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Original Research Article

Effect of 20-hydroxyecdysone and its metabolites in the absence or presence of IGF-1 on regulation of skeletal muscle cell growth

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Abstract

Purpose: *To investigate the effect of 20-hydroxyecdysone (20E) and its metabolites and their synergistic effect with IGF-1 on regulation of skeletal muscle cell growth.*
Methods: *Mouse skeletal muscle cell line (C2C12) was solely treated with 20E and its metabolites (14-deoxy-20-hydroxyecdysone, poststerone, and 14-deoxypoststerone) at doses of 0.1, 1, and 10 μM or co-treated with IGF-1 (10 ng/ml). Cell viability and proliferative capacity were evaluated using MTT and BrdU incorporation assays, respectively. Myogenic differentiation proteins [embryonic myosin heavy chain (EbMHC) and MHC], androgen receptor (AR), and IGF-1 receptor (IGF-1R) protein expression were investigated using immunocytochemistry.*
Results: *Treatments of 20E and its metabolites had no toxicity on skeletal muscle cells or induced AR/IGF-1R expression. In addition, solely treatment of 20E and its metabolites or co-treatment with IGF-1 had no significant effect on cell proliferation and myogenic differentiation capacity. In contrast, IGF-1 treatment alone significantly increased EbMHC expression ($p<0.0001$), MHC expression ($p<0.05$), and myotube number ($p<0.05$).*
Conclusion: *These results indicate that 20E and its metabolites have no direct or synergistic effect with IGF-1 on skeletal muscle cell growth. Nevertheless, the pharmacological effects of 20E on skeletal muscle mass and strength in vivo that raises its therapeutic potential may associate with its indirect action.*

Keywords: *20-hydroxyecdysone, androgen receptor, C2C12, IGF-1, metabolite, skeletal muscle, Vitex glabrata*

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INTRODUCTION

Ecdysteroids are arthropod steroid hormone that contribute significant roles in development and biological processes of insects via ecdysone receptor-mediated signal transduction [1]. Among ecdysteroids, 20-hydroxyecdysone (20E) is the most common found in arthropods whose

analogues were subsequently discovered in certain plant species, i.e., *Vitex glabrata* (http://ecdybase.org/) [2]. Since ecdysteroids are not endogenously synthesized in rodents and humans, its receptor is not present in mammalian species. Nevertheless, the pharmacological effects of 20E in mammals have been documented [3-6]. Various studies demonstrated the effects of 20E on skeletal muscle including increased muscle mass and fiber size [7-9], increased protein synthesis and associated protein signaling [10,11], increased myonuclear number during muscle regeneration [7], alleviated disuse muscle atrophy [12], and increased muscle strength [10].

Currently, the anabolic effect of 20E raises its therapeutic potential to increase muscle mass and strength. However, this effect originates from 20E itself is uncertain. Since the effect of 20E supplementation *in vitro* is transient [11] and it requires high volume with prolonged ingestion of 20E *in vivo* [9,10]. To support this notion, daily gavage of 20E at 50 mg/kg for 28 days increased grip strength in rats [10]. Additionally, skeletal muscle fiber size is increased in rats treated with 5 mg/kg of 20E by daily subcutaneous injection for 21 days [9]. On the contrary, oral ingestion with 200 mg/kg of 20E for a short period (4 h) in overnight fasted rats did not activate skeletal muscle protein synthesis signaling, i.e., phosphorylations of Akt, mTOR, and 4E-BP1 proteins [13]. Indeed, 90% of 20E injected intravenously was eliminated from blood plasma within 8.15 min in mice [14]. In addition, plasma 20E concentration in overnight fasted rat was detected at low bioavailability level after oral gavage of 20E at dose 200 mg/kg [13]. In humans, 20E had the effective half-life for 9 h [15] and its main metabolite (deoxyecdysone) was secreted within 21 h [16]. These cumulative evidences suggest the rapid metabolism of 20E with short half-life after administration into rodents and humans. To explore the metabolism of 20E in mammals, Kumpun and her colleague studied the metabolic fate of 20E after intraperitoneal (IP) injection in mice using radiolabeling technique. The results revealed 14-deoxy-20-hydroxyecdysone, poststerone, and 14-deoxypoststerone were the major 20E metabolites [17]. However, the effects of these 20E metabolites on skeletal muscle cell growth are unknown.

Furthermore, ecdysteroids have been reported to use as dietary natural compound for improving physical performance in the athletes by increasing skeletal muscle mass without androgenic side effects [18]. Recent study demonstrated that skeletal muscle hypertrophy after 20E ingestion positively correlated with increasing of serum insulin-like growth factor 1 (IGF-1) in rats [9]. Since the effect of 20E on skeletal muscle protein synthesis was proposed to mediate through rapid Ca^{2+} flux that activates Akt pathway [11] which is the downstream signaling of IGF-1 [19]. These findings raise the possibility of the synergistic effect of 20E and IGF-1 to enhance skeletal muscle mass in mammals. Nevertheless, whether 20E and its metabolites act together with IGF-1 to enhance skeletal muscle cell growth are not yet clarified. Therefore, the aim of this study was to investigate the effect of 20E and its metabolites and their synergistic effect with IGF-1 on regulation of skeletal muscle cell growth.

EXPERIMENTAL

Chemicals and reagents for biological testing

Cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA) including Dulbecco's Modified Eagle's Medium (DMEM) (12800-017), Hyclone® DMEM without phenol red (SH30284.02), fetal bovine serum (10270-106), horse serum (16050-122), normal goat serum (PCN5000), penicillin-streptomycin (15140-122), and Trypsin-EDTA (0.5%) no phenol red (15400-054). The following chemicals and reagents were purchased as indicated: mouse monoclonal anti-myosin heavy chain (05-716) (Upstate, Temecula, CA ,USA); Dimethyl sulfoxide (DMSO) (102952), ethanol for molecular biology (108543) (Merck Millipore, Billerica, MA, USA), Glycine (4810) (Calbiochem, San Diego, CA, USA); Mouse monoclonal anti-MYH3 (F1.652) antibody (sc-53091), mouse monoclonal anti-MyoD (G-1) antibody (sc-377460), and rabbit polyclonal anti-androgen receptor (N-20) antibody (sc-816) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Goat anti-mouse Alexa Fluor® 488 secondary antibody (ab150117) and goat anti-rabbit Alexa Fluor® 568 secondary antibody (ab175695) (Abcam, Cambridge, UK); Rhodamine Phalloidin (R415) and 4',6-diamidino-2-phenylindole (DAPI) (D1306) (Thermo Fisher Scientific, Waltham, MA, USA); 20-hydroxyecdysone (≥93% HPLC) (H5142), LONG®R^3 IGF-I human recombinant analog (I1271), 5α-Androstan-17β-ol-3-one (A8380), Thiazolyl Blue Tetrazolium Bromide (M5655), 5-Bromo-2′-Deoxyuridine (BrdU) (B5002), mouse monoclonal anti-BrdU antibody (B2531), and Triton X-100 (X100) (Sigma-Aldrich, St. Louis, MO, USA); Paraformaldehyde (PFA) (15713) (Electron Microscopy Sciences, Hatfield, PA, USA); Rabbit monoclonal anti-IGF-1 receptor β antibody (9750) (Cell Signaling Technology, Beverly, MA, USA).

