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Research Article
Ursolic Acid Improved Sarcopenia in D-Galactose-Induced Aging Mice

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Abstract

Background and Objective: Aging is an inducement of sarcopenia to cause hypokinesia. Ursolic acid (UA) has various biologic activities in aging and muscle injury. The mechanism and correlation between UA and sarcopenia is unclear. Hence, the purpose of current study was to appraise the protection of UA on aged-relevant sarcopenia. **Materials and Methods:** The aging mouse model was established by D-galactose. Muscle force was tested according to the grip strength meter. The levels of oxidation indices were measured by spectrophotometer. The levels of inflammatory indexes were analyzed by ELISA kits. The expressions of FBXO-32, MURF-1, Collagen-I, TGF-β, BAX and BCL-2 were detected by western blot. **Results:** The UA enhanced muscle mass and grip strength to mitigate D-galactose-induced muscle dysfunction. In serum, UA restrained oxidative stress and inflammatory response to improve internal environment homeostasis and delay body decrepitude. In skeletal muscle, UA reduced FBXO-32, MURF-1, Collegan-I and TGF-β1 expressions to mitigate D-gal-induced muscle dystrophy and fibrosis. In molecular mechanism, UA decreased BAX and increased BCL-2 expressions to inhibit apoptosis. **Concision:** The UA was considered a functional agent due to its amelioration on apoptosis.

Key words: Aging, ursolic acid, sarcopenia, D-galactose, inflammatory reaction, oxidative stress, fibrosis, apoptosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Advancing age was reported to be coupled with somatic dysfunction[1]. During aging process, exercise ability gradually decline with time[2]. Aging easily results in fatigue to impact athletic ability and hypokinesia further aggravate life quality[3]. Aging is a non-reversible process of life characteristics. The population over 60 years old is increasing year by year, which leads to an increasing degeneration of human organism[4]. Hypokinesia will become a major public health problem in the next decades[5]. Therefore, seeking intervention strategies to improve age-relevant motor disturbance is extremely desired. Some researchers confirmed that D-galactose-induced aging was linked to motor disturbance[6]. In this study, the aging model was established by D-gal to develop feasible intervention strategies for age-related hypokinesia.

Multifarious functions of organs show a downward trend in the elderly population[7]. Skeletal muscle as a crucial locomotive organ is closely related to exercise ability[8,9]. Sarcopenia is a phenomenon of aging[10,11]. Some researchers have indicated skeletal muscle disorder is an important pathological manifestation during sarcopenia[12]. Age-related sarcopenia is bound up with muscle atrophy, which results in degeneration of muscle mass and strength[13]. With increasing age, muscle atrophy is a known risk factor exacerbating motor disturbance. Evidence has shown muscle atrophy is associated with protein synthesis and breakdown. The ubiquitin-proteasome system (UPS) was activated to induce protein degradation imbalance[14]. As age-related sarcopenia is a multifactorial disease, the feasible treatment is not fully elucidated. In this study, novel interventions were further explored to prevent muscle atrophy.

Ursolic acid (UA) is a terpenoid and exists in a wide variety of fruits. The UA has multiple active constituents to cure many diseases[15]. Ramos-Hryb *et al.*[16] and Qu *et al.*[17] research shown UA can availably ameliorate age-related conditions. In male fruit flies, UA increased life span, modulated energy metabolism, improved immune function and enhanced climbing activity[18]. In aged type 2 diabetic rats, UA possessed antioxidant effects to enhance exercise training[19]. In senescent mice, UA had an antioxidant activity and improved cognition deficits against D-galactose-induced neurotoxicity[20]. In aged mice, UA increased metabolic sensor protein levels to regulate the aging process[21]. In the present study, the treatment of UA on muscle atrophy and movement ability was investigated in aged mice. The results displayed a possible mechanism for UA to delay age-related sarcopenia.

MATERIALS AND METHODS

Study area: The study was carried out at the Sports Biochemistry Laboratory, Hunan University of Arts and Science (May to October, 2023).

Animals: The 30 male ICR mice were 6-8 weeks old. All mice were purchased from Hunan SJA Laboratory Animal Co. Ltd (Changsha, China). The body weight of mice was 18-22 g. All animals could eat chows and drink water without restriction.

The experiments were approved by the Ethics Committee of the Hunan University of Arts and Science (No. HUAS-2021-TY-063).

