



Ursolic Acid Induces Neomyogenesis through Hyperplasia and Increases of Satellite Cells Number in Mice Skeletal Muscle

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Abstract

Introduction: Ursolic Acid (UA) is the main compound present in apple cuticular wax as well as in the leaves. Hypertrophic effects of UA on the skeletal muscle ventured us to investigate if UA might activate satellite cells proliferation, and also generate new muscle fibers is so-called "hyperplasia".

Methods: To confirm this hypothesis, UA administrated twice daily for 7 days to 8 weeks old mice (C57BL/6) and then skeletal muscle tissues isolated for new muscle fibers and satellite cells determination by histochemical and immunohistochemical tests, respectively.

Results: Our results demonstrated that UA contributed to skeletal muscle rejuvenation through increasing in the satellite cells number and stimulation of these edge cells to new muscle fiber generation.

Conclusion: Taken together, to the best of our knowledge, current findings sharpen our understanding of UA might be as an improvement of skeletal muscle performance and suggest that UA as potential pharmacological purpose to treat the major muscular atrophy diseases such as skeletal muscle atrophy.

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Introduction

Skeletal muscle has a critical role in carry out locomotion, breathing, postural support and heat production during cold stress^[1]. There are about 640 skeletal muscles in human body which it is constituted of ~38% for men and 30% for women total body mass^[2]. Individual muscle fibers are the functional units of skeletal muscle that generate force by contraction. These fibers are constituted of hundreds of thread like protein filaments called myofibrils. During embryonic and fetal development the fusion of many myoblasts lead to formation of a multinucleate myofibers^[3]. The skeletal muscle is so efficient in self-regeneration even after repeated injury, for this thousand of myoblasts on each incident is required^[4].

Hence, satellite cells which resided in a niche on the surface of myofiber play main roles in muscle growth, repair, muscle fiber homeostasis, requiring for myofiber hypertrophy and hyperplasia^[5]. The satellite cells increase the number of nuclei which pointed to greater protein synthesis. In developmental process of skeletal muscle formation from embryonic and foetal times, muscle progenitor and satellite cells require Pax₃ and Pax₇^[6]. Previous studies have been shown that Pax₃ and Pax₇ involved in quiescent and activated satellite cells (so-called myoblast), respectively^[7,8]. In addition, the timing of the embryonic and postnatal periods of muscle development in mouse indicated the dependence of myogenic stem cell populations on Pax genes. On the other hands, the development of skeletal muscle are including: 1- somitogenesis (Pax₃/Pax₇), 2- embryonic and foetal myogenesis (not tested), 3- hyperplastic and hypertrophic growth (Pax₇) and, 4- hypertrophic growth, adult and aging (independent to Pax₇)^[7,8]. As determined



during this time new muscle fibers generation occurred within 2 weeks of birth. Strictly speaking, the number of myofibers does not change after birth.

According to this consideration, UA is a lipophilic compound found in apple peel, such as some folkloric herbal medicines for diabetes^[9]. Previous studies have suggested that UA applied as small molecule inhibitors of skeletal muscle atrophy, it also increased muscle mass and strength, in a laboratory animal model^[10]. Recently, it has been reported that UA increased brown fat and energy expenditure, reduced diet-induced obesity, white adipose tissue, glucose intolerance and fatty liver diseases in a mouse model of diet-induced obesity^[11]. They also declared that in mice fed a high fat diet, UA increased skeletal muscle mass, anabolic mRNA expression, Akt signaling, fast and slow skeletal muscle fibers size and grip strength^[11]. According to these considerations, skeletal muscles are chosen in this research due to their post mitotic tissue and exhibition of tissue-specific aging effects, including differences in the accumulation of mutant mtDNA^[12]. In this regard, we sought to know the effects of UA, as inhibitor of skeletal muscle atrophy and stimulator of muscle hypertrophy, on satellite cells and muscle rejuvenation. In this research we interested to know if UA might affect on muscle hyperplasia rather than hypertrophy.