Synthesis of 20E and its metabolites

The crude extract of the bark of *Vitex glabrata* was purified by column chromatography and crystallization to obtain pure 20E. The structure of 20E was confirmed by spectroscopic means (IR, NMR, and MS) [20] and used as a precursor compound in this study. The processes for synthesis of 20E metabolites were performed as described in the literature method [17]. For 14-deoxy-20-hydroxyecdysone (14D-20E) synthesis, 20E (800 mg, 1.67 mmol) and Zn powder (1.0 g) were dissolved in 8 ml glacial acetic acid and the reaction was stirred at 67^0C overnight. The progress of reaction was monitored by thin-layer chromatography (TLC). The reaction mixture was filtered through cotton to remove Zn powder and washed with MeOH. Solvent was evaporated under reduced pressure to dryness. The crude extract was purified by column chromatography using CH_2Cl_2-MeOH as solvent by gradually increase the more polar component. 14D-20E (347.8 mg, 45%) was obtained as amorphous solid. Poststerone (POST) (300 mg, 0.83 mmol), which was prepared from 20E was subjected to reduction in the same manner as described in case of 20E. The product was purified by column chromatography using CH_2Cl_2-MeOH as solvent by gradually increase the more polar component to give 14-deoxypoststerone (14D-POST) (176.4 mg, 62%). The structures of synthesized 14D-20E, POST, and 14D-POST were characterized by spectroscopic methods (IR, NMR, and MS) which were consistent with the previously reported values [17].

In vitro study model

Mouse skeletal muscle cell line (C2C12) was obtained from American Type Culture Collection (ATCC) (CRL-1772). This cell line has been widely used to study skeletal muscle cell proliferation and differentiation [21-27]. Myogenic characteristic of C2C12 cell line was determined using myoblast determination protein (MyoD) [28]. C2C12 myoblasts (undifferentiated cells) were cultured in growth medium with reduced serum condition (DMEM + 5% fetal bovine serum) for cell proliferation study [25]. For myogenic differentiation capacity assessment, C2C12 myoblasts were cultured in regular growth medium (DMEM + 10% fetal bovine serum) until reach 70-80% confluency, then growth medium was changed to differentiation medium (DMEM + 2% horse serum) to induce differentiation of C2C12 myoblasts to form differentiated myotubes [29]. 20E derived from the bark of *Vitex glabrata* [20E (VG)] and its metabolites (14D-20E, POST, and 14D-POST) were used to test the effect of 20E and its metabolites on skeletal muscle cell growth during proliferation and differentiation stages. Moreover, 20E commercially available from Sigma-Aldrich [20E (SM)] (≥93% High-performance liquid chromatography, HPLC) was also tested to compare its effect with 20E (VG) (≥99% HPLC) in this study. For co-treatment with IGF-1, cells were co-treated with 20E and its metabolites plus IGF-1 at concentration 10 ng/ml. Ethanol (0.1% final concentration) served as vehicle for 20E and its metabolites.

MTT assay

To evaluate the toxicity of 20E and its metabolites on skeletal muscle cells, cells were seeded in a 96-well plate for cell viability assessment. Cells were daily treated with 20E and its metabolites (14D-20E, POST, and 14D-POST) at 0.1, 1, and 10 µM (final concentration) for 48 h in growth medium with reduced serum condition or daily treated for 5 days in differentiation medium for an evaluation of the toxicity on C2C12 myoblasts and myotubes, respectively. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were rinsed with PBS and incubated with DMEM without phenol red containing 10% MTT solution for 4 h at 37ºC [30]. Thereafter, medium containing MTT was removed and incubated with DMSO for 15 min. Spectrophotometric absorbance at 570 nm wavelength was determined using Wallac 1420 VICTOR2™ microplate reader (PerkinElmer, Waltham, MA, USA).

BrdU incorporation assay

Cell proliferation was examined after C2C12 myoblasts were daily treated with 20E and its metabolites (14D-20E, POST, and 14D-POST) at 0.1, 1, and 10 µM (final concentration) in growth medium with reduced serum condition for 48 h (solely treatment vs. co-treatment with IGF-1). The number of proliferating cells was determined using BrdU incorporation assay [31]. Briefly, BrdU at 10 µM (final concentration) was applied to the cells at 47 h after treatments for 1 h duration before immunocytochemical (ICC) analysis. The processes of fixation and denaturing DNA for BrdU staining were performed as previously described. The percentage of changes of $BrdU^+$ cells/total cell number compared to vehicle- or IGF-1-treated group was used to determine the effect of 20E and its metabolites on skeletal muscle cell proliferation. Six images were randomly captured at magnification ×100 using Olympus Inverted Fluorescence Microscope Model IX83 (Olympus, Tokyo, Japan). The quantitative analysis of the

ratio of $BrdU^+$ cells to the total cell number was performed using cellSens Dimension Desktop software (Olympus, Tokyo, Japan).

Cell differentiation assessment

To assess the myogenic differentiation capacity, 20E and its metabolites (14D-20E, POST, and 14D-POST) at 0.1, 1, and 10 µM (final concentration) were daily treated to the cells for 5 days during differentiation (solely treatment vs. co-treatment with IGF-1). The first treatment was conducted on the initial day of switching the culture medium from growth medium to differentiation medium. Myogenic differentiation capacity was determined by quantifying the expression of differentiation marker protein according to the previously studied [29]. Fifteen images were randomly captured at magnification ×100 using Olympus Inverted Fluorescence Microscope Model IX83 for quantitative analysis of embryonic myosin heavy chain (EbMHC) and MHC protein expression. Moreover, histological features of differentiated myotubes that are characterized as the cells with minimum of five nuclei fusion and positively expressed differentiation marker protein [32] were evaluated. Myotube diameter was determined by measuring the widest diameter of differentiated myotubes and the number of myotubes per field was counted per treatment condition. Quantitative analysis for cell differentiation assessment was performed using cellSens Dimension Desktop software (Olympus, Tokyo, Japan).

Androgen receptor and IGF-1 receptor protein expression analysis

To investigate the localization and expression of androgen receptor (AR) [33] and IGF-1 receptor (IGF-1R) [34] proteins that have been reported in the differentiated myotubes. 20E and its metabolites (14D-20E, POST, and 14D-POST) at 1 µM (final concentration) were daily treated to the differentiated myotubes for 5 days during differentiation stage. The effective doses of dihydrotestosterone (DHT) (100 nM) [35] and IGF-1 (10 ng/ml) [36] were used as positive controls. Double-labelling of IGF-1R and EbMHC proteins was performed to clarify the expression of IGF-1R in differentiated myotubes after 20E and its metabolites were co-treated with IGF-1. DAPI was applied to visualize nuclei. Representative images were taken at magnification ×200 using Olympus Inverted Fluorescence Microscope Model IX83 (Olympus, Tokyo, Japan).

Immunocytochemical analysis

For immunostaining processes, cells were fixed with 4% PFA for 10 min, permeabilized with 0.1% Triton X-100 for 5 min (additional step for nuclear localization protein) as previously described [37], and followed by blocking non-specific reactivity with 5% normal goat serum for 30 min. Thereafter, primary antibodies were incubated for 1 h and followed with incubation of goat anti-mouse Alexa Fluor® 488 or goat anti-rabbit Alexa Fluor® 568 for 1 h. For double-labelling of MyoD and F-actin, cells were post-fixed with 4% PFA for 5 min after MyoD staining followed by quench excess aldehyde with 0.1 M glycine for 5 min. Thereafter, tetramethylrhodamine isothiocyanate (TRITC)–conjugated phalloidin (Rhodamine Phalloidin) (1:100) was incubated for 15 min [37]. Nuclei were stained with DAPI for 5 min and the stained cells were preserved in the mounting medium prior to being visualize under Olympus Inverted Fluorescence Microscope Model IX83 (Olympus, Tokyo, Japan).

