICAR; C2C12 cells

30 **Introduction**

31 The aging process involves the natural degeneration of all the organs of the body over time. In this
32 process, muscular atrophy is an important influencing factor. Muscular atrophy involves the impairment
33 of the major nerves of muscles or a decrease in the absolute volume and strength of muscles under
34 prolonged under certain conditions (Bonewald, 2019; Szentesi et al., 2019).

35 Muscle cells are involved in the regeneration of muscle fibers containing mechanical, chemical, or
36 degenerative lesions through activation, differentiation, and proliferation (Ceafalan et al., 2014;
37 Karalaki et al., 2009). Differentiation of myoblasts is essential for the formation of muscle fibers
38 involved in the development and regeneration of skeletal muscle (Grefte et al., 2007). These monocyte
39 myoblasts proliferate and differentiate, following which, they fuse with existing muscle fibers to form
40 multinucleated myotubes and myofibers. Proliferation and differentiation of muscle cells occur
41 similarly during development and postnatal birth (Schiaffino et al., 2013). Therefore, promotion of
42 myoblast proliferation and differentiation and induction of myotube hypertrophy should be beneficial
43 for muscle regeneration and control of muscle mass.

44 Muscle atrophy with aging is accompanied by muscle damage caused by oxidative damage and
45 malnutrition caused by lack of muscle metabolism. Thus, muscle atrophy is triggered by increasing
46 muscle protein degradation and decreased protein synthesis (Jackson et al., 2007; Zhang et al., 2018).
47 In recent studies, activation of AMPK increased muscle atrophy F-box (MAFbx)/atrogen-1 and muscle
48 RING finger-1 (MuRF-1) via the transcription factor forkhead box O3a (FoxO3a) (Jaitovich et al.,
49 2015). This process has been reported to be directly involved in muscle atrophy by muscle protein
50 degradation through the activation of the ubiquitin-proteasome pathway (Jaitovich et al., 2015;
51 Nakashima, 2008; Tong et al., 2009). Since muscle mass represents a balance between muscle cell
52 replication, protein synthesis, muscle cell death and protein degradation (Scicchitano et al., 2018),
53 increased muscle mass can prevent muscle atrophy by causing an increase in muscle differentiation and
54 inhibiting muscle loss.

55 Deer antler (*Cervi Parvum Cornu*) is a non-osteolytic horn of *Cervus nippon* Temminck or Marcus
56 (*Cervus elaphus* L) and has long been used in oriental countries, such as China and Korea. Deer antler
57 has been reported to contain hexose, pentose, uronic acid, sialic acid, free amino acids, minerals,
58 prostaglandins, and gangliosides in glycolipids (Jhon et al., 1999; Sunwoo et al., 1995). Water-soluble
59 proteins, polypeptides, and free amino acids have been shown to be the main biologically active
60 components of deer antler (Moreau et al., 2004). In addition, there is evidence that deer antler extract
61 has strong potential for promoting bone growth and development (Chen et al., 2015; Shi et al., 2010).
62 Additionally, Chen et al. (2014) estimated through microarray analysis that the anti-fatigue effect of
63 collagen and protein from deer antler extracts is mediated by the increased expression of genes involved
64 in muscle contraction and development.

65 By evaluating the effect of strengthening muscle differentiation and inhibiting muscle atrophy by
66 antler extract having various activities, it was attempted to investigate the possibility of application as
67 a functional material to suppress muscle atrophy due to aging. Therefore, the effects of deer antler
68 extract on the promotion of myoblast differentiation and AICAR-induced muscle atrophy were
69 examined using C2C12 fibroblast.

70

71 **Materials and methods**

72 **Materials**

73 Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (HS), and
74 penicillin/streptomycin (PS) were purchased from WELGENE (Daegu, Korea) and were used for cell
75 culture and differentiation. AICAR and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
76 (MTT) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dried antler (*Cervus*
77 *canadensis*) from adult male Korean elk was obtained from Kwang Dong Pharmaceutical Co., (Seoul,
78 Korea). According to the previous report, antler water extract (HWE) and fermented antler (FE) were

79 prepared. The antler powder was extracted by refluxing for 3 h at 95°C after adding 8 times the amount
80 of water. Fermented antler was extracted by adding 8 times the amount of phosphate buffer (PBS, 50
81 mM, pH 6.0) the antler powder, fermenting it with *Bacillus subtilis* for 48 h, and refluxing for 3 h at
82 95°C. The ultrasonic extract (UE) was extracted by adding 8 times the amount of PBS to the antler
83 powder for 30 minutes at 120 kHz ultrasonic waves (AUG-R3-900, ASIA ULTRASONIC, Korea). After
84 ultrasonic extraction, reflux extraction was performed at 95°C to obtain an ultrasonic extract. After
85 adding 8 times the amount of PBS (50 mM, pH 7.8) to the antler powder and suspending it, Alcalase
86 equivalent to 0.5% of the antler powder was added, followed by hydrolysis at 50°C for 8 h. After
87 hydrolysis, reflux extraction was performed at 95°C to obtain an antler enzyme hydrolysate (ET). The
88 antler extracts were concentrated and lyophilized to be used in the further experiment.

89 **Analysis of collagen**

90 According to the Sunwoo et al (1998) method, the collagen content was calculated by multiplying
91 the content of hydroxyproline by 7. According to the method of Stegemann and Stalder (1967), the
92 content of hydroxyproline before and after acid hydrolysis with 6 N HCl was measured using HPLC. A
93 Phenomenex Luna C18 Column (250 × 4.6 mm, 5 µm) was employed, using following mobile phase:
94 sodium acetate buffer (pH 4.3) containing 3% glacial acetic acid and acetonitrile were mixed with 650
95 and 350 mL, respectively. The flow rate was 1 mL/min, hydroxyproline was identified at a wavelength
96 of ex 260/em 330 nm, and the injection quantity was 20 µL. The collagen content was calculated by
97 multiplying the difference by 7 to the hydroxyproline content before and after acid hydrolysis.

98 **Cell culture and differentiation**

99 The myoblast cell line C2C12 used in this study was purchased from the American Type Culture
100 Collection (Manassas, VA, USA). C2C12 myoblasts were maintained at 37°C and 5% CO₂ using a
101 growth medium containing 90% DMEM, 10% FBS, and 100 unit/mL PS. To maintain cells at an
102 appropriate density, they were passaged every 48 h. Differentiation medium containing 90% DMEM,

103 10% HS, and 100 unit/mL PS was used to differentiate C2C12 myoblasts into myotubes. To confirm
104 the morphological changes during the differentiation process, an inverted microscope (Inverted
105 microscope, Carl Zeiss, Gottingen, Germany) was used to observe cells at a magnification of 200 \times .