Material and Methods

Mouse protocols

Male C57BL/6 mice, aged 8 weeks (~ 20 g weight), were received from Pasteur Institute, Iran, held in colony cages with 12 hr light/12 hr dark cycles, and used for tests within 3 weeks of their onset. Moreover, otherwise designated, the mice were kept on standard chow (Harlan Teklad formula 7013). UA (Enzo Life Sciences, BML-CT105-0250) was dissolved in corn oil at a 20 mg/ml concentration for intra-peritoneal injection (i.p). UA (200 mg/kg) or placebo alone (corn oil) was administrated via i.p. Injection was done twice daily for 7 days^[13]. Each mouse was subjected to three consecutive tests to obtain the peak. Histochemical and immunohistochemical tests for determination of tissue regeneration and satellite cells detection, respectively. All animal procedures were approved by the Institutional Animal Care and use Committee of the Endocrinology and Metabolism Research Center, Tehran University of Medical Sciences. Finally, for IHC and histochemical test the animals should be weighted and anaesthetized by an i.p of ketamine/xylazine and then fixation with 4% PFA, 2.5% glutaraldehyde.

Hematoxylin & eosine staining for evaluation of muscle regeneration

Tissue samples was incubated in the 30% sucrose, and stored at 4°C for H & E (0.1 mg/ml) and immunohistochemical tests. Then, they were fixed with OCT compound (Tissue-Tek). Serial cross-sections (8 µm thicknesses) were performed using a cryostat microtome at -25°C, mounted on cover slips, and then stained for cytoplasm and nucleus detection. In this method, prior to staining, slides must be blocked at room temperature for 10 minutes. Following, cytoplasmic staining by hematoxyline for 5 min, washing with PBS and water, nuclei staining by eosine for 5 min and then washing with PBS and water, dehydration by descending alcohol and, and mounting a cover slips and visualize staining of tissue under a microscope using a bright-field illumination.

Immunohistochemical test for satellite cells identification

Staining of the frozen muscle sections (8 µm) was performed as described by Sherwood et al^[14]. In this procedure, we acted as follows: thaw the slides at room temperature for 10 - 20 minutes, when staining cryostat sections stored in a freezer, rehydrated the slides in the wash buffer for 10 minutes, surround the tissues with a hydrophobic barrier using a barrier pen (ab2601, Abcam). To quench endogenous peroxidase activity, incubate the sample with 1-3 drops peroxidase blocking reagent (3% H₂O₂ in water) for 5 - 15 minutes. Rinse the sample, and gently wash in wash buffer for 5-minutes. Blocking the section with 1 - 3 drops of serum blocking reagent (0.5% BSA for 30 min at 37°C) for 15 minutes. Then, samples were incubated by primary antibody Pax₇ (ab6107, Abcam) (1/100 in 1% BSA + 0.5% skim milk) for 1h at 37°C. Rinse the samples with wash buffer. Incubating the samples with Horse Raddish Peroxidase (HRP) - conjugate goat anti-rabbit polyclonal IgG (SC-J2811). Rinsing and washing 3 times in the wash buffer for 2 minutes. Adding 1-5 drops of DAB/AEC (CTS011) chromogen solution to cover the entire tissue section, and incubate for 20 minutes. Rinsing the samples with the wash buffer. Rinsing in deionized H₂O and mounting a cover slips and visualize staining of tissue under a microscope using a bright-field illumination.

Results

New muscle fiber generation by UA

Since, the process of two adjacent membranes fusion accomplishes many goals that are crucial to the development and maintenance of the skeletal muscles; we directly tested if UA might trigger myoblast fusion in adult mice. Therefore, cross-sections of myofibers from the control and treated mice stained by H & E. As shown in Figure 1A and 1B, the presence of centrally nucleated myofibers and collectivity of nuclei in the UA-treated mice exhibited a hallmark of muscle regeneration which illuminates a new fiber generation.