Statistical Analysis

Data are presented as mean±SEM. Normal distribution and the homogeneity of variance were analyzed prior to comparison the data among groups. One-way ANOVA with Turkey post-hoc test or Kruskal-Wallis test was used to determine the significant difference between groups where appropriate. Statistical significance was analyzed with SPSS and $p<0.05$ was considered a significant difference among groups.

RESULTS

20E and its metabolites had no toxicity on skeletal muscle cells

In the current study, 20E from *Vitex glabrata* was used as a starting material to synthesize 20E metabolites (14D-20E, POST, and 14D-POST) and their chemical structures are illustrated in Figure 1A. Nevertheless, the toxicity of 20E and its metabolites on skeletal muscle cell has not been reported elsewhere. Therefore, skeletal muscle cell viability in response to the treatments of 20E and its metabolites was determined prior conducting the subsequent experiments. Myogenic characteristic of mouse skeletal muscle cell line (C2C12) used in this study was identified using MyoD staining and double-labelled with F-actin as illustrated in Figure 1B. The results revealed that 20E and its metabolites had no toxicity effect on both undifferentiated skeletal muscle cells (myoblast) (Figure 1C) and differentiated skeletal muscle cells (myotube)

(Figure 1D) compared to vehicle as assessed by MTT assay. Of these, non-toxicity effect was similar observed when the cells treated with 20E (SM) (≥93% HPLC) vs. 20E (VG) (≥99% HPLC) (Figure 1C-1D).

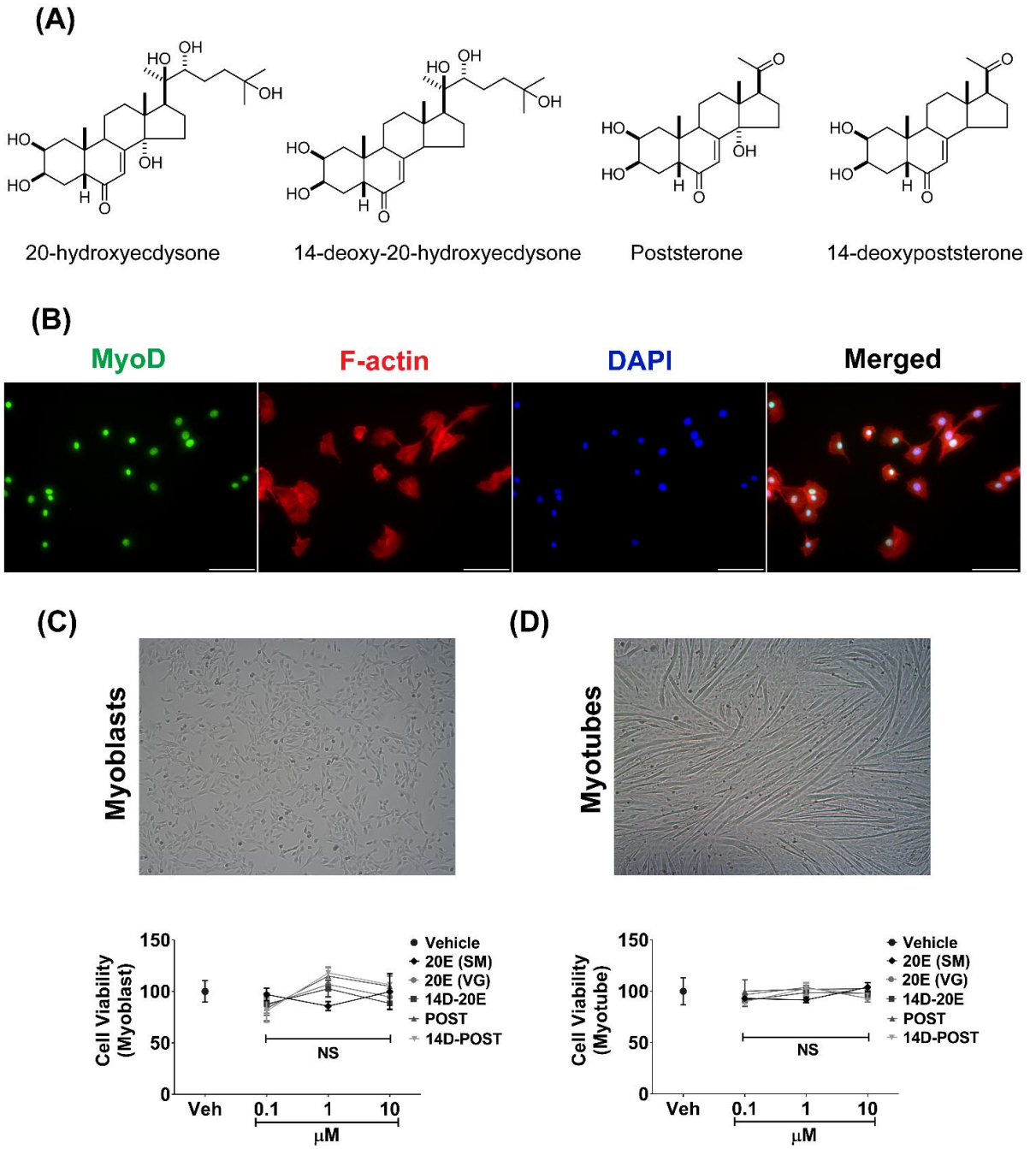

Figure 1 Structures of 20E and its metabolites and the toxicity testing on skeletal muscle cell
(A) Chemical structures of 20-hydroxyecdysone (20E), 14-deoxy-20-hydroxyecdysone (14D-20E), poststerone (POST), and 14-deoxypoststerone (14D-POST). (B) Nuclear localization of MyoD protein indicates myogenic characteristic of C2C12 cell line. F-actin and nuclei were visualized using Rhodamine Phalloidin and DAPI staining, respectively. Images were taken at magnification ×200, scale bars = 100 μm. (C-D) Viability of C2C12 myoblasts and myotubes after treated with 20E [commercially available from Sigma-Aldrich; 20E (SM) or extracted from the bark of *Vitex glabrata*; 20E (VG)], and its metabolites (14D-20E, POST, and 14D-POST) at 0.1, 1, and 10 μM (final concentration). Representative images of C2C12 myoblasts and myotubes (C-D) were taken at magnification ×40 using Nikon Eclipse TS100 (Nikon, Tokyo, Japan). Ethanol (0.1% final concentration) served as vehicle. Data are expressed as percentage of changes compared to vehicle group (n=3 independent experiments). NS indicates not statistically significant

Effects of 20E and its metabolites on skeletal muscle cell proliferation and differentiation

The effect of 20E and its metabolites on myoblast proliferation was evaluated using BrdU incorporation assay (Figure 2A). The results demonstrated that no significant changes in the percentage of $BrdU^+$ cell after solely treatments of 20E, 14D-20E, POST, and 14D-POST compared to vehicle (Figure 2B). To further assess the effect of 20E, 14D-20E, POST, and 14D-POST on myogenic differentiation capacity, the differentiation markers of skeletal muscle cells including EbMHC and MHC were investigated using ICC analysis (Figure 2C and 2E). The results revealed no significant differences of