106 **Cell viability**

107 The cell viability of C2C12 cells was measured using an MTT assay. C2C12 cells were seeded into
108 a 96-well plate at a concentration of 1×10^6 cells/mL the day before the experiment. Deer antler extracts
109 (0-1,000 μ g/mL) were incubated with cells for 24 h. Subsequently, the culture supernatant was discarded
110 and 5 mg/mL MTT dissolved in DMEM was added to each well; the plate was then covered with
111 aluminum foil to create dark conditions and incubated for 2 h. After removing the MTT solution, 100
112 μ L of dimethyl sulfoxide was added to the cells and left for 2 h. The absorbance was measured at 540
113 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

114 **Jenner–Giemsa staining and measurement of myotube length and diameter**

115 Myotube length and diameter were measured using images obtained through Jenner-Giemsa staining
116 of C2C12 differentiated cells (Velica and Bunce, 2011). The cultured cells were washed with PBS, fixed
117 with 100% methanol for 5 min, and air dried for 10 min. The Jenner staining solution (Sigma-Aldrich,
118 St. Louis, MO) was diluted 3-fold with 1 mM sodium phosphate buffer (pH 5.6); 1 mL of this solution
119 was incubated in the wells for 5 min and then the cells were washed with distilled water. The cells were
120 then incubated for 10 min at 25 $^{\circ}$ C with 1 mL Giemsa stain diluted 1:20 with 1 mM sodium phosphate
121 buffer (pH 5.6) and then washed again with water. Cells in each well were observed using a MM-400
122 microscope (Nikon; Kawasaki, Kanagawa, Japan) and photographed in nine randomly selected areas
123 using a digital microscope camera JENOPTIK ProgRes speed Xt^{core} 3 and the ProgRes Image Capture
124 Software (Jenoptik Optical Systems GmbH, Jena, Germany). Myotube length and diameter were
125 measured and quantified using the Image-J software (Scion, Frederick, MD).

126 **RNA isolation and quantitative real-time reverse-transcription-polymerase chain reaction (qRT-**

127 **PCR)**

128 Total RNA from C2C12 cells was extracted using the TRIzol® reagent (Invitrogen, CA, USA),
129 according to the manufacturer's protocol. Quality controlled RNA samples with high optical density
130 ratios were treated with RQ1 RNase-free DNase I (Promega, WI, USA). One microgram of total RNA
131 was reverse transcribed using SuperScript® III Reverse Transcriptase (Invitrogen) with oligo d(T)
132 primers. qRT-PCR was performed using the Taqman Gene Expression Master Mix (Applied Biosystems,
133 CA, USA), and quantitative analyses were conducted using the StepOne plus Software V. 2.0 (Applied
134 Biosystems). All results were normalized against the expression of the control gene, GAPDH
135 (NM_008084.2), using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). The information of primers for
136 the qRT-PCR of tested genes related to muscle differentiation and atrophy were as follows: myogenin
137 differentiation 1 (MyoD1) (NM_010866.2), myogenic factor 5 (Myf5) (NM_008656.5), AMP-activated
138 protein kinase (AMPK) (NM_001013367.3), forkhead box O3 (FoxO3a) (NM_019740.2), muscle
139 RING-finger protein 1 (MuRF-1) (NM_001039048.2), and myogenin (NM_031189.2).

140 **Statistical analysis**

141 All experimental results were obtained using the SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA)
142 statistical program. Data represent the mean \pm standard deviation (SD). Differences among the groups
143 were evaluated by one-way analysis of variance (ANOVA) and Tukey's multiple test. Student's *t*-test
144 was used to analyze differences between groups with normally distributed data.

145 **Results and discussion**

146 **Collagen composition and C2C12 cells viability**

147 As a result of analyzing the collagen content of HWE, ET, UE and FE, it was 20.09 ± 0.19 , $29.07 \pm$
148 0.12 , 27.32 ± 0.09 , 23.58 ± 0.11 $\mu\text{g}/\text{mg}$ of extract, respectively, and it was confirmed that the collagen
149 content of the extract increased due to fermentation, enzyme, and ultrasonication treatment (Table 1).
150 In particular, the content of hydroxyproline (4.15 ± 0.02 $\mu\text{g}/\text{mg}$ of extract) and protein (295.05 ± 11.22

151 mg/g of extract) as well as collagen in ET was higher than that of other antler extracts.

152 Deer antler extracts, including HWE, FE, ET, and UE were used for the evaluation of myoblast
153 C2C12 cells differentiation activity. These extracts had protein contents of 225.1-295.1 mg/g extract
154 (data not shown). Upon evaluation of the cytotoxicity of the antler extracts against C2C12 cells (Fig.
155 1), all extracts except the ultrasonic extract (UE) did not show a cytotoxicity of up to 1000 µg/mL. UE-
156 treated cells showed a cell viability of 90.7-88.9% upon treatment with 200-1000 mg/mL extract. Deer
157 antler extracts were found to have no or low cytotoxicity at 1000 µg/mL. Therefore, further experiments
158 were conducted using up to 200 µg/mL of extracts, with little cytotoxicity.

159 Deer antler extracts have been widely used in traditional Chinese medicine for centuries and are
160 generally believed to provide nourishment, strengthen the kidneys, strengthen the spleen, strengthen
161 bones and muscles, and promote blood flow (Wu et al., 2013). Previous studies have reported that the
162 collagen and protein components of deer antler extracts are major bioactive substances with effects,
163 such as anti-fatigue, anti-stress (Fengyan et al., 2001), and bone growth effects (Niu et al., 2012) and
164 stimulation of hematopoietic function (Lee et al., 2012). Kitakaze et al. (2016) reported that collagen
165 hydrolysates promoted differentiation and increased size of myoblast C2C12 cells. Hydroxypropyl-
166 glycine, a collagen hydrolysate, has been reported to induces myogenic differentiation and root canal
167 hypertrophy by increasing the size of the root canal and the expression of root canal specific MyHC
168 and tropomyosin structural proteins. Hyp-Gly presumably promotes myogenic differentiation by
169 activating the PI3K/Akt/mTOR signaling pathway according to peptide/histidine transporter 1 for entry
170 into cells (Kitakaze et al., 2016; Li et al., 2016; Niu et al., 2016).

171 **Changes in myotube length and diameter caused by C2C12 cells differentiation**

172 To determine the degree of myotube differentiation during C2C12 cells differentiation, Jenner-
173 Giemsa staining was performed on the second and fourth days of C2C12 cells differentiation to measure
174 changes in myotube morphology, such as myotube length and diameter (Fig. 2 and 3).

175 On the second day of cell differentiation, myotube length was increased in cells treated with deer
176 antler extracts compared with the control group. On the fourth day, myotube length was increased in
177 cells treated with all deer antler extracts except for FE. The cells treated with FE showed increased
178 myotube length at extract concentrations of 50 and 100 µg/mL and shortened myotube length when
179 treated with extract concentration of 200 µg/mL (Fig. 3).

180 On day two of cell differentiation, cells treated with deer antler extract showed a similar diameter to
181 that of control group, but on day four of cell differentiation, cells showed a tendency to have increased
182 myotube diameter compared to control group (Fig. 3). Upon treatment of cells with deer antler extract,
183 myotube length was increased by promoting the myotube differentiation capacity, and the myotube
184 diameter also tended to increase with increasing myotube length.

185 In the differentiation phase, the length of myoblasts become increased, fused with localized cells and
186 converted into tubular multinuclear cells. When differentiation is induced from myoblasts to myotubes,
187 the number of muscle fibers increases due to the fusion between cells and the thickening of muscle
188 fibers also occurs (Bentzinger et al., 2012; Burattini et al., 2004). Net protein balance affects the size of
189 individual myotubes and myofibers, where an increase in net protein levels leads to muscle hypertrophy
190 (White et al., 2010) and contributes to an increase in skeletal muscle mass (Ontell et al., 1984).

