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Role of gallic acid in sarcopenia in D-galactose-treated mice

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ABSTRACT

Objective(s): Aging is a natural phenomenon that results in sarcopenia to impair living quality in humans. Gallic Acid (GA) has many biological activities against aging and muscle injury. Our purpose was to clarify possible mechanisms of GA on aged-relevant sarcopenia.

Materials and Methods: Senescent model was established by D-galactose in mice. The senescent model was treated with GA for 10 weeks. Skeletal muscle was stained via HE to assess muscle fiber size. Oxidation indicators and inflammatory factors were detected using a spectrophotometer and ELISA. The protein expressions were tested by Western blot.

Results: Our data unveiled GA enhanced muscle mass to improve grip strength and fatigue in D-galactose-induced aging mice. In serum, GA increased T-AOC, SOD and CAT levels, and decreased MDA, TNF-α, and IL-6 levels to exert its anti-oxidative and anti-inflammatory properties. In skeletal muscle, GA decreased atrogin 1, Murf-1, collagen I, and Tgf-β1 expressions to improve atrophy and fibrosis. Furthermore, GA increased Pgc-1α, Nrf-1, Beclin-1, and Bcl-2 expressions, and decreased P62 and Bax expressions to prevent skeletal muscle damage.

Conclusion: GA exerted its protective effects on sarcopenia concomitant aging via moderating mitochondrial biology, enhancing autophagy and restraining apoptosis.

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Introduction

Sarcopenia is one of age-related degenerative conditions, which is also related to multiple co-morbidities, such as cognitive impairment and diabetes mellitus (1, 2). Skeletal muscle is a primary tissue responsible for movement, whole-body metabolism and overall health. With increasing age, skeletal muscle gradually degenerates and motor capacity displays a downwards trend (3). Sarcopenia is linked to a reduction in muscle weight and grip strength, which influence life quality and athletic ability (4). These alterations are closely connected with the functional state of skeletal muscles. Sarcopenia leads to increased risk of falls and disability. In addition, the problem of aging is getting worse year by year. The proportion of elderly people will reach one in six individuals in 2050. Accordingly, the morbidity of aged-relevant sarcopenia increases with time (5). However, the feasible therapeutic method underlying aged-relevant sarcopenia remains unclear.

There are various kinds of factors that may result in sarcopenia. One of the reasons for aged-relevant sarcopenia is skeletal muscle dystrophy caused by ubiquitin-proteasome system disorders (6). Ubiquitin-mediated proteolysis plays a crucial role in muscle atrophy (7). Disturbance of proteostasis intensifies proteolysis to lessen muscle weight (8). Atrogin 1 and Murf-1 are crucial ubiquitin ligases, which are involved in regulating protein homeostasis. Ubiquitin-proteasome system is activated as represented by increased atrogin 1 and Murf-1 to induce muscle proteolysis (9). Fibrosis is another vital pathological characteristic of skeletal muscle aging, which accelerates aged-relevant sarcopenia process (10). Collagen I and Tgf-β1 as important regulatory factors for fibrosis are activated in various organs of the elderly (11). Likewise, advancing age is proved to be connected with a condition of fibrosis in skeletal muscles, which is characterized by regulating the TGF-β pathway. Hence, suppression of muscle atrophy and fibrosis may be an effective way in the treatment of aged-relevant sarcopenia.

Gallic Acid (GA) is a plant polyphenolic compound and can be extracted from natural plants, such as strawberries and red wines. Recently, GA has been applied in medicine and functional food because of its extensive biological function, such as anti-oxidative stress potential and anti-inflammatory effects (12, 13). Several researches revealed GA possessed anti-aging effect in many experiments. The Alzheimer model research revealed GA as a dual α/β-secretase modulator mitigated cerebral amyloidosis and reversed senescence-associated cognitive impairment (14). Another research demonstrated GA regulated senescence-related molecular and biochemical indicators (15). GA was uncovered to be strongly associated with mitigation of d-galactose induced thymic involution via its anti-apoptosis capacity (16). Recent research has suggested GA alleviated

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D-galactose-induced cardiac hypertrophy via modulation of mitochondrial biogenesis (17). However, the anti-aging effect of GA on skeletal muscles is not expounded. Therefore, we hypothesized GA might play a vital in improvement of muscle atrophy and fibrosis during aging, and explored its biologic activity and mechanism in the development of sarcopenia in D-galactose-induced aged mice.

Materials and Methods

Animals

Forty male ICR mice were 6-8 weeks old. The body weight of mice was 18-22 g. The experiments were approved by Ethics Committee of Hunan University of Arts and Science, China (No. HUAS-2021-TY-085).

