

Ajuga turkestanica increases Notch and Wnt signaling in aged skeletal muscle

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Abstract. – BACKGROUND: The declining myogenic potential of aged skeletal muscle is multifactorial. Insufficient satellite cell activity is one factor in this process. Notch and Wnt signaling are involved in various biological processes including orchestrating satellite cell activity within skeletal muscle. These pathways become dysfunctional during the aging process and may contribute to the poor skeletal muscle competency. Phytoecdysteroids are natural adaptogenic compounds with demonstrated benefit on skeletal muscle.

AIM: To determine the extent to which a phytoecdysteroid enriched extract from *Ajuga turkestanica* (ATE) affects Notch and Wnt signaling in aged skeletal muscle.

MATERIALS AND METHODS: Male C57BL/6 mice (20 months) were randomly assigned to Control (CT) or ATE treatment groups. Chow was supplemented with either vehicle (CT) or ATE (50 mg/kg/day) for 28 days. Following supplementation, the triceps brachii muscles were harvested and immunohistochemical analyses performed. Components of Notch or Wnt signaling were co-labelled with Pax7, a quiescent satellite cell marker.

RESULTS: ATE supplementation significantly increased the percent of active Notch/Pax7+ nuclei ($p = 0.005$), Hes1/Pax7+ nuclei ($p = 0.038$), active B-catenin/Pax7+ nuclei ($p = 0.011$), and Lef1/Pax7+ nuclei ($p = 0.022$), compared to CT. ATE supplementation did not change the resting satellite cell number.

CONCLUSIONS: ATE supplementation in aged mice increases Notch and Wnt signaling in triceps brachii muscle. If Notch and Wnt benefit skeletal muscle, then phytoecdysteroids may provide a protective effect and maintain the integrity of aged skeletal muscle.

Key Words:

Phytoecdysteroids, Satellite cell, Myocyte, Active Notch, β -catenin.

Abbreviations

ATE = *Ajuga turkestanica* extract; DAPI = 40-6-diamidino-2-phenylindole; EtOH = ethanol; Lef1 = Lymphoid enhancer-binding factor 1; Hes1 = hairy and enhancer of split-1; NGS = Normal goat serum; PFA = Paraformaldehyde; PBS = Phosphate buffer saline; Pax7 = Paired box 7.

Introduction

Sarcopenia is a major health concern as its prevalence in persons aged 80 years and older can exceed 50%¹. There are numerous contributors to sarcopenia, including environmental factors such as sedentary lifestyle and inadequate dietary intake of nutrients². Although researchers who study aging are only at the beginning of delineating the cellular and molecular causes of sarcopenia, age-induced physiological changes including chronic inflammation, decreased circulating hormone levels, and cellular senescence contribute to poor skeletal muscle integrity³⁻⁵ and sarcopenia. Recently, added to the pathogenesis list of sarcopenia is a diminished myogenic potential and an impaired ability to successfully respond to skeletal muscle injury, both of which play a role in age-associated muscle dysfunction.

tion⁵⁻⁷. Traditional treatments for sarcopenia, such as hormone replacement therapy, are not entirely effective or have negative side effects⁸. Thus, developing safe, natural countermeasures to the pathogenesis of sarcopenia would increase the quality of life for those suffering from this debilitating disorder.

Improving the myogenic potential of aged skeletal muscle, including enhanced satellite cell activity, would better prepare the muscle to respond to injurious stimuli (e.g., walking down stairs)⁵. Notch and Wnt are developmental signaling pathways that regulate key biological processes including stem cell pool maintenance, cell fate determination, cell survival and apoptosis⁹⁻¹³. During skeletal muscle remodeling, Notch and Wnt are critical for satellite cell activity and the muscle repair process^{14,15}. Since aged skeletal muscle experiences impaired regeneration and overall poor competency^{5,16}, characterizing Notch and Wnt's role in age-associated muscle dysfunction, will determine if these pathways provide therapeutic opportunities or if they further weaken aged skeletal muscle competency.