Chemicals and reagents: Ursolic acid (UA, Purity $\geq$95.0%) was purchased from Sangon Biotech. The UA was stored at 4°C. The detection kits were purchased from Nanjing Jiancheng Biotechnology Institute. The ELISA kits were purchased from BOSTER Biological Technology. All primary and secondary antibodies were purchased from Proteintech, Servicebio and Sangon Biotech.

Experimental design: The control groups were treated with a normal saline solution. The model groups were subcutaneously injected with D-galactose (500 mg/kg b.wt.)[22,23]. The UA groups received D-galactose (500 mg/kg b.wt., for 10 weeks) and ursolic acid (200 mg/kg b.wt., for 10 weeks), respectively.

Grip strength test: At the end of the experiment, the control, model and GA groups executed grip strength test[24]. The grip tester was purchased from Anhui Zhenghua Bioinstrumentation. Mice pulled the stick to acquire experiment data. The grip strength was recorded by multiple metering.

Biochemical assay: After the grip strength test, mice were sacrificed. The blood was acquired from the eyeball. Next, blood was further centrifuged to separate the supernatant. The absorbance values of ALT, AST, BUN and CK were tested at 505, 510, 640 and 660 nm.

Oxidation indices assay: Gastrocnemius were dissected and weighed. Then, skeletal muscle was rapidly frozen by liquid nitrogen. The SOD was determined by the hydroxylamine method. The CAT was determinate by the ammonium molybdate spectrometric method. The MDA was determined

by the thiobarbituric acid method. The absorbance values of T-AOC, SOD, CAT and MDA were tested at 520, 550, 405 and 532 nm.

ELISA: Biotin was labeled in TNF-α and IL-6 antibodies. Tetramethylbenzidine was used as chromogenic substrate. The absorbance value was tested at 405 nm.

Western blot: Skeletal muscle was lysed with a RIPA lysis buffer. The homogenates were centrifuged to separate protein extraction of skeletal muscle. Protein concentration was detected by the BCA method. The SDS-PAGE was used to separate tissue protein samples. The method of wet electroblot technology was used to transfer different molecular weights of proteins onto PVDF. The PVDF was incubated with non-fat milk, primary antibodies and peroxidase-conjugated secondary antibodies. Signals were visualized by the ECL system. The β-actin was the reference gene to normalize protein levels. The results were analyzed via Quantity One v4.6.2.

Statistical analysis: All results were expressed as Mean±SD. The significance was demonstrated by a one-way ANOVA followed by Duncan's multiple range test. The $p<0.05$ were deemed significant.

RESULTS

Roles of UA on D-galactose-induced skeletal muscle dysfunction: In Fig. 1a, D-gal reduced gastrocnemius weight ($p<0.05$), while UA visibly augmented muscle mass to alleviate sarcopenia ($p<0.05$). In Fig. 1b, D-gal decreased grip strength ($p<0.05$), while UA visibly augmented grip strength to increase muscle function ($p<0.05$). These results suggested UA potentially ameliorated skeletal muscle dysfunction in aged mice.

Roles of UA on D-galactose-induced biochemical indexes in serum: In Fig. 2a-d, D-gal induced ALT, AST, BUN and CK levels ($p<0.05$), while UA visibly reduced biochemical indexes ($p<0.05$). These results suggested UA potentially ameliorated liver, kidney and muscle injury in senescence mice.

Roles of UA on D-galactose-induced oxidation damage in serum: In Fig. 3(a-c), D-gal decreased T-AOC, SOD and CAT concentrations ($p<0.05$), while UA visibly reversed these adverse alterations to enhance antioxidant capacity ($p<0.05$). In Fig. 3d, D-gal increased MDA levels ($p<0.05$), while UA visibly decreased MDA levels to relieve oxidative stress ($p<0.05$). These results suggested UA potentially alleviated oxidative damage in senescence mice.

Roles of UA on D-galactose-induced inflammatory response in serum: In Fig. 4(a-b), D-gal strengthened TNF-α and IL-6 contents ($p<0.05$). However, UA visibly suppressed adverse inflammatory cytokine contents ($p<0.05$). These results suggested UA potentially alleviated inflammatory response in senescence mice.