191 **Effect of deer antler extract on the expression of myogenic differentiation-related genes in C2C12** 192 **myoblasts**

193 Muscle differentiation is regulated by myogenic regulatory factors (MRFs), such as MyoD1 and
194 Myf5 (Braun et al., 1992; Hyatt et al., 2003). The effect of the antler extract on the expression of muscle
195 differentiation factors MyoD1 and Myf5 was measured on days two and four of C2C12 cell
196 differentiation, respectively (Fig. 4). The expression levels of MyoD1 were not significantly affected
197 by myoblast differentiation on days two and four, whereas Myf5 expression levels were high on days
198 two and four compared with the control group. Deer antler extracts significantly increased the
199 expression level of Myf5 ($p < 0.05$). In particular, it was found that the level of Myf5 increased in a

200 concentration-dependent manner when the HWE was added on day four of myogenic differentiation.
201 However, increasing concentrations of UE and FE tended to decrease the expression level of Myf5.
202 These results indicated that antler extract was not only involved in increasing the size (length and
203 diameter) of myotubes, but also in increasing the expression level of myogenic differentiation factor
204 that promotes myoblast C2C12 cell differentiation.

205 Muscle differentiation is regulated mainly by myogenic regulatory factors (MRFs), such as MyoD1
206 and Myf5, which are involved in establishing myogenic lineages (Braun et al., 1992). Later in muscle
207 differentiation, there is an increase in the expression of myosin heavy chain (MyHC), a major structural
208 protein of myotubes (Soundharajan et al., 2019). Deer antler extracts were also found to be involved
209 in the increase in the expression levels of muscle differentiation factors (Fig. 4), which may increase
210 the size of the myotubes (Fig. 3).

211 **Inhibitory activity of deer antler extracts on muscle atrophy factors in AICAR-induced muscle** 212 **atrophied C2C12 cells**

213 As a result of a 0.25-2 mM treatment of AICAR to create a muscle atrophy model in myoblast cells,
214 AMPK expression increased in an AICAR concentration-dependent manner (Fig. 5). C2C12 cells
215 showed no cytotoxicity when treated with 2 mM of AICAR (data not shown). Therefore, C2C12 cells
216 were treated with 2 mM AICAR to induce muscle atrophy, and the effect of the inhibition of muscle
217 atrophy through the addition of antler extracts was confirmed.

218 The effects of deer antler extracts (50 and 200 µg/mL) on the expression levels of muscle atrophy
219 factors (FoxO3a and MuRF-1) were measured in AICAR-induced muscle-atrophy cells (Fig. 6). Upon
220 AICAR treatment, the expression of AMPK increased in C2C12 cells and deer antler extract-treated
221 muscle-atrophy cells compared to the control group (CON). The expression levels of AMPK in deer
222 antler extract-treated cells were higher than those in cells treated with AICAR alone (CON group). The
223 muscle atrophy factors FoxO3a and MuRF-1 showed significantly higher expression levels in the

224 AICAR treated group (CON) than in the normal group (NOR). In deer antler extract-treated muscle-
225 atrophied cells, the increase in the expression level of the muscle atrophy factor MuRF-1 tended to be
226 lower than that in the CON group cells. In particular, the expression level of FoxO3a in deer antler
227 extract-treated atrophied cells was significantly lower than that in cells treated with AICAR alone ($p <$
228 0.05).

229 Recent studies have shown that muscle atrophy occurs due to increased proteolysis due to the
230 activation of the ubiquitin-proteasome pathway (Jagoe and Goldberg, 2001; Bodine and Baehr, 2014;
231 Liu et al., 2016). In particular, muscle atrophy F-box (MAFbx)/atrogen-1 and muscle RING finger-1
232 (MuRF-1), which are muscle-specific ubiquitin ligases, are expressed early in the muscle atrophy
233 process and are directly involved in muscle protein degradation (Bodine and Baehr, 2014; Liu et al.,
234 2016). In addition, when muscle atrophy is induced, expression of genes, such as myogenin and MyoD,
235 muscle-specific transcription factors involved in myogenic differentiation, is reduced by activating
236 muscle-specific gene expression (Hyatt et al., 2003; Tintignac et al., 2005).

237 **Inhibitory activity of deer antler extracts on muscle differentiation factors in AICAR-induced** 238 **muscle-atrophied C2C12 cells**

239 The expression levels of MyoD1 and myogenin, which are muscle differentiation factors, were lower
240 than those of normal cells upon treatment with AICAR alone (Fig. 7). In deer antler extract-treated
241 muscle-atrophied cells, the expression levels of muscle differentiation factors MyoD1 and myogenin
242 were higher than those in cells treated with AICAR alone. The expression levels of MyoD1 in the
243 enzyme-treated deer antler extract (ET, 200 $\mu\text{g}/\text{mL}$)-treated muscle-atrophied cells were significantly
244 higher than those of normal cells ($p < 0.05$). In addition, the expression levels of myogenin in the
245 enzyme-treated deer antler extract (ET, 50 and 200 $\mu\text{g}/\text{mL}$) and fermented deer antler extract (FE, 200
246 $\mu\text{g}/\text{mL}$)-treated muscle-atrophied cells were significantly higher than that in normal cells. The treatment
247 of AICAR-induced atrophied cells with deer antler extract inhibited the expression of the muscle
248 atrophy factors FoxO3a and MuRF1. In addition, deer antler extract treatment restored the expression

249 levels of myoD1 and myogenin to the same or higher levels as those of normal cells.

250 In Figure 6, muscle atrophy factors FoxO3a and MuRF-1 can be seen to be increased upon AICAR
251 treatment, and their increase results in muscle atrophy. In addition, in Figure 7, the expression levels of
252 MyoD and myogenin, which are myogenic factors, can be seen to decrease upon AICAR treatment.
253 However, MuRF-1 expression decreased upon treatment with antler extract, and the expression factor
254 of myogenesis markers increased. Deer antler extracts seemed to contribute more to the inhibition of
255 muscle atrophy by increasing the expression of muscle differentiation factors than by decreasing the
256 expression of muscle atrophy factors.

257 The increase in skeletal muscle mass occurs due to an increase in muscle protein synthesis (MPS)
258 compared to muscle protein breakdown (Rennie, 2007). To increase skeletal muscle or inhibit muscle
259 loss, it is important to have both an increase in myogenic capacity and an inhibition of muscle loss.
260 Therefore, the supply of high-quality dietary protein, which is an essential nutrient for promoting
261 muscle and overall metabolic health, is important (Arentson-Lantz et al., 2015). Deer horn extract is an
262 excellent source of protein that is especially rich in collagen type I (Li et al., 2016). Therefore, the active
263 substances of antler extract to myogenesis and inhibit the muscle atrophy are presumed to be protein,
264 collagen and hydroxyproline. It has also been reported that collagen breakdown products, which form
265 a major component of antlers, are involved in the activation of mTORC1 and muscle protein synthesis.
266 The mechanistic/mammalian target of rapamycin (mTORC1) pathway is the central molecular pathway
267 of muscle protein synthesis and is activated by a variety of stimuli, including, but not limited to,
268 resistance exercise (Bolster et al., 2003), insulin (Conejó et al., 2001), and dietary amino acids (Gordon
269 et al., 2013). We found four distinct effects of treating C2C12 cells with antler extract: 1) increase in
270 myotube size, 2) increase in muscle differentiation factor (MyoD1 and Myf5) expression, 3) suppression
271 of muscular atrophy factor (FoxO3a and MuRF-1) expression, and 4) increase in the expression of the
272 muscle differentiation factors (MyoD1 and myogenin), in a muscle atrophy model.