During skeletal muscle repair, Notch signaling is initiated when Delta1 ligand and Notch receptor interact. The resultant active Notch translocates to the myonucleus and up-regulates target genes such as hairy and enhancer of split-1 (Hes1) to activate satellite cells¹⁴. Active Wnt signaling results in myonuclear translocation of β -catenin and interaction with transcription factors such as, lymphoid enhancer-binding factor 1 (Lef1) to promote the later stages of postnatal myogenesis¹⁴. In aged skeletal muscle, Notch signaling is weakened which negatively affects skeletal muscle competency^{5,15,17}. The role of Wnt signaling in aged skeletal muscle is equivocal¹⁸⁻²², some investigators report that Wnt promotes fibrogenesis in aged muscle¹⁸, while others suggest that Wnt is critical for muscle repair²¹. However, successful muscle repair and overall muscle competency only occurs when Notch and Wnt are well orchestrated and this coordination is impaired in aged skeletal muscle^{5,15}. Notch and Wnt are imperative for biological functions, that span from cell fate determination to apoptosis and yet there are many unanswered questions regarding Notch and Wnt's role in age-related dysfunctions. Therefore, developing therapeutic strategies to restore functional Notch and Wnt signaling in aged skeletal muscle is clinically important.

The plant *Ajuga turkestanica* contains many bioactive phytochemicals, including the phy-

toecdysteroids 20-hydroxyecdysone (20E) and turkesterone²³. Phytoecdysteroids possess systemic anti-inflammatory^{24,25} and adaptogenic^{25,26} properties, with little to no known negative side effects in mammals. Oral supplementation of phytoecdysteroids (50 mg/kg/day) for 28 days has been demonstrated to increase muscle strength and mass in young rats^{27,28}. However, additional studies are required to support the use of phytoecdysteroids as a therapeutic tool to maintain satellite cell competency in aged skeletal muscle. Therefore, the purpose of this study was to test the efficacy of a phytoecdysteroid enriched extract from *A. turkestanica* (ATE) on Notch and Wnt signaling in resting satellite cells of aged skeletal muscle. Our overall hypothesis was that oral supplementation of ATE (50 mg/kg/day) for 28 days would affect Notch and Wnt signaling components in aged rodent skeletal muscle.

Materials and Methods

Preparation of *A. Turkestanica* and Phytoecdysteroid Enrichment

A crude phytoecdysteroid extract (a kind gift from PL Thomas, Inc., Morristown, NJ, USA) was produced from dried ground whole *A. turkestanica* material using a 30:70 water-ethanol solvent solution (PoliNat; Las Palmas, Spain). High Pressured Liquid Chromatography (HPLC) analysis (Agilent Technologies, Inc.; Wilmington, DE, USA) with autosampler, DAD (247 nm) and Synergi 4 μ m Hydro-RP 80A reversed phase column (Phenomenex; Torrance, CA, USA) confirmed the total phytoecdysteroid content of the crude extract to be 5.72% dry weight. The method of Cheng et al^{29,30} was modified to further purify and enrich the crude extract to produce the *A. turkestanica* extract (ATE) in 100% non-denatured ethanol (EtOH). The final phytoecdysteroid concentration was determined by HPLC analysis. The dose of ATE was based on the sum of all phytoecdysteroids present (Figure 1).

Animals

Aged (20 months) male C57BL/6 mice (~33 g; Charles River Laboratories; Wilmington, MA, USA) were housed within the David H. Murdock Research Institute (DHMRI) Center for Laboratory Animal Sciences (vivarium) at the North Carolina Research Campus under standard conditions (21°C; 50% humidity; 12:12h reverse

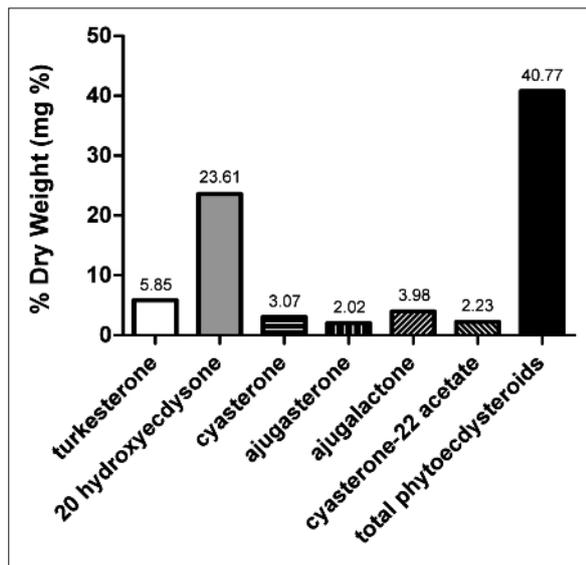


Figure 1. HPLC analysis of final ATE and 20E. The percent dry weight of the components within the supplemented ATE.