Roles of UA on D-galactose-induced protein degeneration in skeletal muscle: In Fig. 5(a-c), the parameters of protein degeneration were detected by western blot. In Fig. 5(b-d), D-gal enhanced FBXO-32 and MURF-1 expressions ($p<0.05$). However, UA visibly relieved D-galactose-evoked ubiquitin E3 ligases to improve muscular dystrophy ($p<0.05$). These results

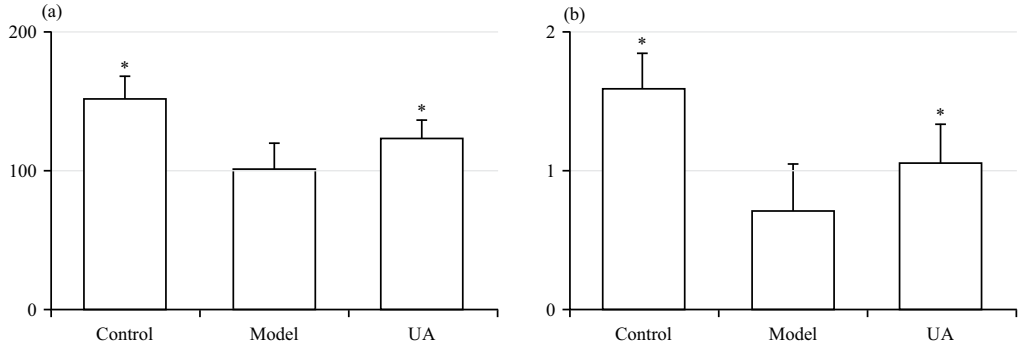

Fig. 1(a-b): Roles of UA on skeletal muscle dysfunction in D-galactose-treated mice, (a) Gastrocnemius weight (mg) and (b) Grip strength (N) in senescence mice

*$p<0.05$ vs model group

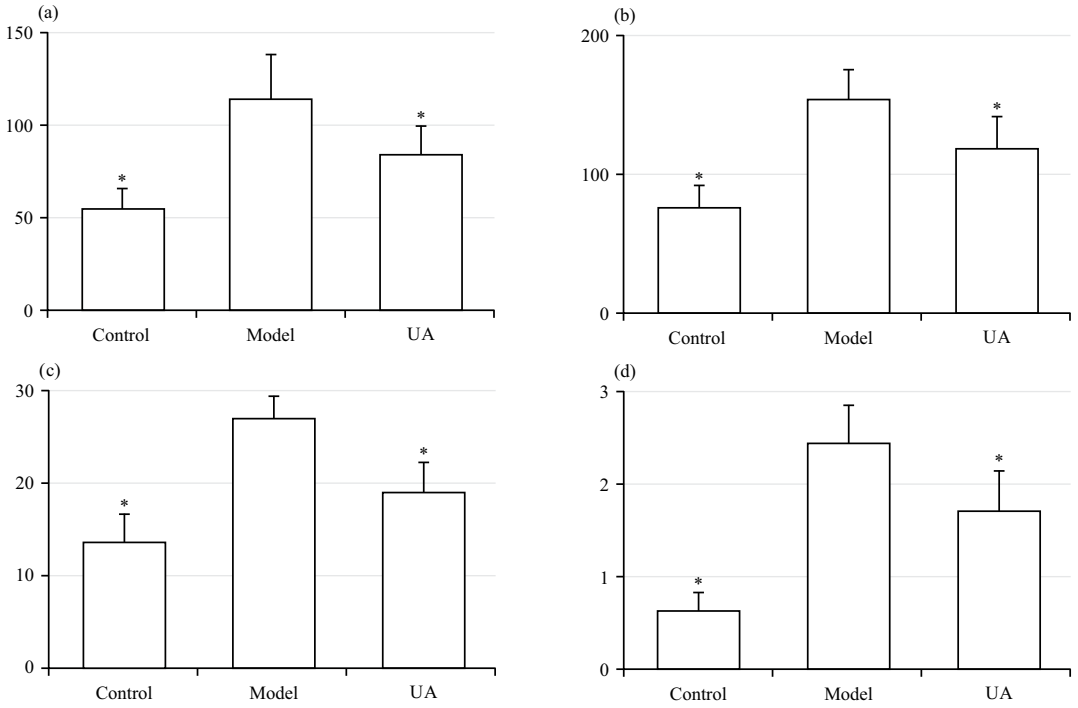

Fig. 2(a-d): Roles of UA on biochemical indexes in D-galactose-treated mice. Serum levels of (a) ALT (U/L), (b) AST (U/L), (c) BUN (mmol/L) and (d) CK (U/mL)