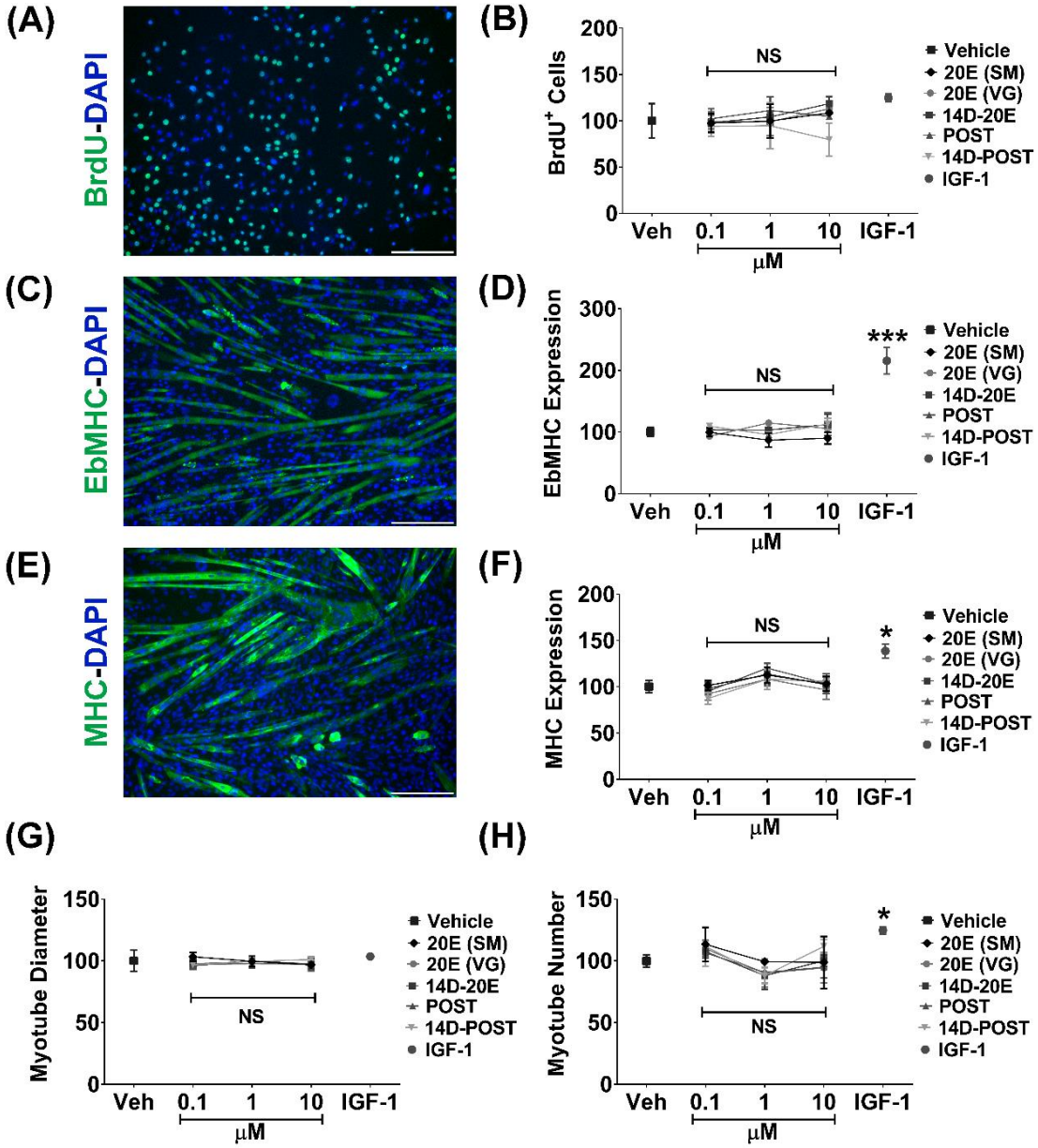

Figure 2: Effects of 20E and its metabolites on skeletal muscle cell proliferation and differentiation Cells were treated with 20E [commercially available from Sigma-Aldrich; 20E (SM) or extracted from the bark of *Vitex glabrata*; 20E (VG)], and its metabolites (14D-20E, POST, and 14D-POST) at 0.1, 1, and 10 µM (final concentration). (A) Representative image of $BrdU^+$ cell (C2C12 myoblasts); (C and E) Representative images of EbMHC and MHC protein expression (C2C12 myotubes). Nuclei were visualized with DAPI and images were taken at the magnification ×100, scale bars = 200 µm. Quantitative analysis on the percent changes in (B) $BrdU^+$ cell, (D and F) EbMHC and MHC protein expression, and (G-H) histological features of differentiated myotubes. Ethanol (0.1% final concentration) and IGF-1 (10 ng/ml) represent vehicle and positive control, respectively. Data are expressed as percentage of changes compared to vehicle group (n=3 independent experiments). *p<0.05 and ***p<0.0001 indicates significant difference compared to vehicle group. NS indicates not statistically significant

EbMHC (Figure 2D) and MHC (Figure 2F) protein expression after the cells were treated with 20E and its metabolites during differentiation stage compared to vehicle-treated. Furthermore, no significant changes of myotube diameter (Figure 2G) and myotube number (Figure 2H) support the absence of positive effect of 20E, 14D-20E, POST, and 14D-POST on myogenic differentiation capacity. In contrast, IGF-1 treatment enhanced myogenic differentiation capacity by increasing EbMHC (p<0.0001) (Figure 2D), MHC (p<0.05) (Figure 2F), and myotube number (p<0.05) (Figure 2H). Altogether, solely treatment of 20E and its metabolites had no effects on skeletal muscle cell proliferation and differentiation.

20E and its metabolite treatments had no effect on androgen receptor protein expression

Since 20E has no endogenous receptor in the mammalian tissues, however, the similarity of its core structure as steroid backbone raise the possibility of mammalian steroid receptor activation. The result demonstrated that 20E, 14D-20E, POST, and 14D-POST had no effect on androgen receptor protein expression compared to DHT treatment. Nuclear localization of AR protein expression was evident after DHT treatment in which none of 20E and its metabolites elicit the similar effect (Figure 3). These results indicate AR protein expression in skeletal muscle cell is not regulated by 20E and its metabolites.