273 **Conclusion**

274 Several studies have reported that collagen, a major component of deer antler, helps myogenesis. In
275 this study, experimental results on C2C12 cells suggested that antler extract has the ability to inhibit
276 muscle atrophy and promote muscle differentiation by increasing the expression of myoblast
277 differentiation factors MyoD, Myf5 and myogenin. Further experiments will be conducted to identify
278 muscle differentiation promoting substances.

279

ACCEPTED

280 **References**

- 281 Arentson-Lantz E, Clairmont S, Paddon-Jones D, Tremblay A, Elango R. 2015. Protein: A nutrient in
282 focus. *Appl Physiol Nutr Metab* 40:755-761.
- 283 Bentzinger CF, Wang YX, Rudnicki MA. 2012. Building muscle: molecular regulation of myogenesis.
284 *Cold Spring Harb Perspect Biol* 4.
- 285 Bodine SC, Baehr LM, 2014. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and
286 MAFbx/atrogen-1. *Am J Physiol Endocrinol Metab* 307:469-484.
- 287 Bolster DR, Kubica N, Crozier SJ, Williamson DL, Farrell PA, Kimball SR, Jefferson LS. 2003.
288 Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following
289 acute resistance exercise in rat skeletal muscle. *J Physiol* 553:213-220.
- 290 Bonewald L. 2019. Use it or lose it to age: A review of bone and muscle communication. *Bone* 120:212-
291 218.
- 292 Braun T, Rudnicki MA, Arnold HH, Jaenisch R. 1992. Targeted inactivation of the muscle regulatory
293 gene Myf-5 results in abnormal rib development and perinatal death. *Cell* 71:369-382.
- 294 Burattini S, Ferri P, Battistelli M, Curci R, Luchetti E, Falcieri E. 2004. C2C12 murine myoblasts as a
295 model of skeletal muscle development: morpho-functional characterization. *Eur J Histochem* 48:
296 223-233.
- 297 Ceafalan LC, Popescu BO, Hinescu ME. 2014. Cellular players in skeletal muscle regeneration. *Biomed*
298 *Res Int* 957014.
- 299 Chen JR, Yang YF, Abbasi S, Hajinezhad D, Kontulainen S, Honaramooz A. 2015. The effects of elk
300 velvet antler dietary supplementation on physical growth and bone development in growing rats.
301 *Evid Based Complement Alternat Med* 819520.
- 302 Chen JC, Hsiang CY, Lin YC, Ho TY. 2014. Deer antler extract improves fatigue effect through altering
303 the expression of genes related to muscle strength in skeletal muscle of mice. *Evid Based*
304 *Complement Alternat Med* 540580.
- 305 Conejo R, Valverde AM, Benito M, Lorenzo M. 2001. Insulin produces myogenesis in C2C12

306 myoblasts by induction of NF-kappa B and downregulation of AP-1 activities. J Cell Physiol 186:
307 82-94.

308 Fengyan S, Huiping L, Yanmei W, Yuxia H, Souming X, Yuqi X. 2001. Protein component extraction
309 and its bioactivity determination of sika deer antler base. Anim Sci Vet Med 2.

310 Gordon BS, Kelleher AR, Kimball SR. 2013. Regulation of muscle protein synthesis and the effects of
311 catabolic states. Int J Biochem Cell Biol 45:2147-2157.

312 Grefte S, Kuijpers-Jagtman AM, Torensma R, Von den Hoff JW. Skeletal muscle development and
313 regeneration. Stem Cells Dev 16: 857-868.

314 Hyatt JPK, Roy RR, Baldwin KM, Edgerton VR. 2003. Nerve activity-independent regulation of
315 skeletal muscle atrophy: role of MyoD and myogenin in satellite cells and myonuclei. Am J Physiol
316 Cell Physiol 285:1161-1173.

317 Jackson MJ, Pye D, Palomero J, Kabayo T, Close G, Vasilaki A, McArdle A. 2007. Free radicals in
318 skeletal muscle: Essential signals or mediators of damage. Free Radic Res 41:S6.

319 Jagoe RT, Goldberg AL. 2001. What do we really know about the ubiquitin-proteasome pathway in
320 muscle atrophy? Curr Opin Clin Nutr Metab Care 4: 183-190.

321 Jaitovich A, Angulo M, Lecuona E, Dada LA, Welch LC, Cheng Y, Gusarova G, Ceco E, Liu C,
322 Shigemura M, Barreiro E, Patterson C, Nader GA, Sznajder JI. 2015. High CO₂ levels cause skeletal
323 muscle atrophy via AMP-activated kinase (AMPK), FoxO3a protein, and muscle-specific ring
324 finger protein 1 (MuRF1). J Biol Chem 290:9183-9194.

325 Jhon GJ, Park SY, Han SY, Lee S, Kim Y, Chang YS. 1999. Studies of the chemical structure of
326 gangliosides in deer antler, *Cervus nippon*. Chem Pharm Bull 47:123-127.

327 Karalaki M, Fili S, Philippou A, Koutsilieris M. 2009. Muscle regeneration: Cellular and molecular
328 events. In Vivo 23:779-796.

329 Kitakaze T, Sakamoto T, Kitano T, Inoue N, Sugihara F, Harada N, Yamaji R. 2016. The collagen
330 derived dipeptide hydroxyprolyl-glycine promotes C2C12 myoblast differentiation and myotube
331 hypertrophy. Biochem Biophys Res Commun 478:1292-1297.

332 Lee SH, Suh HJ, Lee HS, Park Y, Park JW, Jung EY. 2012. Hematopoietic effect of *Bacillus subtilis*-
333 fermented antler extract on phenylhydrazine-induced hemolytic anemia in Sprague-Dawley rats. *J*
334 *Med Food* 15:774-780.

335 Li N, Zhang M, Drummen GPC, Zhao Y, Tan YF, Luo S, Qu XB. 2016. Sika deer antler collagen type
336 I-accelerated osteogenesis in bone marrow mesenchymal stem cells via the Smad pathway. *Evid*
337 *Based Complement Alternat Med* 2109204.

338 Liu Y, Du MT, Li X, Chen L, Shen QW, Tian JW, Zhang DQ. 2016. Role of the ubiquitin-proteasome
339 pathway on proteolytic activity in postmortem proteolysis and tenderisation of sheep skeletal
340 muscle. *Int J Food Sci Tech* 51:2353-2359.

341 Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative
342 PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 25:402-408.

343 Moreau M, Dupuis J, Bonneau NH, Lécuyer M. 2004. Clinical evaluation of a powder of quality elk
344 velvet antler for the treatment of osteoarthritis in dogs. *Can Vet J* 45:133-139.

345 Nakashima K. 2008. AMPK activation stimulates myofibrillar protein degradation and expression of
346 atrophy-related ubiquitin ligases by increasing FOXO transcription factors in C2C12 myotubes.
347 *Biosci Biotechnol Biochem* 72:1650-1656.