*p<0.05 vs model group

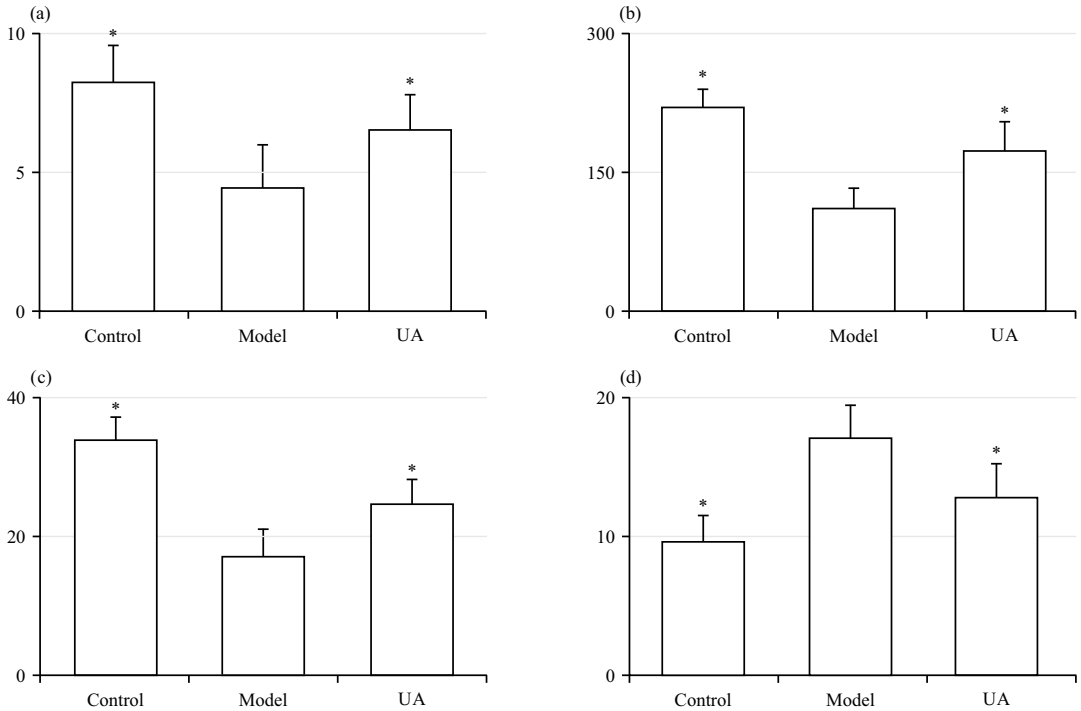

Fig. 3(a-d): Roles of UA on oxidation damage in D-galactose-treated mice. Serum concentrations of (a) T-AOC (U/mL), (b) SOD (U/mL), (c) CAT (U/mL) and (d) MDA (nmol/mL)

*p<0.05 vs model group

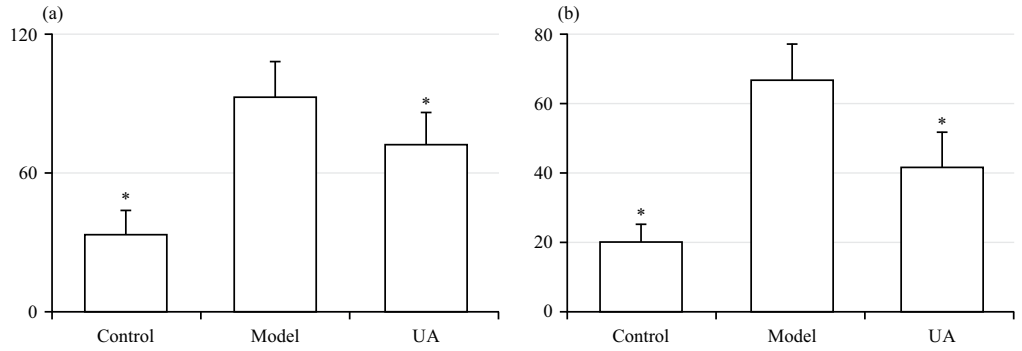

Fig. 4(a-b): Roles of UA on inflammatory response in D-galactose-treated mice. Serum contents of (a) TNF-α (pg/mL) and (b) IL-6 (pg/mL)
*p<0.05 vs model group