No synergistic effect of 20E and its metabolites after co-treatment with IGF-1 on skeletal muscle cell growth

To clarify the potential synergistic effect of 20E with IGF-1, 20E and its metabolites were co-treated with IGF-1 to investigate the proliferative and myogenic differentiation capacities. The results revealed co-treatment of 20E and its metabolites with IGF-1 had no synergistic effect on the changes of percentage of $BrdU^+$ cell (Figure 4A), EbMHC and MHC protein expression (Figure 4B-C), and histological

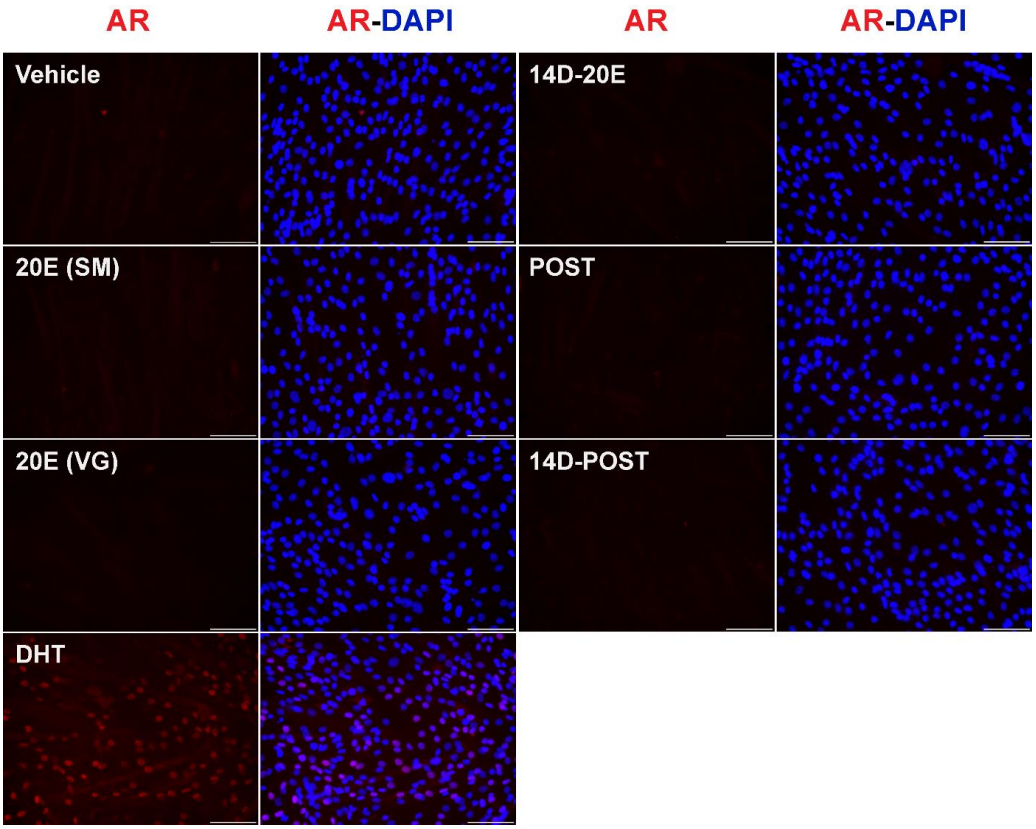

Figure 3: AR protein expression in response to the treatments of 20E and its metabolites
Representative images of AR protein expression in differentiated myotubes after treated with 20E [commercially available from Sigma-Aldrich; 20E (SM) or extracted from the bark of *Vitex glabrata*; 20E (VG)], and its metabolites (14D-20E, POST, and 14D-POST) at 1 µM (final concentration) during differentiation stage. Nuclei were visualized with DAPI and images were taken at the magnification ×200, scale bars = 100 µm. Ethanol (0.1% final concentration) and DHT (100 nM) represent vehicle and positive control, respectively

features of differentiated myotubes (Figure 4D-E) compared with IGF-1 treatment alone. This anabolic effect of IGF-1 was associated with an increase of IGF-1R in the differentiated myotubes. Nevertheless, none of 20E and its metabolites had an effect on IGF-1R protein expression and histological features of differentiated myotubes when co-treated with IGF-1. The representative images of IGF-1R in differentiated myotubes after co-treatment of 20E and its metabolites with IGF-1 compared with IGF-1 treatment alone are illustrated in Figure 5.

DISCUSSION

This study investigated the effects of 20E and its metabolites and their synergistic effect with IGF-1 on skeletal muscle cell growth. The major findings are (1) 20E and its metabolites had no toxicity effect on skeletal muscle cell; (2) treatments of 20E and its metabolites had no effects on skeletal muscle cell proliferation and myogenic differentiation capacity; (3) no synergistic effect of 20E and its metabolites when co-treated with IGF-1 on skeletal muscle cell growth. The lack of anabolic effect of 20E and its metabolites was associated with no effects on AR and IGF-1R protein expression.

In the current study, 20E had no effect on skeletal muscle cell viability at any stage of skeletal muscle cell growth (proliferation and differentiation). These results support very low toxicity of 20E in mammal, as LD_{50} of 20E was 6.4 g/kg after IP administration and no fatality in mice up to 9 g/kg oral administration was reported [38]. However, 20E has been found to be metabolized rapidly in mice and human [14,15]. Therefore, 20E metabolites rather than

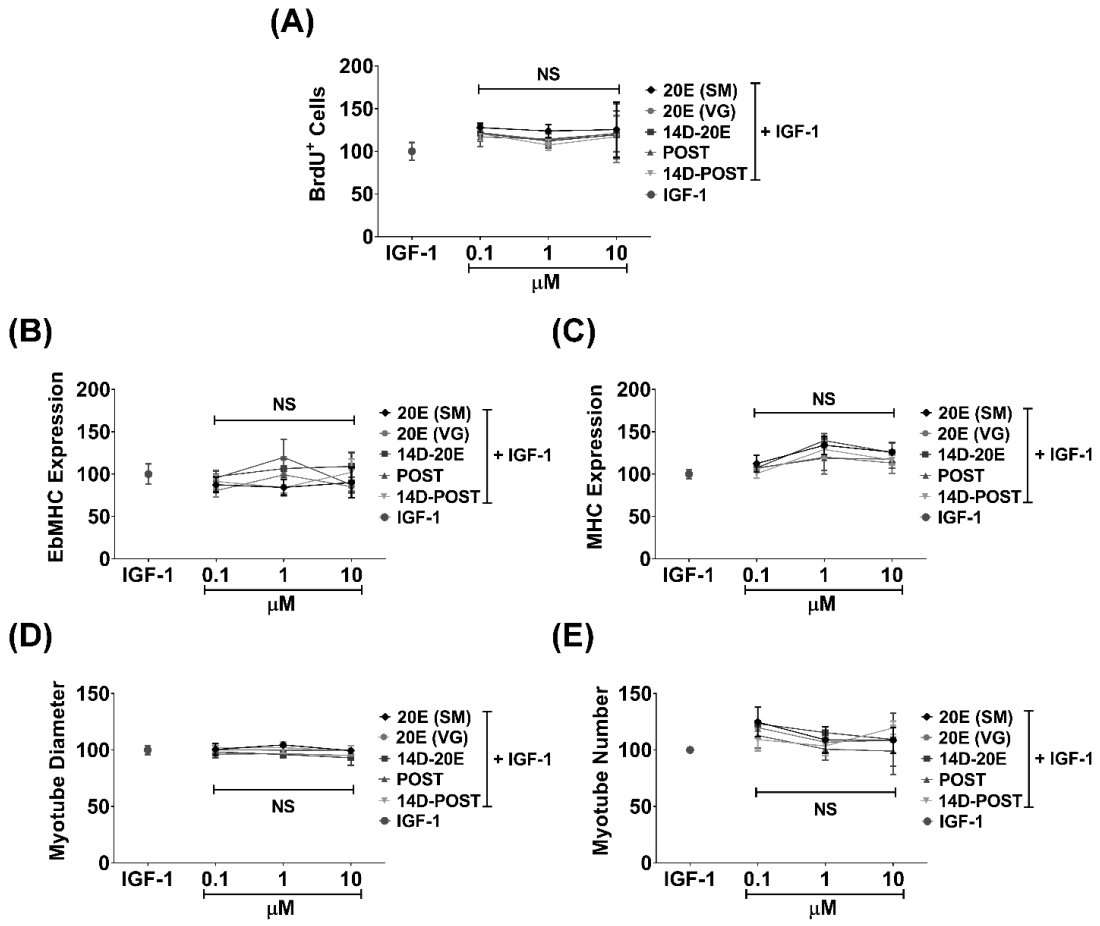

Figure 4: Effects of 20E and its metabolites co-treated with IGF-1 on skeletal muscle cell proliferation and differentiation