348 Niu F, Zhao Y, Xu Y, Zhang H, Lin Y. 2012. Therapeutic effects of collagen of antler plate on
349 osteoporosis in ovariectomized rats. *Chin J Mod Appl Pharm* 2.

350 Ontell M, Feng KC, Klueber K, Dunn RF, Taylor F. 1984. Myosatellite cells, growth, and regeneration
351 in murine dystrophic muscle: a quantitative Study. *Anat Rec* 208:159-174.

352 Rennie MJ. 2007. Exercise- and nutrient-controlled mechanisms involved in maintenance of the
353 musculoskeletal mass. *Biochem Soc Trans* 35:1302-1305.

354 Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M. 2013. Mechanisms regulating skeletal muscle
355 growth and atrophy. *FEBS J* 280:4294-4314.

356 Scicchitano BM, Dobrowolny G, Sica G, Musaro A. 2018. Molecular insights into muscle homeostasis,
357 atrophy and wasting. *Curr Genomics* 19:356-369.

358 Stegemann H, Stalder K. 1967. Determination of hydroxyproline. Clin Chim Acta 18:267-273.

359 Sunwoo HH, Nakano T, Sim JS. 1998. Isolation and characterization of proteoglycans from growing
360 antlers of wapiti (*Cervus elaphus*). Comp Biochem Physiol B Biochem Mol Biol 121:437-442.

361 Shi B, Li G, Wang P, Yin W, Sun G, Wu Q, Yu G. 2010. Effect of antler extract on corticosteroid-induced
362 avascular necrosis of the femoral head in rats. J Ethnopharmacol 127:124-129.

363 Soundharrajan I, Kim DH, Kuppusamy P, Choi KC. 2019. Modulation of osteogenic and myogenic
364 differentiation by a phytoestrogen formononetin via p38MAPK-dependent JAK-STAT and Smad-
365 1/5/8 signaling pathways in mouse myogenic progenitor cells. Sci Rep 9:9307.

366 Sunwoo HH, Nakano T, Hudson RJ, Sim JS. 1995. Chemical-composition of antlers from wapiti
367 (*Cervus-elaphus*). J Agric Food Chem 43:2846-2849.

368 Szentesi P, Csernoch L, Dux L, Keller-Pintér A. 2019. Changes in redox signaling in the skeletal muscle
369 with aging. Oxid Med Cell Longev 4617801.

370 Tintignac LA, Lagirand J, Batonnet S, Sirri V, Leibovitch MP, Leibovitch SA. 2005. Degradation of
371 MyoD mediated by the SCF (MAFbx) ubiquitin ligase. J Biol Chem 280:2847-2856.

372 Tong JF, Yan X, Zhu MJ, Du M. 2009. AMP-activated protein kinase enhances the expression of
373 muscle-specific ubiquitin ligases despite its activation of IGF-1/Akt signaling in C2C12 Myotubes.
374 J Cell Biochem 108:458-468.

375 Velica P, Bunce CM. 2011. A quick, simple and unbiased method to quantify C2C12 myogenic
376 differentiation. Muscle Nerve 44:366-370.

377 White RB, Bierinx AS, Gnocchi VF, Zammit PS. 2010. Dynamics of muscle fibre growth during
378 postnatal mouse development. BMC Dev Biol 10:21.

379 Wu F, Li H, Jin L, Li X, Ma Y, You J, Li S, Xu Y. 2013. Deer antler base as a traditional Chinese
380 medicine: a review of its traditional uses, chemistry and pharmacology. J ethnopharmacol 145:403-
381 415.

382 Zhang Y, Li CZ, Zhang H, Li WH, Li JW, Wang XB. 2018. Color-coded digital subtraction angiography

383 for assessing acute skeletal muscle ischemia-reperfusion injury in a rabbit model. Acad Radiol 25:
384 1609-1616.
385

ACCEPTED

386 **Figure legends**

387 **Fig. 1. Cell viability of deer antler extracts on C2C12 cells. CON, normal control; HWE, hot water**
388 **extract; ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE:**
389 **how water extract of fermented deer antler. Values are presented as the mean \pm standard deviation**
390 **(SD) for each group.**

391 **Fig. 2. Changes in myotube structure during C2C12 cell differentiation, as observed through**
392 **Jenner-Giemsa staining. CON, normal control; HWE, hot water extract; ET, enzyme-derived**
393 **extract, UE: extract prepared by ultrasonication of deer antler, FE: how water extract of**
394 **fermented deer antler. Values are presented as the mean \pm standard deviation (SD) for each group.**

395 **Fig. 3. Changes in myotube length and diameter during C2C12 cell differentiation. CON, normal**
396 **control; HWE, hot water extract; ET, enzyme-derived extract, UE: extract prepared by**
397 **ultrasonication of deer antler, FE: how water extract of fermented deer antler. Values are**
398 **presented as the mean \pm standard deviation (SD) for each group. The different letters indicate**
399 **statistically significant ($p < 0.05$) differences among groups, as determined through Tukey's test.**

400 **Fig. 4. Effects of deer extracts on the relative expression of MyoD1 and Myf5 mRNA in C2C12**
401 **cells at two and four days of cell differentiation. CON, normal control; HWE, hot water extract;**
402 **ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE: how**
403 **water extract of fermented deer antler. Values are presented as the mean \pm standard deviation**
404 **(SD) for each group. The different letters indicate statistically significant ($p < 0.05$) differences**
405 **among groups, as determined by Tukey's test.**

406 **Fig. 5. Effects of AICAR on relative AMPK mRNA expression in C2C12 cells. Values are**
407 **presented as the mean \pm standard deviation (SD) for each group. The different letters indicate**
408 **statistically significant ($p < 0.05$) differences among groups, as determined by Tukey's test.**

409 **Fig. 6. Effects of deer antler extract on relative mRNA expression of AMPK, FoxO3a, and MuRF-**

410 1 in AICAR-induced muscle-atrophied C2C12 cells. CON, normal control; HWE, hot water
411 extract; ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE:
412 hot water extract of fermented deer antler. Values are presented as the mean \pm standard deviation
413 (SD) for each group. Asterisks indicate significant differences: *** $p < 0.001$ indicates significance
414 of difference compared with the control, as determined by Student's *t* test. The different letters
415 indicate statistically significant ($p < 0.05$) differences among groups, as determined by Tukey's
416 test.

417 Fig. 7. Effects of deer antler extract on relative mRNA expression of MyoD1 and Myogenin in
418 AICAR-induced muscle atrophy in C2C12 cells. CON, normal control; HWE, hot water extract;
419 ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE: hot
420 water extract of fermented deer antler. Values are presented as the mean \pm standard deviation
421 (SD) for each group. Asterisks indicate significant differences: *** $p < 0.001$ indicates significance
422 of difference compared with the control, as determined by Student's *t* test. The different letters
423 indicate statistically significant ($p < 0.05$) differences among groups, as determined by Tukey's
424 test.