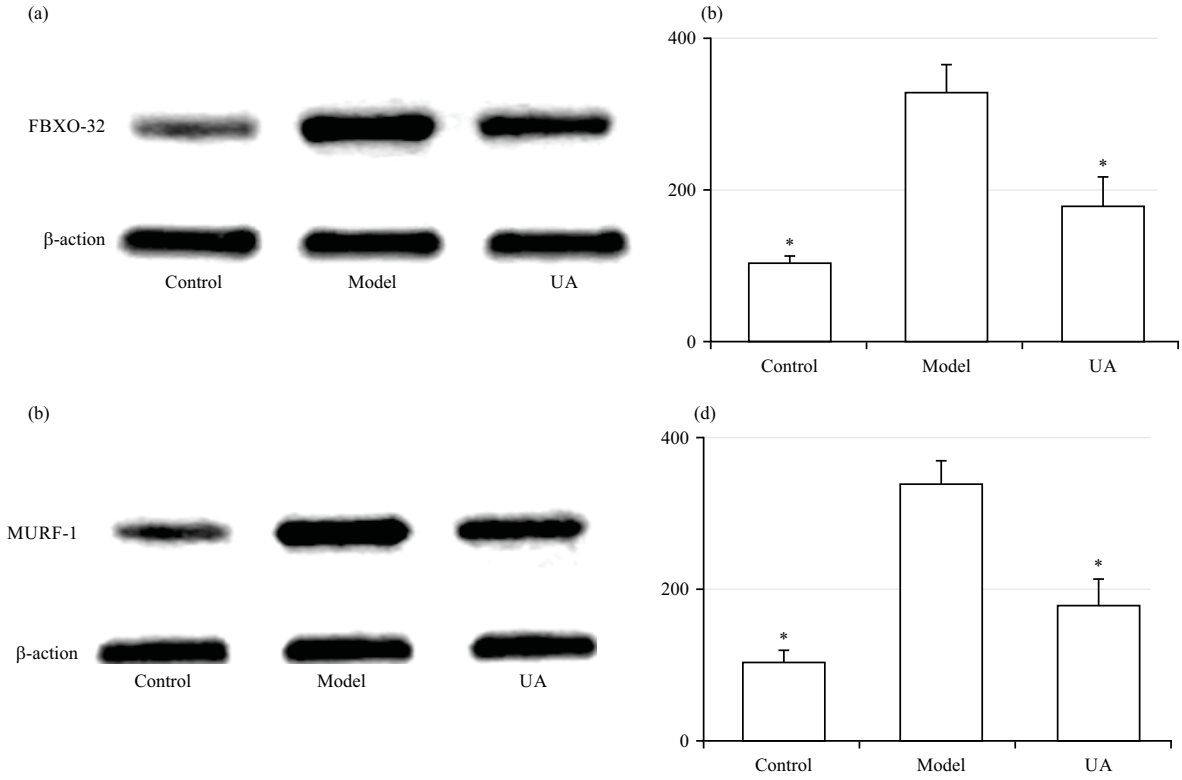

Fig. 5(a-d): Roles of UA on muscle atrophy in D-galactose-treated mice. Western blot and semiquantitative analysis of (a-b) FBXO-32 protein expression (control%) and (c-d) MURF-1 protein expression (control%)
*p<0.05 vs model group

suggested UA potentially ameliorated ubiquitin-proteasome system in senescence mice.

Roles of UA on D-galactose-induced fibrosis in skeletal muscle: In Fig. 6(a-c), the indicators of fibrosis were detected by Western blot. In Fig. 6(b-d), D-gal enhanced TGF-β1 and Collegan-I expressions ($p<0.05$). However, UA visibly reduced TGF-β1 and Collegan-I expressions to improve muscle fibrosis ($p<0.05$). These results suggested UA potentially regulated TGF-β signaling pathway in senescence mice.

Roles of UA on D-galactose-induced excessive apoptosis in skeletal muscle: In Fig. 7(a-c), the indexes of apoptosis were detected by western blot. In Fig. 7(b-d), D-gal heightened Bax