Cells were treated with 20E [commercially available from Sigma-Aldrich; 20E (SM) or extracted from the bark of *Vitex glabrata*; 20E (VG)], and its metabolites (14D-20E, POST, and 14D-POST) at 0.1, 1, and 10 μM (final concentration) plus IGF-1 (10 ng/ml). Quantitative analysis on the percent changes of (A) $BrdU^+$ cell, (B-C) EbMHC and MHC protein expression, and (D-E) histological features of differentiated myotubes. Data are expressed as percentage of changes after the cells were treated with 20E and its metabolites plus IGF-1 compared to IGF-1 treatment alone (n=3 independent experiments). NS indicates not statistically significant

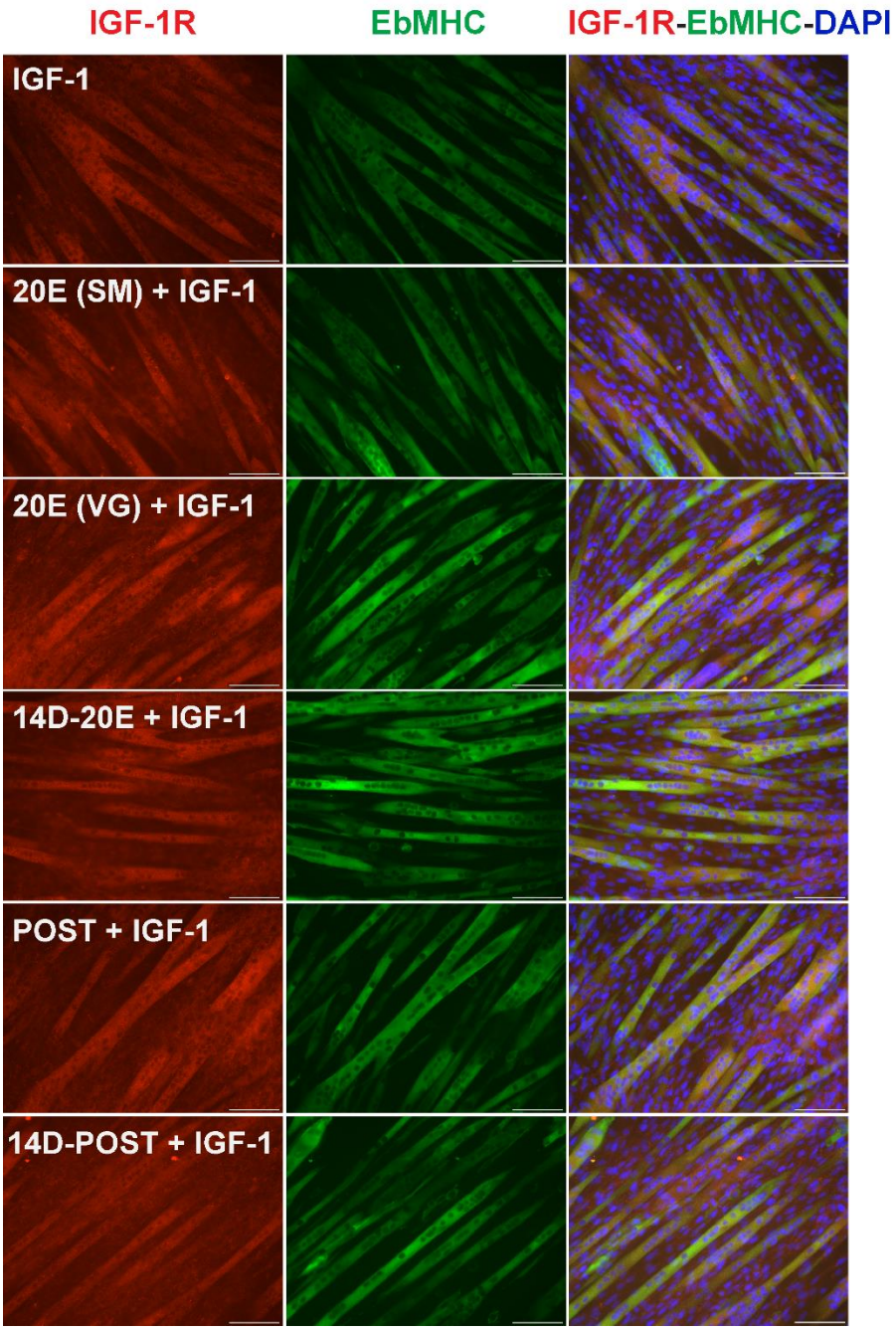

Figure 5: IGF-1R protein expression in response to 20E and its metabolites co-treated with IGF-1
Representative images of IGF-1R protein expression in differentiated myotubes after treated with 20E [commercially available from Sigma-Aldrich; 20E (SM) or extracted from the bark of *Vitex glabrata*; 20E (VG)], and its metabolites (14D-20E, POST, and 14D-POST) at 1 µM (final concentration) plus IGF-1 (10 ng/ml) compared to IGF-1 treatment alone. EbMHC protein expression and DAPI were used to indicate the differentiated myotubes and nuclei, respectively. IGF-1R protein mainly localized in the cytoplasm of the differentiated myotubes. Images were taken at the magnification ×200, scale bars = 100 µm

20E itself could play a major role of 20E action in the body. Unfortunately, no available information on the toxicity of 20E metabolites on skeletal muscle cell viability has been demonstrated. Herein, 20E metabolites (14D-20E, POST, and 14D-POST) that could occur after several reaction steps by the liver and gut bacteria also had no toxicity comparable to 20E. These results suggest that 20E and its metabolites are non-toxic substances to skeletal muscle cell and they have a potential to use targeting skeletal muscle.

Skeletal muscle cell proliferation is an important process on skeletal muscle cell growth, i.e., expansion of skeletal muscle stem cell number during muscle regeneration after injury [39]. Hence, the proliferative effect of 20E and its metabolites on skeletal muscle cell was

evaluated. The result revealed no proliferative effect was observed after the cells treated with 20E. In addition, the removal of the side chain or hydroxyl group from the 14-position of 20E structure as occurs during 20E metabolism also had no effect on cell proliferation. The lacking effect on skeletal muscle cell proliferation suggest 20E and its metabolites are not the growth-promoting factor that regulate cell division to enhance skeletal muscle cell growth. In contrast, the proliferative effect of 20E in silkworm (*Bombyx mori*) during pupal wing development was reported [40]. These contradict results between insect and mammalian cell suggest 20E action on cell proliferation is species specific.