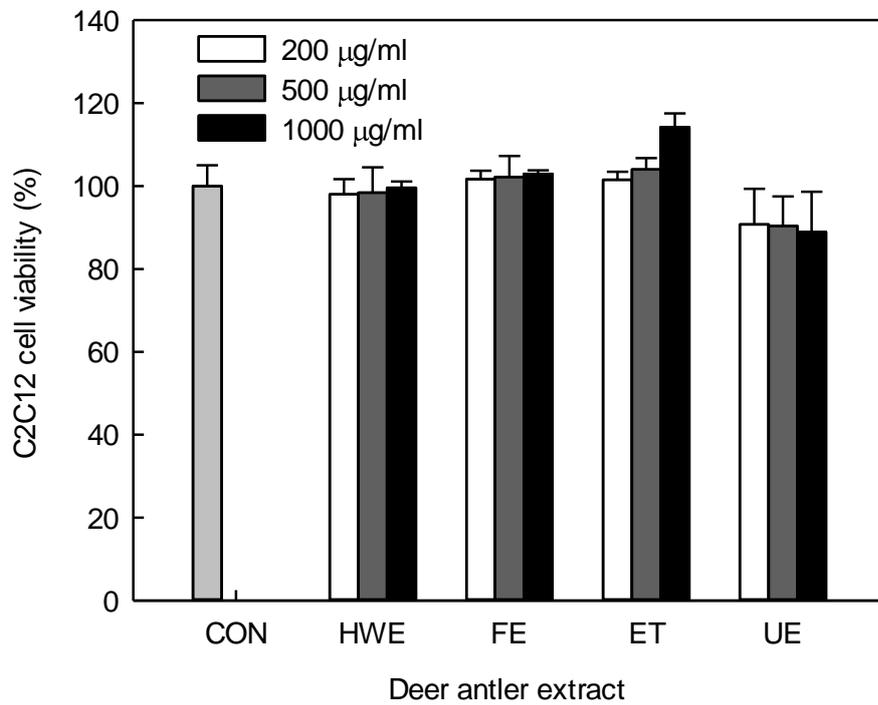
425 **Table 1. Protein, hydroxyproline, and collagen content in antler extracts**

Sample	Protein (mg/g)	Hydroxyproline ($\mu\text{g}/\text{mg}$)	Collagen ($\mu\text{g}/\text{mg}$)
HWE	240.66 \pm 10.31	2.87 \pm 0.03	20.09 \pm 0.19
ET	295.05 \pm 11.22	4.15 \pm 0.02	29.07 \pm 0.12
UE	225.08 \pm 7.22	3.90 \pm 0.01	27.32 \pm 0.09
FE	284.68 \pm 6.15	3.37 \pm 0.02	23.58 \pm 0.11

426 Content value represents the mean \pm SD.

427

ACCEPTED



428

429 **Fig. 1. Cell viability of deer antler extracts on C2C12 cells. CON, normal control; HWE, hot water**
 430 **extract; ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE:**
 431 **how water extract of fermented deer antler. Values are presented as the mean \pm standard deviation**
 432 **(SD) for each group.**

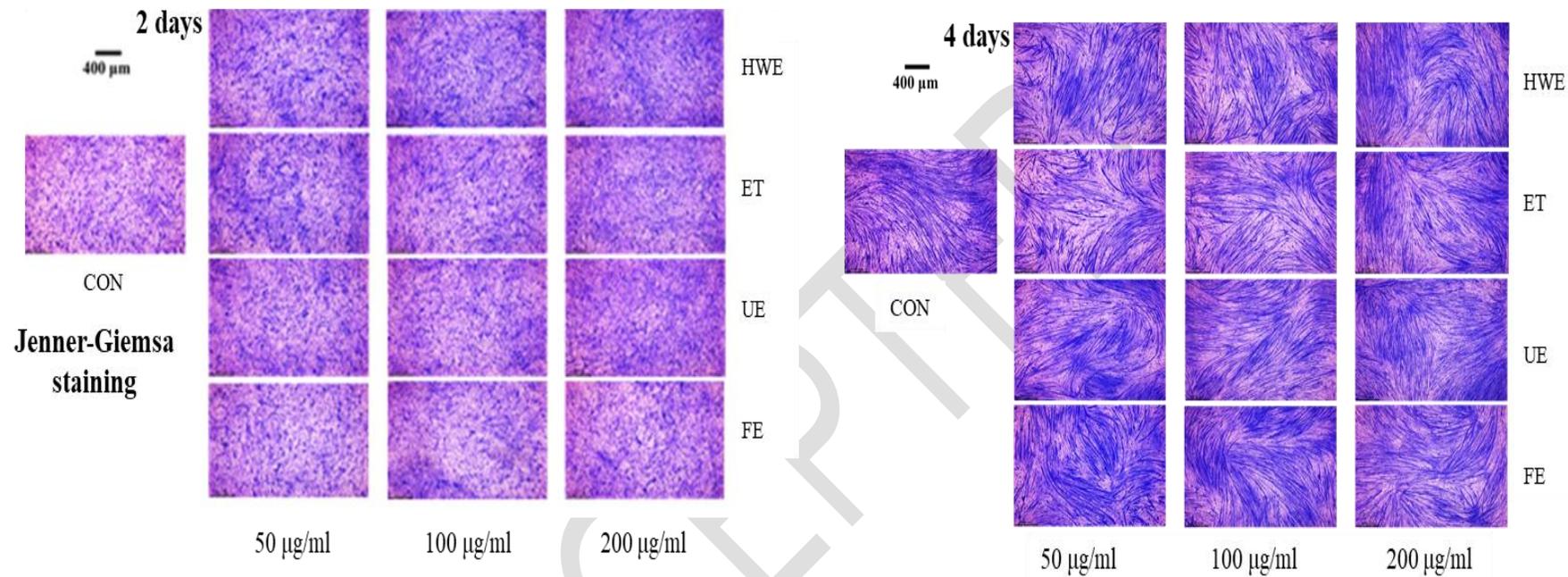


Fig. 2. Changes in myotube structure during C2C12 cell differentiation, as observed through Jenner-Giemsa staining. CON, normal control; HWE, hot water extract; ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE: hot water extract of fermented deer antler. Values are presented as the mean \pm standard deviation (SD) for each group.