light cycle), and were provided standard chow and water *ad libitum*. All procedures were approved by the North Carolina Research Campus Institutional Animal Care and Use Committee and conducted in adherence to the American Physiological Society animal care guidelines. Mice were randomly assigned to one of two treatment groups: CT (vehicle, 100% non-denatured EtOH; $n = 12$) or ATE (50 mg/kg/day; $n = 12$; from the enriched phytoecdysteroid extract described above). ATE was suspended in EtOH. For each individual mouse, the ATE was aliquoted onto a single food enrichment treat (Fruit Crunchies; Bio-Serv, Frenchtown, NJ, USA) to achieve a dose of 50 mg/kg body weight. An equivalent volume of vehicle (100% non-denatured ethanol) was aliquoted onto the food enrichment treat for the CT mice. The ATE and vehicle were applied approximately 18 h prior to feeding in order to allow the ethanol to evaporate. At the end of each light cycle (0800) body mass was measured. Mice were then placed individually in an empty cage without bedding and fed one treated Fruit Crunchie. Consumption of the treated Fruit Crunchie was visually confirmed for each mouse. All mice were supplemented once per day for 28 consecutive days.

Whole Tissue Preparation

Following euthanasia, triceps brachii were excised, coated with optimal cutting temperature

gel (Tissue Tek; Fisher Scientific; Pittsburgh, PA, USA), frozen in isopentane-cooled in liquid nitrogen and stored at -80°C until analysis. Transverse sections (10 μm) from the entire muscle of the triceps brachii were cut using a microtome (HM 505E; Microm, Germany). One section for every 15 cuts of the mid-belly of the triceps brachii was mounted onto gelatin-coated slides and stored at -20°C until processed for immunofluorescence.

Notch and Wnt Signaling Characterization

Protein markers associated with Notch and Wnt signaling in satellite cells from resting skeletal muscle were quantified using immunofluorescence on triceps brachii cross-sections with antibodies directed to either Notch signaling (Delta1, full length Notch receptor, active Notch, and Hes1) or Wnt signaling (active β -catenin or Lef1) and co-stained with the quiescent satellite cell marker, Pax7. Triceps brachii cross-sections were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich; St. Louis, MO, USA) for 10 minutes at room temperature, washed with a standard washing buffer (1% normal goat serum (NGS) in phosphate buffer saline (PBS)/0.1% Triton-X 100 detergent (Sigma-Aldrich; St. Louis, MO, USA) and nonspecific sites were subsequently blocked for 1 h with 5% NGS/0.1% Triton-X100/PBS. Primary antibodies used were mouse anti-Pax7 (1:30; Developmental Studies Hybridoma Bank; Iowa City, IA, USA), rabbit anti-Delta1 antibody (1:40; Santa Cruz Biotechnology; Dallas, TX, USA; cat H265), rabbit anti-full length Notch receptor (1:100; Upstate; Lake Placid, NY, USA; cat 07-220), active Notch (1:500; Abcam; Cambridge MA, USA; cat ab8925), Hes1 (1:1,000; Abcam; cat ab71559), active β -catenin (1:100; Cell Signaling Technology; Boston, MA, USA; cat D13A1) and Lef1 (1:500; Cell Signaling Technology; cat 2230). The isotype control antibodies of mouse IgG (BD Biosciences; San Jose, CA, USA; cat # 553445) or rabbit IgG (BD Biosciences; cat # 550326) were used at the same concentration as the primary antibodies. All primary antibodies were prepared in washing buffer and incubated overnight at 4°C . Following washes, sections were then incubated for 1 hour at room temperature with goat anti-mouse Texas Red (1:1,000; Invitrogen/Life Technologies; Grand Island, NY, USA; cat # T862) or goat anti-rabbit FITC (1:1,000; Invitrogen/Life Technologies; cat # L43001) and nuclei staining 40-6-Diamidino-2-

phenylindole (DAPI; 1:10,000; Sigma Aldrich), each prepared in washing buffer. Immunofluorescence images were visualized using an inverted fluorescent microscope (Olympus IX-71; Parkway Valley, PA, USA). The percentage of Notch or Wnt-expressing nuclei was expressed relative to Pax7-positive nuclei within an entire section.