Increasing of skeletal muscle fiber size (i.e., muscle hypertrophy) requires stimulation of muscle protein synthesis and enhance myogenic differentiation capacity. Initially, the anabolic effect of 20E was proposed via activation of G protein-coupled receptor that raising intracellular Ca^{2+} and lead to induce Akt phosphorylation, resulting in increased protein synthesis of skeletal muscle cell [11]. Increasing of C2C12 myotube diameter and skeletal muscle fiber size after 20E treatment was also reported in the following studies [9,41]. Nevertheless, another investigations revealed no anabolic effect after treatment of 20E to either skeletal muscle cell or rodent [13,42]. Akt phosphorylation was not changed after skeletal muscle cells treated with 20E at various concentrations (0.1, 1, 10, and 60 µM) [42]. Moreover, 20E did not activate Akt phosphorylation or increase phosphorylation of mTOR and 4E-BP1 proteins in hindlimb muscle of rats when compared to leucine [13]. These results suggest ambiguous evidence about the effects of 20E on increasing skeletal muscle protein synthesis to support its effect on skeletal muscle growth.

On the contrary to the previous investigations that focused on protein synthesis signaling, myogenic differentiation capacity was verified in this study to evaluate the effect of 20E and its metabolites on skeletal muscle cell growth. The results revealed no significant changes of EbMHC and MHC protein expression and histological features of differentiated myotubes after treatments of 20E and its metabolites. These results support the lacking of anabolic effect of 20E on skeletal muscle cell growth [13,42]. Nonetheless, the anabolic effect of 20E in some of the previous studies could not be excluded. Since the effects of ecdysteroid-containing plant extracts, i.e., spinach and A.turkestanica extracts could stimulate protein synthesis of C2C12 myotubes, although the high concentrations were required to elicit the significant effect [10]. These results suggest an important of mixture of the other compounds in the extracts with 20E to enhance the effect on skeletal muscle protein synthesis. Moreover, the modification at specific sites of 20E chemical structures demonstrated the significant impact on Akt signaling which is the regulatory pathway of skeletal muscle protein synthesis compared to 20E precursor [42]. This latter finding indicates 20E metabolites (14D-20E, POST, and 14D-POST) in the current study may not have the polarity or chemical structure that could enhance skeletal muscle cell growth.

Since ecdysone receptors are specifically expressed in arthropods, hence the effects of 20E on skeletal muscle cell growth in mammals remain unclear. ICC analysis in this study revealed lack of an increased in AR protein expression after the differentiated myotubes were treated with 20E and its metabolites. This result was supported by barely detection of 20E binding affinity on AR using receptor-binding assay [10]. Nevertheless, recent studies revealed the effects of 20E on skeletal muscle hypertrophy mediated through estrogen receptor beta [9,41]. Unfortunately, the present results on myogenic differentiation capacity and histological features of differentiated myotubes after treated with 20E and its metabolites do not support the anabolic effect of 20E on skeletal muscle hypertrophy.

Despite lacking the strong evidence on how 20E enhances skeletal muscle cell growth, previous studied report that 20E increased muscle fiber size and its anabolic effect correlated with increasing serum IGF-1 *in vivo* [9]. This evidence suggest the possible synergistic effect of 20E with IGF-1 to enhance skeletal muscle growth. Herein, IGF-1 enhanced myogenic differentiation capacity of skeletal muscle cell supports the well-documented anabolic effect of IGF-1 through PI3K/Akt pathway [19]. However, no synergistic effect of 20E and its metabolites when co-treated with IGF-1 were evident in this study, although the underlying mechanism of 20E on regulation of skeletal muscle protein synthesis via raising intracellular Ca^{2+} and induction of Akt phosphorylation has been proposed [11]. Therefore, increase in skeletal muscle mass and serum IGF-1 after 20E supplementation *in vivo* may associate with indirect action of 20E on modulating IGF-1 level rather than its direct effect on skeletal muscle cell growth. To support this hypothesis, 20E treatment increased gene expression and secretion of *Bombyx mori* IGF-like peptide in the fat body culture [43]. The effect of 20E on insect IGF-like peptide secretion was seen in the culture medium within 24 h and substantially increased at 48 h. This insect IGF-

like peptide has the functions on growth and development comparable to the mammal IGF-1. Nonetheless, the modulation of IGF-1 level in mammals on regulating skeletal muscle growth occurs via action of 20E or its metabolites remain warranted.

CONCLUSION

Currently, the pharmacological effects of 20E on skeletal muscle that raises its therapeutic potential to increase muscle mass and strength have been documented. However, the direct anabolic effect of 20E on skeletal muscle cell growth is still ambiguous. The present results suggest that 20E and its metabolites have no direct or synergistic effect with IGF-1 on regulation of skeletal muscle cell growth. Nevertheless, an increasing of skeletal muscle mass and strength in mammals after 20E supplementation *in vivo* may associate with its indirect action on modulating IGF-1 level rather than the direct effect on skeletal muscle cell.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. R.S. conceived and designed the study; B.Y. and S.K. synthesized and characterized the compounds; R.S. and K.S. performed experiments, analyzed the data, and interpreted the results; R.S. wrote the manuscript. All authors read and approved the final version of manuscript.

REFERENCES

1. *Spindler KD, Honl C, Tremmel C, Braun S, Ruff H, Spindler-Barth M. Ecdysteroid hormone action. Cell Mol Life Sci. 2009;66(24):3837-3850.*
2. *Lafont R, Harmatha J, Marion-Poll F, Dinan L, Wilson I. Ecdybase, a free ecdysteroid database. Retrieved May. 2002;26:2014.*
3. *Lafont R, Dinan L. Practical uses for ecdysteroids in mammals including humans: an update. J Insect Sci. 2003;3:7.*
4. *Dinan L, Lafont R. Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. J Endocrinol. 2006;191(1):1-8.*
5. *Bathori M, Toth N, Hunyadi A, Marki A, Zador E. Phytoecdysteroids and anabolic-androgenic steroids--structure and effects on humans. Curr Med Chem. 2008;15(1):75-91.*
6. *Dinan L. The Karlson Lecture. Phytoecdysteroids: what use are they? Arch Insect Biochem Physiol. 2009;72(3):126-141.*
7. *Toth N, Szabo A, Kacsala P, Heger J, Zador E. 20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat. Phytomedicine. 2008;15(9):691-698.*
8. *Cheng DM, Kutzler LW, Boler DD, Drnevich J, Killefer J, Lila MA. Continuous infusion of 20-hydroxyecdysone increased mass of triceps brachii in C57BL/6 mice. Phytother Res. 2013;27(1):107-111.*
9. *Parr MK, Zhao P, Haupt O, Ngueu ST, Hengevoss J, Fritzemeier KH, Piechotta M, Schlorer N, Muhn P, Zheng WY, Xie MY, Diel P. Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone. Mol Nutr Food Res. 2014;58(9):1861-1872.*
10. *Gorelick-Feldman J, Maclean D, Ilic N, Poulev A, Lila MA,*

Cheng D, Raskin I. Phytoecdysteroids increase protein synthesis in skeletal muscle cells. J Agric Food Chem. 2008;56(10):3532-3537.