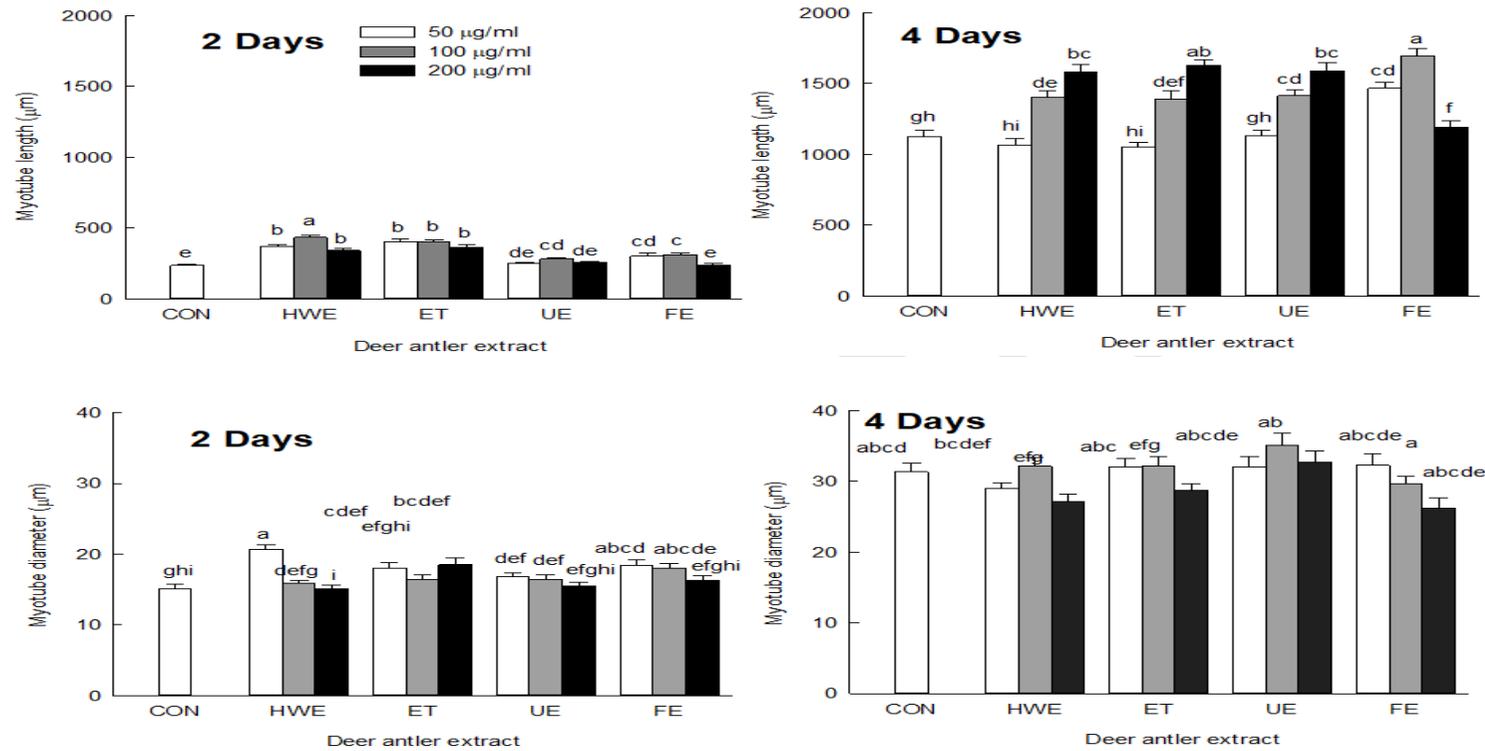


Fig. 3. Changes in myotube length and diameter during C2C12 cell differentiation. CON, normal control; HWE, hot water extract; ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE: hot water extract of fermented deer antler. Values are presented as the mean \pm standard deviation (SD) for each group. The different letters indicate statistically significant ($p < 0.05$) differences among groups, as determined through Tukey's test.

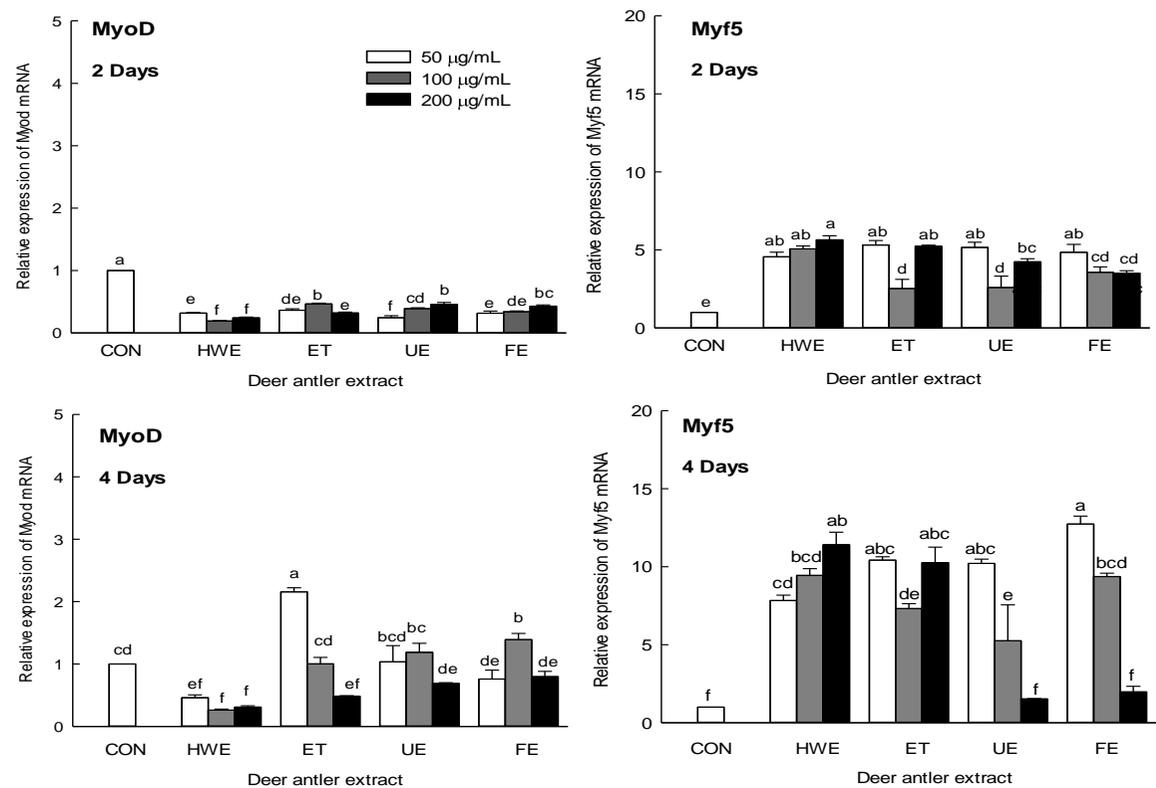


Fig. 4. Effects of deer extracts on the relative expression of MyoD1 and Myf5 mRNA in C2C12 cells at two and four days of cell differentiation. CON, normal control; HWE, hot water extract; ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE: how water extract of fermented deer antler. Values are presented as the mean \pm standard deviation (SD) for each group. The different letters indicate statistically significant ($p < 0.05$) differences among groups, as determined by Tukey's test.

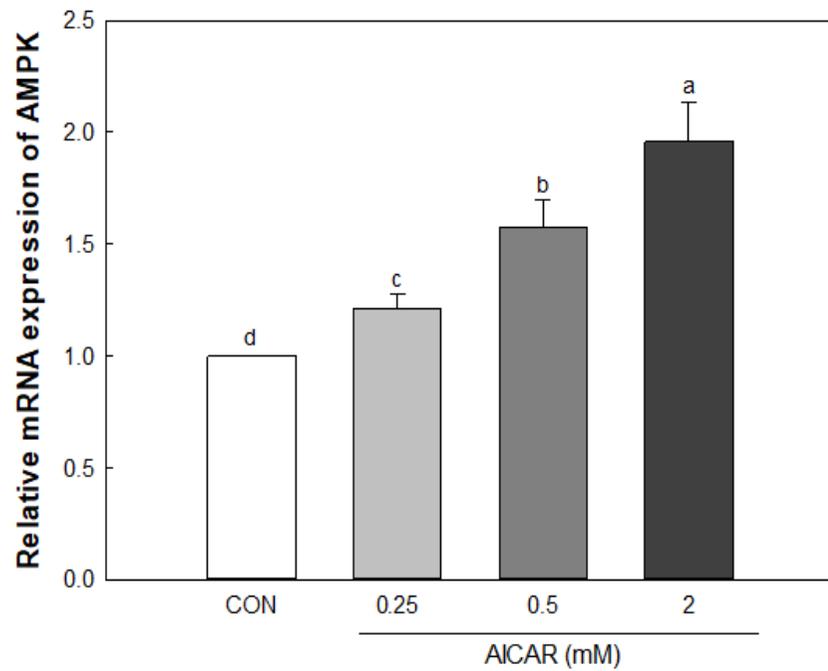


Fig. 5. Effects of AICAR on relative AMPK mRNA expression in C2C12 cells. Values are presented as the mean \pm standard deviation (SD) for each group. The different letters indicate statistically significant ($p < 0.05$) differences among groups, as determined by Tukey's test.