Statistical Analysis

Statistical analysis was performed using a two-tailed independent *t*-test statistic (SigmaPlot v. 12; Systat, Inc.; San Jose, CA) to determine the extent that ATE affected Notch and Wnt signaling in resting satellite cells. Data are reported as the mean \pm SEM.

Results

ATE Does Not Affect Resting Satellite Cell Number

Resting satellite cell number in triceps brachii was not altered in aged mice treated with ATE, relative to CT. The mean percentage of nuclei expressing the quiescent satellite cell marker Pax7 in CT and ATE were 12.5% \pm 1.2 and 13.4% \pm 2.5, respectively ($p = 0.745$).

ATE Increases Components of Notch Signaling in Aged Skeletal Muscle

ATE did not affect Delta1 ligand expression in Pax7-positive nuclei from resting triceps brachii muscles ($p = 0.505$). The mean percentage of Pax7-positive nuclei expressing Delta1 ligand in CT and ATE were 81.2% \pm 2.5 and 79.0% \pm 1.9, respectively. Similarly, expression of full length Notch receptor was not altered in triceps brachii of mice treated with ATE, relative to CT ($p = 0.143$). The mean percentage of Pax7-positive nuclei expressing full length Notch receptor in CT and ATE was 60.5% \pm 4.6 and 50.8% \pm 4.4, respectively. The percentage of Pax7-positive nuclei expressing active Notch in ATE-treated triceps brachii was significantly greater ($p = 0.005$) than those in the CT treated triceps brachii (Figure 2A). The downstream target of active Notch signaling, Hes1, increased in Pax7-positive nuclei (Figure 2B) of ATE-treated muscles, relative to CT ($p = 0.038$).

ATE increases Wnt signaling in Aged Skeletal Muscle

The percentage of Pax7-positive nuclei expressing active β -catenin in ATE-treated triceps