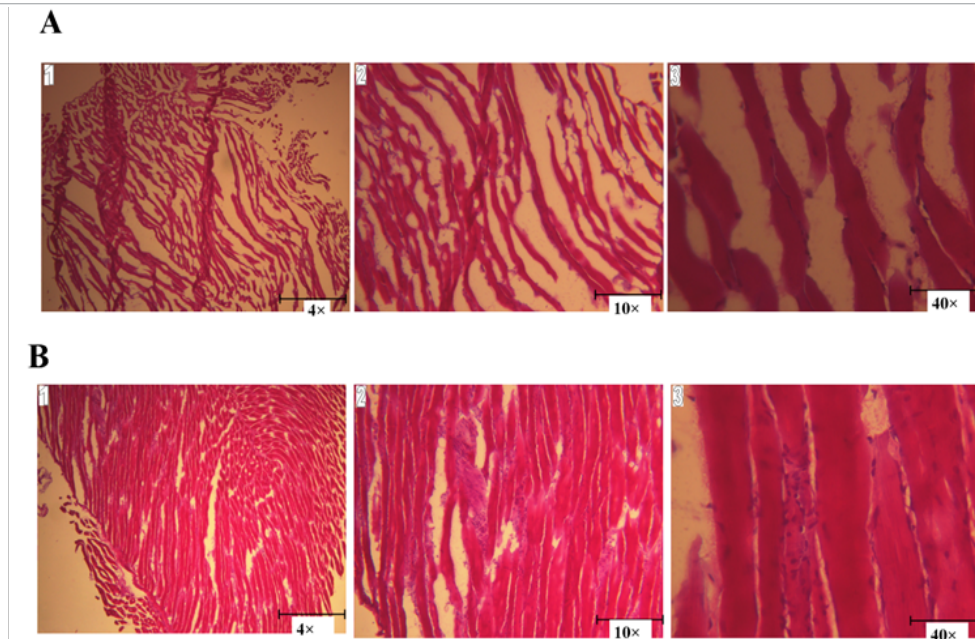


Figure 1: UA neomyogenesis stimulation through satellite cell proliferation and myoblast fusion: The mice received corn oil and UA+ corn oil (ip) containing 200 mg/kg UA for 7 days; (A-B) 8 μ m cross-sections of gastrocnemius muscle for observation of myofiber regeneration stained by H & E using 3 different magnifications (1) 4X, (2) 10X and (3) 40X. The presence of centrally nucleated myofibers indicates a hallmark of muscle regeneration; (A): Corn oil; and (B) UA+ corn oil.

Satellite cells waking up induction in adult mouse muscle by UA

To access the effects of UA on the muscle rejuvenation, next we sought to consider the UA influences on the satellite cell proliferation. Therefore, we examined the PAX₇ expression in the treated and control mice muscles by immunohistochemistry method. Specifically, as illustrated in Figure 2A and 2B, there was a noteworthy alteration in the number of myonuclei following the UA administration to the mice.