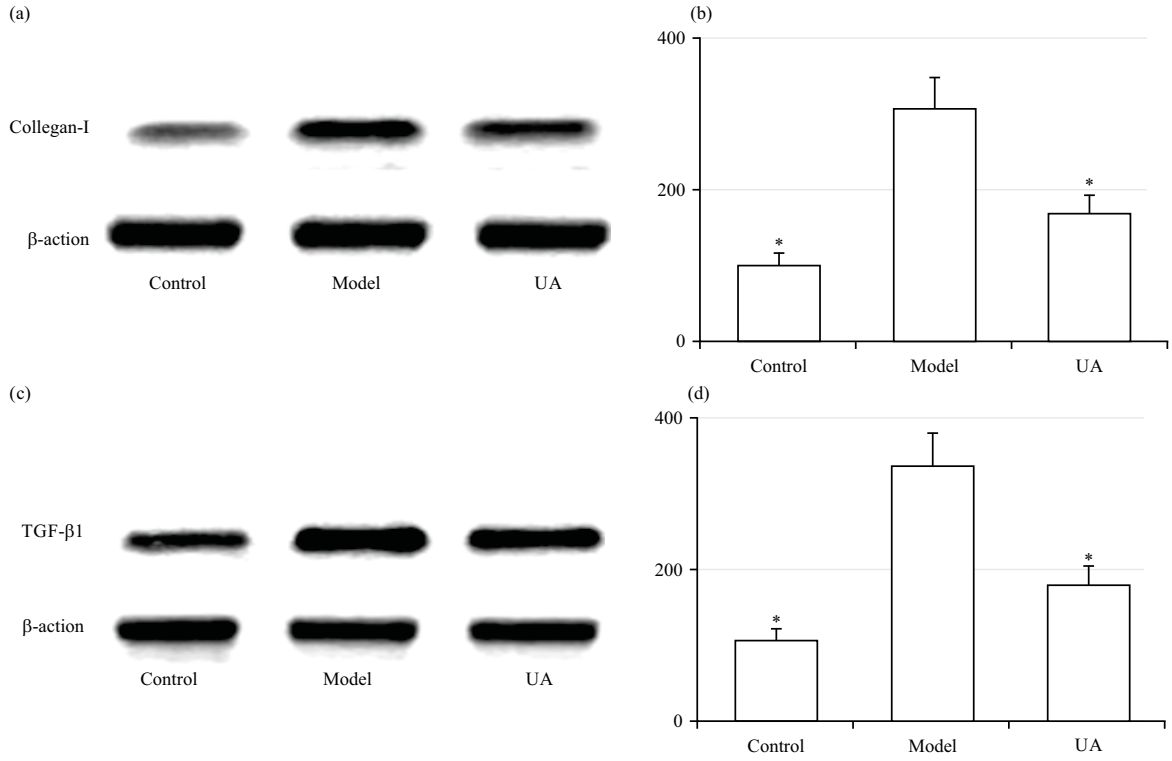

Fig. 6(a-d): Roles of UA on muscle fibrosis in D-galactose-treated mice. Western blot and semiquantitative analysis of (a-b) Collegan-I protein expression (control%) and (c-d) TGF-β1 protein expression (control%)
*p<0.05 vs model group

expression and restrained BCL-2 expression (p<0.05). However, UA visibly reversed these adverse alterations in skeletal muscle (p<0.05). These results suggested UA potentially relieved D-galactose-evoked excessive apoptosis in senescence mice.

DISCUSSION

The UA has pharmacological action on muscle function. The UA was proved to enhance mitochondrial mass and increase endurance exercise capacity[25]. In hypobaric hypoxia exposed rats, UA attenuated oxidative damage inflammatory response, enhanced Akt pathway-related proteins to relieve skeletal muscle damage[26]. In obese mice, UA increased Akt activity in skeletal muscle to induce hypertrophy and enhance movement ability[27]. In resistance exercise-induced fatigue rats, UA increased mTORC1 activity and facilitated anabolism in skeletal muscle[28]. In resistance-trained men, UA elevated Irisin and alleviated skeletal muscle damage markers in blood to augment muscle strength[29,30]. In addition, UA possessed anti-aging effects. The UA promoted anti-aging biomarkers and satellite cells proliferation to accelerate skeletal muscle rejuvenation[31,32]. We hypothesized that UA improved sarcopenia to sustain muscle function in D-galactose-induced aging mice. In this study, UA relieved skeletal muscle damage in senescent mice as represented by increasing muscle mass and grip strength to mitigate D-galactose-induced muscle dysfunction.

Aging is a risk factor resulting in incapacity of body systems. The liver, kidney and muscle are sensitive organs in aging process, which is associated with numerous effects on structure and function. Aging is linked to a variety of diseases, such as hepatic fibrosis, kidney inflammation and muscular atrophy. The UA was proved to have a therapeutic effect on body systems. In db/db mice, UA reduced liver weight and ALT and AST levels in serum to inhibit nonalcoholic fatty liver disease[33]. In T2DM rats, UA attenuated creatinine and BUN levels to mitigate kidney cell damage[34]. In T1DM rats, UA inhibited CK and LDH levels to improve cardiac structure and function[35]. In this study, UA decreased ALT, AST, BUN and CK levels in serum of D-galactose-induced aging mice. These results indicated that UA ameliorated liver, kidney and muscle injury in aging mice.