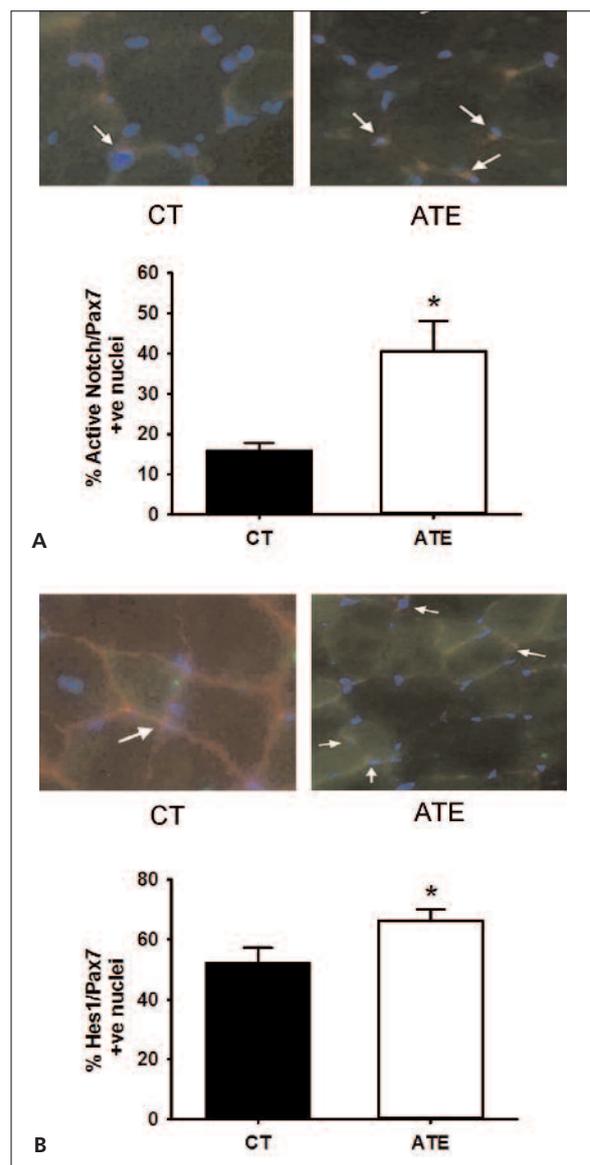


Figure 2. Notch signaling in satellite cells of aged skeletal muscle. **A**, (above) Immunofluorescence representation of active Notch (green), Pax7 (red) and DAPI (blue). Arrows indicate co-localization of active Notch and Pax7. **A**, (below) Immunofluorescence quantification of co-localization of active Notch and Pax7 in nuclei on cross sections of triceps brachii muscle in aged mice supplemented with ATE or CT for 28 days. The percentages of active Notch-positive nuclei to Pax7-positive nuclei are reported. Two sections were counted for every muscle (six muscles/time point). Values are mean \pm SEM. * $p < 0.01$ vs. CT. **B**, (above) Immunofluorescence representation of Hes1 (green), Pax7 (red) and DAPI (blue). Arrows indicate co-localization of Hes1 and Pax7. **B**, (below) Immunofluorescence quantification of co-localization of Hes1 and Pax7 in nuclei on cross sections of triceps brachii muscle in aged mice supplemented with ATE or CT for 28 days. The percentages of Hes1-positive nuclei to Pax7-positive nuclei are reported. Two sections were counted for every muscle (six muscles/time point). Values are mean \pm SEM. * $p < 0.05$ vs. CT.

brachii (Figure 3A) was significantly greater, compared to CT ($p = 0.011$). Downstream Wnt signaling target, Lef1, also increased in ATE-treated triceps brachii (Figure 3B), relative to CT ($p = 0.022$).

Discussion

The coordination of Notch and Wnt is impaired in aged skeletal muscle and may contribute to the reduced muscle competency experienced with aging^{5,22,31}. The purpose of this study was to determine if an extract from *A. turkestanica*, enriched in phytoecdysteroids, affected Notch and Wnt signaling in resting, non-injured satellite cells of aged skeletal muscle. The novel findings of this study were that phytoecdysteroid supplementation increased components of both Notch and Wnt signaling pathways in triceps brachii skeletal muscle of aged mice.

The reported beneficial effects of *A. turkestanica* on skeletal muscle may extend to restoring Notch and Wnt orchestration in aged skeletal muscle.

Twenty-eight days of ATE supplementation induced a 2.5-fold and 1.3 fold increase in active Notch and Hes1 expression respectively in triceps brachii over CT. ATE did not affect the expression of Delta1 ligand or full length Notch receptor in resting satellite cells within triceps brachii muscle. One possible explanation for this may be that components of ATE enhanced the affinity between Delta1 and Notch receptors, thereby increasing activation of Notch signaling, independent of increased ligand and/or receptor expression. Alternatively, other ligands for Notch receptor could have increased in response to ATE treatment, thereby increasing active Notch and Hes1 expression. However, we did not measure other ligands for Notch receptor, such as Jagged1, in this study and this warrants further investigation. It is also possible that ATE supplementation increased the expression and/or activity of the proteolytic enzymes metalloprotease and/or g secretase; both enzymes cleave the full length Notch receptor to activate Notch signaling. Previous investigators had shown that force activating Notch in aged myogenic cells results in improved skeletal muscle integrity including a restored regenerative potential suggesting that increased Notch levels is needed to restore aged skeletal muscle to normal³². The ability of ATE to increase Notch signaling in resting aged satellite