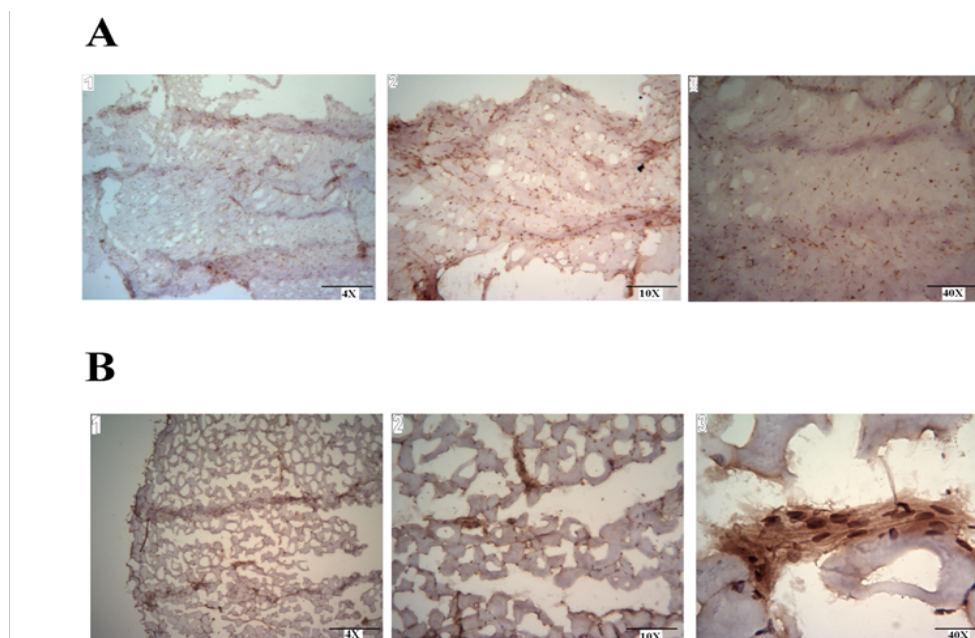


Figure 2: UA increases satellite cells proliferation by up-regulation of Pax₇. The mice were received corn oil and UA+corn oil (i.p) containing 200 mg/kg UA for 7 days. (A-B) 8 μ m Cross-sections of gastrocnemius muscle for observation of satellite cells by Pax₇, as an identifier of these cells using 3 different magnifications (1) 4X, (2) 10X and (3) 40X; (A): Corn oil; and (B) UA+ corn oil.

Discussion

Satellite cells are small mononuclear progenitor cells with responsibility in the repair of injured fibers, differentiate and fuse to augment existing muscle fibers and proliferate to form new fiber generation^[15]. There are two ways in new fibers formation. First, fiber splitting from large fibers into two or smaller fibers^[16,17] Second satellite cells activation^[18-20].

According to the recent study that showed the hypertrophic effects of UA on the skeletal muscle^[13], we more motivated to investigate if UA might stimulate skeletal muscle hyperplasia as well. Remarkably in the H & E-stained samples of UA-treated mice, the results indicated the elevation in the number of nuclei, which astonishingly were centrally located in the cytoplasm and also new fibers have been generated (Figure 1A and 1B). In support of this hypothesis, a previous study has been shown that the fusion of myoblast promotes new fibers development (multinucleation, or so-called neomyogenesis), which plays an essential role in the muscle function^[21,22]. Furthermore, There are two ways for promoting new fibers formation i.e. fiber splitting from large fibers into two or smaller fibers^[16,17] and satellite cells activation^[18-20]. It has been reported that enhanced SIRT1 expression in the satellite cells stimulates their proliferation by inhibiting the expression of the cell cycle inhibitor P21^{Waf/Cip1} and P27^{Kip1}^[23]. On the other hand, SIRT1 by promoting PAX₇ expression prohibit the muscle formation by suppressing the myogenic gene program (MyoD and Myf5)^[24]. Further, provoked by these findings, we set out to explore the immediate crucial question if UA might induce satellite cells proliferation. To answer this question, our observations identified novel populations of cells that express PAX₇, which are resided in the skeletal muscles, gastrocnemius myofibril (Figure 2B). Satellite cells are characterized by the expression of the paired box transcription factor PAX₇^[25]. On the other hand, it has been reported that PAX₇ is required for satellite cell survival and proliferation, and for prohibiting differentiation progress of myogenic progenitor cells^[26]. Satellite cells of the adult skeletal muscle fibers are myogenic mono-nucleated cells that are strictly adhered to the muscle fibers. They are more numerous in slow-twitch than in fast-twitch muscles IIB. Besides, the highest turnover rate of these quiescent cells in normal muscles is 1% per week but injury, extreme muscle activity, mechanical stretching, and also androgens induce proliferation and ultimately fusion into myotubes^[27]. As we comprehended about UA, it seems that it by increasing satellite cells activation leads to generate large fibers (hypertrophy), and also inducing in fiber splitting into smaller fibers to prevent muscle atrophy. Furthermore, recent studies showed that there is a molecular network between PGC-1 α , neuromuscular junction (NMJ) and the skeletal muscle satellite cells^[28,29]. The co-activator PGC-1 α allows recruitment of GA-binding protein and led to up-regulation of a broad NMJ gene program^[28]. Accordingly, the results showed that satellite cells were more observed to concentrate at the NMJ^[28].

Taken together; our findings demonstrate that UA by recruiting in the satellite cells activation and fusion to each other result in new myofiber generation, strictly speaking hyperplasia versus hypertrophy. Finally, our results strongly proposed UA as an excellent candidate for pharmacological purpose in treatment of skeletal muscle myopathy such as, Duchenes muscular dystrophy, skeletal muscle atrophy (SMA).

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Conflict of Interest: We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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