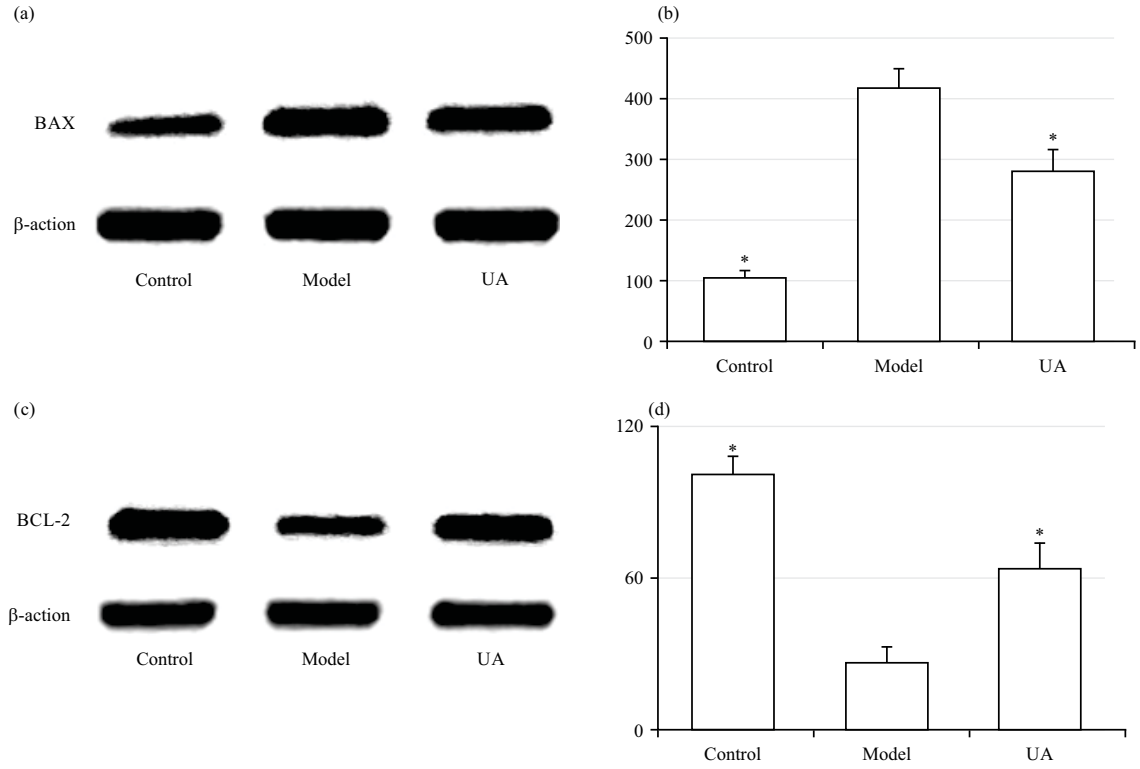

Fig. 7(a-d): Roles of UA on excessive apoptosis in D-galactose-treated mice. Western blot and semiquantitative analysis of (a-b) BAX protein expression (control%) and (c-d) BCL-2 protein expression (control%)
*p<0.05 vs model group

Oxidative stress was related to aging. The SOD and CAT are antioxidant enzymes, which can scavenge oxygen free radical to improve internal environment homeostasis. The MDA, as lipid peroxidation production, causes biological macromolecule disorder in aging process. Ma *et al.*[36] and Ma *et al.*[37] showed that UA had oxidation resistance to cure various diseases. In CCl4-treated mice, UA enhanced T-AOC level to relieve hepatotoxicity and nephrotoxicity. In dextran sulfate sodium-treated mice, UA enhanced SOD activity to protect against ulcerative colitis[38]. In isoproterenol-administered rats, UA enhanced CAT activity to delay heart damage[39]. In diabetic rats, UA reduced MDA activity to alleviate renal injury[40]. Moreover, previous studies showed that D-gal disturbed disequilibrium of oxidation and antioxidation to aggravate organ dysfunction. In this study, UA increased T-AOC, SOD and CAT levels and decreased MDA levels in serum of D-galactose-induced aging mice. These results indicated that UA ameliorated oxidative stress to improve internal environment homeostasis in aging mice.