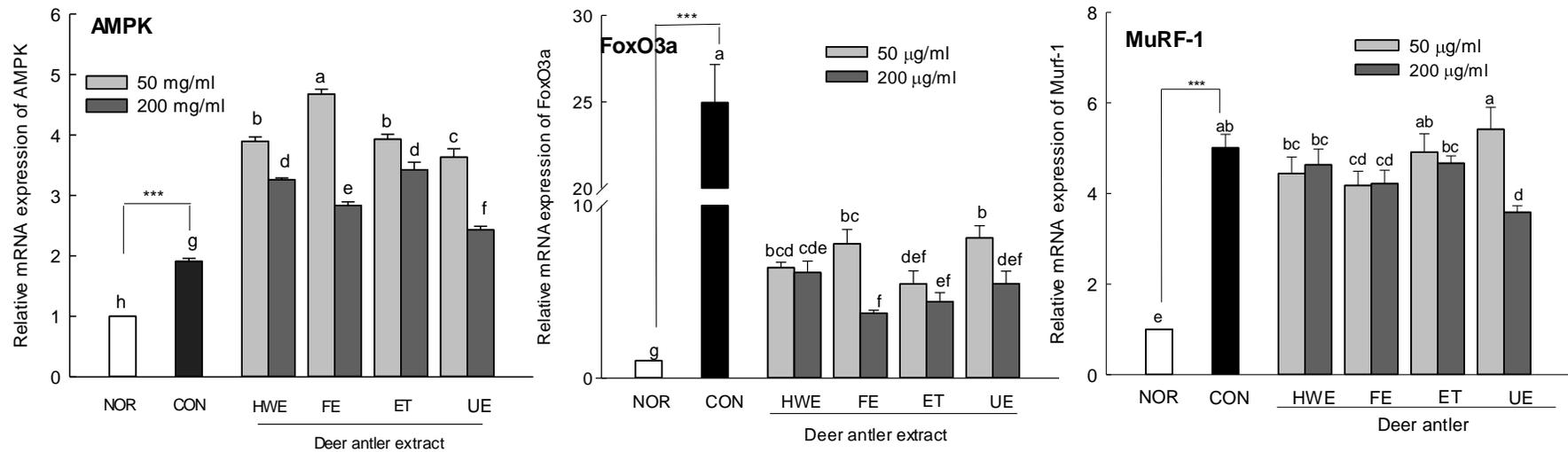


Fig. 6. Effects of deer antler extract on relative mRNA expression of AMPK, FoxO3a, and MuRF-1 in AICAR-induced muscle-atrophied C2C12 cells. CON, normal control; HWE, hot water extract; ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE: hot water extract of fermented deer antler. Values are presented as the mean \pm standard deviation (SD) for each group. Asterisks indicate significant differences: *** $p < 0.001$ indicates significance of difference compared with the control, as determined by Student's t test. The different letters indicate statistically significant ($p < 0.05$) differences among groups, as determined by Tukey's test.

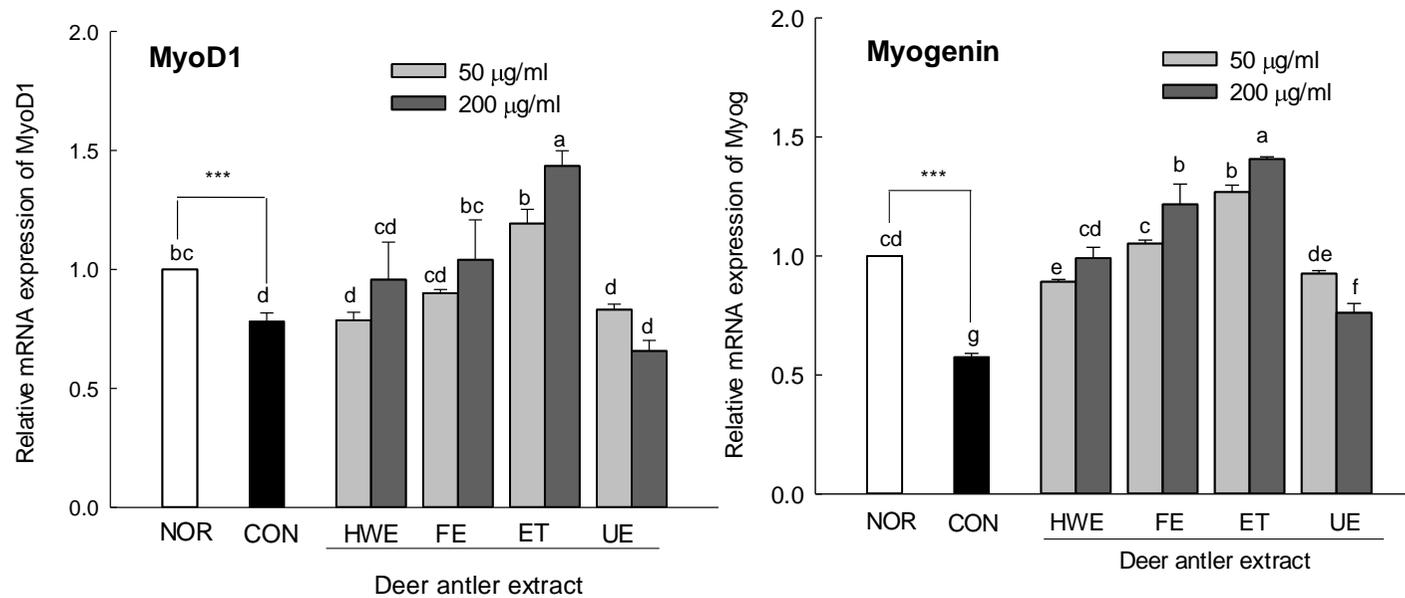


Fig. 7. Effects of deer antler extract on relative mRNA expression of MyoD1 and Myogenin in AICAR-induced muscle atrophy in C2C12 cells. CON, normal control; HWE, hot water extract; ET, enzyme-derived extract, UE: extract prepared by ultrasonication of deer antler, FE: how water extract of fermented deer antler. Values are presented as the mean \pm standard deviation (SD) for each group. Asterisks indicate significant differences: *** $p < 0.001$ indicates significance of difference compared with the control, as determined by Student's t test. The different letters indicate statistically significant ($p < 0.05$) differences among groups, as determined by Tukey's test.