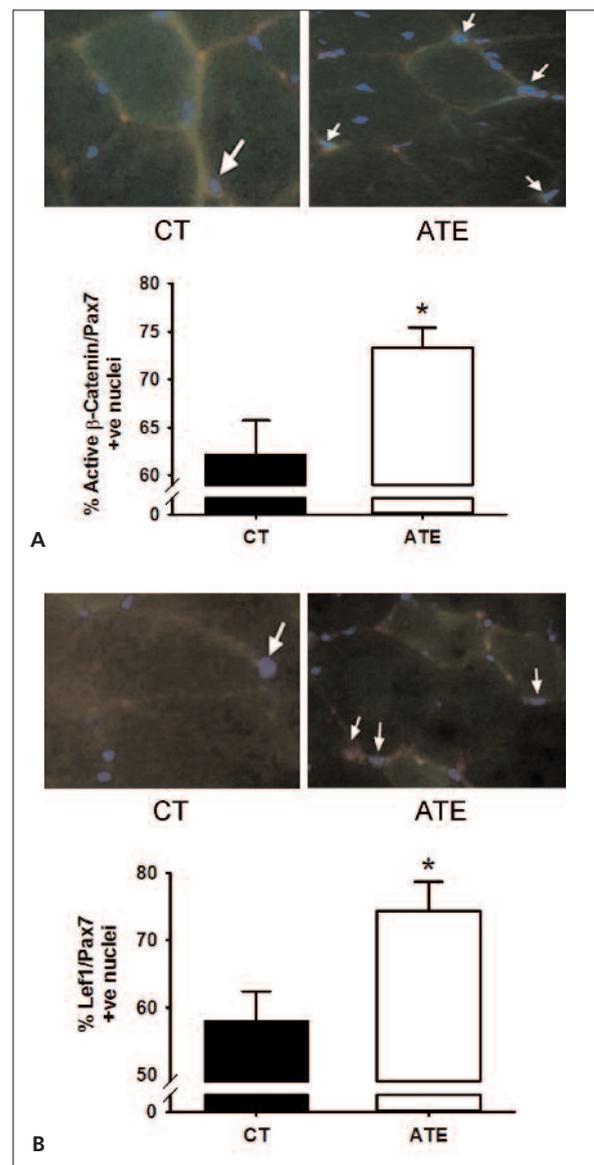


Figure 3. Wnt signaling in satellite cells of aged skeletal muscle. **A**, (above) Immunofluorescence representation of active β -catenin (green), Pax7 (red) and DAPI (blue). Arrows indicate co-localization of active β -catenin and Pax7. **A**, (below) Immunofluorescence quantification of co-localization of active β -catenin and Pax7 in nuclei on cross sections of triceps brachii muscle in aged mice supplemented with ATE or CT for 28 days. The percentages of active β -catenin-positive nuclei to Pax7-positive nuclei are reported. Two sections were counted for every muscle (six muscles/time point). Values are mean \pm SEM. * $p < 0.05$ vs. CT. **B**, (above) Immunofluorescence representation of Lef1 (green), Pax7 (red) and DAPI (blue). Arrows indicate co-localization of Lef1 and Pax7. **B**, (below) Immunofluorescence quantification of co-localization of Lef1 and Pax7 in nuclei on cross sections of triceps brachii muscle in aged mice supplemented with ATE or CT for 28 days. The percentages of Lef1-positive nuclei to Pax7-positive nuclei are reported. Two sections were counted for every muscle (six muscles/time point). Values are mean \pm SEM. * $p < 0.05$ vs. CT.

cells may provide a protective effect to aged muscle and assist in maintaining muscle competency during aging.

Furthermore, 28 days of ATE supplementation increased components of Wnt signaling in the triceps brachii muscle of aged mice. ATE supplementation elevated both active β -catenin and Lef1 expression in resting satellite cells, compared to CT. The role of Wnt signaling in aged skeletal muscle remains controversial^{5,18-22,33,34}. Some investigators report that Wnt may provide beneficial effects to maintaining myogenic potential and competency in skeletal muscle during aging²¹. Fujimaki et al²¹ reported an exercise-induced elevation of Wnt signaling in aged skeletal muscle, which modulated chromatin structures associated with myogenic regulatory factors and induced satellite cell activation. Decreased Wnt signaling was inversely correlated with cell senescence in aged human fibroblasts suggesting Wnt may play a role in longevity³⁴. However, other investigators demonstrated that increased Wnt signaling in aged skeletal muscle inhibited myogenic potential and lend preference to a fibrogenic cell fate of aged satellite cells^{18,20,35}. In addition, there is evidence of Wnt-induced DNA damage³⁶, cell senescence and impaired life span in aged models³⁷.

To further complicate the understanding of Wnt function in aging cells, there is contradiction in the role of Wnt during apoptosis^{12,38-41}. Wnt has been demonstrated to facilitate apoptosis in hematopoietic stem cells through Annexin V and Caspase 3⁴² and to induce apoptosis of osteoblasts during bone remodeling³⁸. On the contrary, apoptosis was elevated in prostate cancer cells when Wnt/ β -catenin was inhibited by ursolic acid⁴³. These differing data suggest that Wnt may either upregulate or inhibit apoptosis depending on the model under investigation. With Wnt's diverse roles in biological processes, it is difficult to establish the function of Wnt in aged skeletal muscle competency. The true role of Wnt in aged skeletal muscle may be dependent upon the micro-environment stimuli¹⁰, the context in how the cell received the signal¹², or the dosage of Wnt signaling activation⁴¹. High levels of β -catenin resulted in apoptosis of intestinal epithelia, moderate levels of β -catenin inducing cell proliferation intestinal epithelia and low levels of β -catenin resulted in no cellular proliferation and differentiation of intestinal epithelia⁴⁴⁻⁴⁶. More work is needed to determine if elevated Wnt signaling in aged muscle treated

with ATE results in improved skeletal muscle competency or if it has deleterious effects and exacerbates the aging process.