Inflammatory response is one of critical precipitating factors of aging. Overmuch inflammation level is harmful for regeneration and accelerates decrepitude of the human body. Previous studies showed that UA had anti-inflammatory effects in many diseases. In aged rats, UA decreased proinflammatory cytokines IL-6 and IL-1â levels to ameliorate adipose tissue insulin resistance[41]. In CCl4-induced liver fibrosis mice, UA inhibited inflammatory factor TNF-α level to attenuate intestinal injury and protect the intestinal barrier[42]. Moreover, previous studies showed that D-gal activated inflammatory response to induce senescence. In this study, UA decreased TNF-α and IL-6 levels in the serum of D-galactose-induced aging mice. These results indicated that UA ameliorated inflammatory response to delay decrepitude of the human body in aging mice.

Aging intensifies the ubiquitin-proteasome system to disturb protein synthesis and breakdown. Protein degradation imbalance results in muscle atrophy. Muscle atrophy is a common pathological phenomenon during aging. The UA was proven to be involved in mitigating muscle atrophy. Kang *et al.*[43] observed UA improved muscle mass in immobilization-induced muscle atrophy. In emphysema rats, UA alleviated cigarette smoke-induced musculi soleus

atrophy[44]. In addition, UA prevented muscle atrophy and activated SIRT1 against cancer cachexia[45]. The FBXO-32 and MURF-1 are important factors of the ubiquitin-proteasome system and crucial proteins which is relevant to protein degradation. Previous research demonstrated that a decrease in FBXO-32 and MURF-1 is a feasible approach for improving atrophy in skeletal muscle. In this study, UA relieved skeletal muscle atrophy by decreasing FBXO-32 and MURF-1 expressions in D-galactose-induced aging mice.

Skeletal muscle fibrosis is a common and typical phenomenon during aging. Excessive fibrosis influences muscle fiber regeneration and motor capacity. The TGF-β1 signaling has been proven to play an important role in fibrosis. Previous studies showed that UA could alleviate fibrosis in many experimental models. In CCl_4-induced mice, UA reduced Collegan-I expression to reverse liver fibrosis[46]. In diabetic nephropathy, UA reduced SMA-α expression to relieve renal fibrosis[47]. In diabetic cardiomyopathy, UA reduced TGF-β1 expression to alleviate myocardial interstitial fibrosis[34]. In isoproterenol-induced myocardial infarction, UA reduced Collegan-I and TGF-β1 expressions to inhibit myocardial fibrosis[48]. Previous research showed that aging aggravates fibrosis in many organs. In skeletal muscle, the expressions of Collegan-I and TGF-β1 were increased accompanying aging. In this study, UA relieved skeletal muscle fibrosis by decreasing Collegan-I and TGF-β1 expressions in D-galactose-induced aging mice.

Apoptosis imbalance is harmful to muscle mass. Previous studies showed UA had anti-apoptotic effects. In alcohol-treated mice, UA decreased apoptosis to mitigate liver injury[49]. In LPS-exposed mice, UA prevented apoptotic imbalance to relieve asthenozoospermia disease[50]. In chronic unpredictable stress-treated mice, UA increased the BCL-2/BAX ratio to abrogate depressive-like behavior[51]. Aging facilitates the abnormal apoptosis process. During aging, apoptosis imbalance was activated in skeletal muscle. Previous studies showed restraint of apoptosis can efficiently alleviate age-related muscle decline[52]. In this study, UA decreased BAX and increased BCL-2 to relieve sarcopenia in D-galactose-induced aging mice. These results indicated that UA was involved in the inhibition of D-galactose-induced apoptosis to improve aging-associated sarcopenia.

CONCLUSION

Current results displayed UA increased muscle mass and grip strength in senescence mice. In serum, UA exhibited anti-oxidative and anti-inflammatory effects to protect against organic damage. In skeletal muscle, UA reduced dystrophy and fibrosis via ubiquitin-proteasome system and TGF-β pathway against D-gal-induced muscle injury. The possible mechanism of UA on aged-associated sarcopenia was ascribed to its amelioration on apoptosis.

SIGNIFICANCE STATEMENT

Current study revealed the improvement of UA against muscle atrophy in D-galactose-induced aging mice. The UA enhanced muscle mass and grip strength to alleviate muscle atrophy. The protective effects of GA on sarcopenia concomitant aging were associated with its biological functions on restraint of apoptosis. The present study indicated a new discovery in understanding of UA on treating aged-associated sarcopenia.

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