11. *Gorelick-Feldman J, Cohick W, Raskin I. Ecdysteroids elicit a rapid Ca^{2+} flux leading to Akt activation and increased protein synthesis in skeletal muscle cells. Steroids. 2010;75(10):632-637.*
12. *Hirunsai M, Yimlamai T, Suksamrarn A. Effect of 20-hydroxyecdysone on proteolytic regulation in skeletal muscle atrophy. In Vivo. 2016;30(6):869-877.*
13. *Anthony TG, Mirek ET, Bargoud AR, Phillipson-Weiner L, DeOliveira CM, Wetstein B, Graf BL, Kuhn PE, Raskin I. Evaluating the effect of 20-hydroxyecdysone (20HE) on mechanistic target of rapamycin complex 1 (mTORC1) signaling in the skeletal muscle and liver of rats. Appl Physiol Nutr Metab. 2015;40(12):1324-1328.*
14. *Dzhukharova MK, Sakhibov AD, Kasymov B, Syrov VN, Takanaev AA, Saatov Z. Pharmacokinetics of ecdysterone in experiments. Pharmaceutical Chemistry Journal. 1987;21(10):689-692.*
15. *Simon P, Koolman J. Ecdysteroids in vertebrates: pharmacological aspects. In: Ecdysone. Verlag, Stuttgart; 1989:254-259.*
16. *Tsitsimpikou C, Tsamis GD, Siskos PA, Spyridaki MH, Georgakopoulos CG. Study of excretion of ecdysterone in human urine. Rapid Commun Mass Spectrom. 2001;15(19):1796-1801.*
17. *Kumpun S, Girault JP, Dinan L, Blais C, Maria A, Dauphin-Villemant C, Yingyongnarongkul B, Suksamrarn A, Lafont R. The metabolism of 20-hydroxyecdysone in mice: relevance to pharmacological effects and gene switch applications of ecdysteroids. J Steroid Biochem Mol Biol. 2011;126(1-2):1-9.*
18. *Borrione P, Luigi LD, Maffulli N, Pigozzi F. Herbal supplements: cause for concern? J Sports Sci Med. 2008;7(4):562-564.*
19. *Schiaffino S, Mammucari C. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. Skelet Muscle. 2011;1(1):4.*
20. *Suksamrarn A, Sommechai C, Charulpong P, Chitkul B. Ecdysteroids from Vitex canescens. Phytochemistry. 1995;38(2):473-476.*
21. *Yang SY, Goldspink G. Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. FEBS Lett. 2002;522(1-3):156-160.*
22. *Joulia D, Bernardi H, Garandel V, Rabenoelina F, Vernus B, Cabello G. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. Exp Cell Res. 2003;286(2):263-275.*
23. *Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet. 2006;38(2):228-233.*
24. *Lee JH, Tachibana H, Morinaga Y, Fujimura Y, Yamada K. Modulation of proliferation and differentiation of C2C12 skeletal muscle cells by fatty acids. Life Sci. 2009;84(13-14):415-420.*
25. *Zhang L, Shi S, Zhang J, Zhou F, ten Dijke P. Wnt/beta-catenin signaling changes C2C12 myoblast proliferation and differentiation by inducing Id3 expression. Biochem Biophys Res Commun. 2012;419(1):83-88.*
26. *Hwang SY, Kang YJ, Sung B, Jang JY, Hwang NL, Oh HJ, Ahn YR, Kim HJ, Shin JH, Yoo MA, Kim CM, Chung HY, Kim ND. Folic acid is necessary for proliferation and differentiation of C2C12 myoblasts. J Cell Physiol. 2018;233(2):736-747.*
27. *Yang DL, Gan ML, Tan Y, Ge GH, Li Q, Jiang YZ, Tang GQ, Li MZ, Wang JY, Li XW, Zhang SH, Zhu L. MiR-222-3p regulates the proliferation and differentiation of C2C12 myoblasts by targeting BTG2. Mol Biol (Mosk). 2019;53(1):44-52.*
28. *Ferri P, Barbieri E, Burattini S, Guescini M, D'Emilio A, Biagiotti L, Del Grande P, De Luca A, Stocchi V, Falcieri E. Expression and subcellular localization of myogenic regulatory factors during the differentiation of skeletal muscle C2C12 myoblasts. J Cell Biochem. 2009;108(6):1302-1317.*
29. *Srikuea R, Hirunsai M. Effects of intramuscular administration of $1\alpha,25(OH)_2D_3$ during skeletal muscle regeneration on regenerative capacity, muscular fibrosis, and angiogenesis. J Appl Physiol (1985). 2016;120(12):1381-1393.*
30. *Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer. 1987;56(3):279-285.*
31. *Srikuea R, Esser KA, Pholpramool C. Leukaemia inhibitory factor is expressed in rat gastrocnemius muscle after contusion and increases proliferation of rat L6 myoblasts via c-Myc signalling. Clin Exp Pharmacol Physiol. 2011;38(8):501-509.*
32. *Sun Y, Ge Y, Drnevich J, Zhao Y, Band M, Chen J. Mammalian target of rapamycin regulates miRNA-1 and follistatin in skeletal myogenesis. J Cell Biol. 2010;189(7):1157-1169.*
33. *Wannenes F, Caprio M, Gatta L, Fabbri A, Bonini S, Moretti C. Androgen receptor expression during C2C12 skeletal muscle cell line differentiation. Mol Cell Endocrinol. 2008;292(1-2):11-19.*
34. *Senesi P, Luzi L, Montesano A, Mazzocchi N, Terruzzi I. Betaine supplement enhances skeletal muscle differentiation in murine myoblasts via IGF-1 signaling activation. J Transl Med. 2013;11:174.*
35. *Chen Y, Lee NK, Zajac JD, MacLean HE. Generation and analysis of an androgen-responsive myoblast cell line indicates that androgens regulate myotube protein accretion. J Endocrinol Invest. 2008;31(10):910-918.*
36. *Latres E, Amini AR, Amini AA, Griffiths J, Martin FJ, Wei Y, Lin HC, Yancopoulos GD, Glass DJ. Insulin-like growth factor-1 (IGF-1) inversely regulates atrophy-induced genes via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. J Biol Chem. 2005;280(4):2737-2744.*
37. *Srikuea R, Zhang X, Park-Sarge OK, Esser KA. VDR and CYP27B1 are expressed in C2C12 cells and regenerating skeletal muscle: potential role in suppression of myoblast proliferation. Am J Physiol Cell Physiol. 2012;303(4):C396-405.*
38. *Ogawa S, Nishimoto N, Matsuda H. Pharmacology of ecdysones in vertebrates. In: Burdette WJ, ed. Invertebrate Endocrinology and Hormonal Heterophylly. Berlin, Heidelberg: Springer Berlin Heidelberg; 1974:341-344.*
39. *Dumont NA, Bentzinger CF, Sincennes MC, Rudnicki MA. Satellite cells and skeletal muscle regeneration. Compr Physiol. 2015;5(3):1027-1059.*
40. *Fujiwara H, Ogai S. Ecdysteroid-induced programmed cell death and cell proliferation during pupal wing development of the silkworm, Bombyx mori. Dev Genes Evol. 2001;211(3):118-123.*
41. *Parr MK, Botre F, Nass A, Hengevoss J, Diel P, Wolber G. Ecdysteroids: A novel class of anabolic agents? Biol Sport. 2015;32(2):169-173.*
42. *Csabi J, Hsieh TJ, Hasanpour F, Martins A, Kele Z, Gati T, Simon A, Toth G, Hunyadi A. Oxidized metabolites of 20-hydroxyecdysone and their activity on skeletal muscle cells: preparation of a pair of desmotropes with opposite bioactivities. J Nat Prod. 2015;78(10):2339-2345.*
43. *Okamoto N, Yamanaka N, Satake H, Saegusa H, Kataoka H, Mizoguchi A. An ecdysteroid-inducible insulin-like growth factor-like peptide regulates adult development of the silkmoth Bombyx mori. FEBS J. 2009;276(5):1221-1232.*