There are plausible theories to explain how phytoecdysteroids could activate Notch and Wnt within skeletal muscle. Phytoecdysteroids within ATE, specifically, 20E, are known to induce calcium influx and activate the serine/threonine-specific protein kinase, Akt⁴⁷, which may upregulate the calcium-sensitive components within Wnt signaling, including β -catenin⁴⁸. Since Akt is also recognized to crosstalk with Wnt⁴⁹, as well as activate Notch signaling⁵⁰ and play a role in muscle repair⁵¹, there may be a link between phytoecdysteroids and Notch/Wnt induced-myogenic competency via Akt. This potential mechanism warrants further investigation.

To our knowledge, this is the first study to examine the effect of phytoecdysteroids on Notch and Wnt signaling in quiescent satellite cells of aged skeletal muscle. Previous reports have demonstrated the beneficial effects of phytoecdysteroids on skeletal muscle competency^{27,28,47,52}. *A. turkestanica* contains multiple phytoecdysteroids including 20E and turkesterone²⁵ which have been reported to increase protein synthesis in C2C12 muscle cells, skeletal muscle strength in rats, and skeletal muscle hypertrophy in young rats and mice^{26-28,52}. Furthermore, our analysis of ATE identified other phytoecdysteroids that have additional putative effects including anti-inflammatory and antioxidant properties that may enhance the stability of aged skeletal muscle integrity^{25,30,53}. The multiple bioactive components within ATE may function synergistically to induce a pluripotent response to enhancing and protecting skeletal muscle health⁵⁴.

These data suggest that ATE increases Notch and Wnt in resting aged skeletal muscle. Characterizing Notch and Wnt expression in ATE-treated skeletal muscle is the first step in determining if these two signaling pathways contribute to the restoration of critical myogenic signaling pathways and enhance competency of aged skeletal muscle. Although it is not known if ATE induces apoptosis in skeletal muscle, it is possible that the observed elevated Wnt signaling could have negative effects on aged muscle including increasing apoptosis and cell senescence. Future work will determine if activated Wnt in resting aged muscle would improve skeletal muscle competency or induce deleterious effects. Future

studies will also determine if the phytoecdysteroids extracted from *A. turkestanica* will induce a protective effect in skeletal muscle following exposure to injurious physiological stimuli. Specifically, future work will ascertain if ATE supplementation rejuvenates impaired skeletal muscle repair or, conversely, if it has deleterious effects, such as converting satellite cells to a fibrogenic line or increasing apoptosis of satellite cells. In addition, these studies will examine if phytoecdysteroid treatment activates Akt, thus rescuing the Notch and Wnt equilibrium during skeletal muscle repair. If ATE-induced elevations of Notch and Wnt in aged skeletal muscle is determined to restore aged skeletal muscle to normal function, then phytoecdysteroids on Notch and Wnt signaling may prove to be a novel non-toxic, non-androgenic therapy to combat poor myogenic capacity in aged skeletal muscle. That information will help determine the extent to which phytoecdysteroid-induced Notch and Wnt signaling rescues the age-associated poor muscle repair.

Conclusions

This study is the first to report that ATE supplementation increases Notch and Wnt signaling components in resting tricep brachii muscle from aged mice. It is possible that the phytoecdysteroid-induced alterations in Notch and Wnt signaling will better prepare aged skeletal muscle for repair following exposure to muscle injury. The results from this study will provide critical information to further the understanding of the cellular changes with aging and sarcopenia as well as to inform the development of interventions and non-toxic, non-androgenic therapies for those suffering from sarcopenia and other disorders associated with muscle wasting (such as AIDS, cancer, metabolic disorders, and cardiovascular diseases).

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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