Experimental design

Mice were divided into 4 groups. Each group included 10 mice. The Control group was administered normal saline solution. Gallic acid (Purity≥99.0%) was purchased from Sangon Biotech. GA was stored at 4 ˚C. The Model group was subcutaneously injected with D-galactose (18). The injected dose of D-galactose was 500 mg/kg body weight. The GA group received D-galactose and gallic acid. Experimental animals of GA-L group and GA-H group were administered intragastric GA at different doses for 10 weeks. The dose of GA was 50 or 100 mg/kg/d.

Grip strength test

Mice pulled stick to test grip strength. Grip tester was purchased from Anhui Zhenghua Bioinstrumentation. Grip strength of experiment data was acquired by multiple metering.

Forced swimming test

After grip strength test, mice underwent a loading swimming. The water depth was 30 cm. For measuring exhaustive time, the mouse was forced to swim with load (19). Exhaustive time of each mouse was calculated when experimental animal could not swim over 10 sec.

Histological analysis

After forced swimming test, animals were sacrificed. Gastrocnemius and soleus were dissected and weighed. Gastrocnemius was fixed, paraffin-embedded and cut into 4 μm (20). The nucleus and cytoplasm of gastrocnemius stained with Hematoxylin and Eosin (HE), respectively. The image was gathered through an optical microscope. The cross-sectional area of gastrocnemius tissue was counted via ImageJ.

Biochemical assay

Blood was acquired from the eyeball. Then, blood was centrifuged to separate the supernatant. The detection kits of biochemical indicators were purchased from Nanjing Jiancheng Biotechnology Institute. The absorbance values of ALT, AST, BUN, and CK were detected at 505, 510, 640, and 660 nm by a spectrophotometer.

Oxidation indices assay

The detection kits of oxidation indices were purchased from Nanjing Jiancheng Biotechnology Institute. Gastrocnemius was ground and centrifuged. The supernatant was used to assay oxidation indices. SOD, CAT, and MDA were determined by the hydroxylamine method, ammonium molybdate spectrometric method, and thibabituric acid method. The absorbance values of CAT, MDA, SOD, and T-AOC were detected at 405, 532, 550, and 520 nm.

ELISA

The inflammatory cytokines were detected by ELISA. The ELISA kits were purchased from BOSTER Biological Technology. Tetramethylbenzidine was used as chromogenic substrate. The absorbance value was tested at 405 nm.

Western blot

Skeletal muscle was rapidly frozen by liquid nitrogen. Protein was stored at -80 ˚C. Different molecular weights of protein were separated by SDS-PAGE. The expression of proteins was tested via western blot. The protein of skeletal muscle was transferred onto PVDF by wet electroblot technology. The antibodies of collagen I (1:500 dilution), Tgf-β1 (1:1000 dilution), BAX (1:1000 dilution), BCL-2(1:1000 dilution), β-actin (1:10000 dilution), and HRP-conjugated anti-mouse (1:3000 dilution) and anti-rabbit (1:3000 dilution) were obtained from Proteintech (Wuhan, China). The antibodies of atrogin-1(1:500 dilution) and MuRF-1(1:500 dilution), NRF-1(1:500 dilution), and PGC-1α (1:500 dilution) were obtained from Sangon Biotech (Shanghai, China). The antibodies of Beclin-1 (1:500 dilution) and P62 (1:500 dilution) were obtained from Servicebio (Wuhan, China). β-actin gene was used as internal reference. Protein signal was captured by ECL system.

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. $P<0.05$ was deemed significant.

Results

Role of GA in skeletal muscle dysfunction

Sarcopenia has a high incidence in the aged adults. Aged-relevant sarcopenia impairs fragility and mobility in skeletal muscles. The dominant characteristic of sarcopenia is muscle loss which leads to decreased muscle strength. In Figure 1A-B, D-gal obviously decreased myocyte cross-sectional area ($P<0.01$), while GA visibly augmented myofiber sizes ($P<0.05$). In Figure 1C-D, D-gal obviously decreased the weight of gastrocnemius and soleus ($P<0.01$), while GA visibly increased muscle mass ($P<0.05$). In Figure 1E-F, D-gal obviously reduced grip strength and exercise performance, while GA visibly increased muscle function ($P<0.01$).

Role of GA in biochemical indexes in serum

Aging is a complex disease with various premonitory symptoms which is relevant to physiological dysfunctions in many organisms. D-galactose is widely used to mimic natural aging in human. In animal experiments, D-galactose was proved to cause multiple organism injuries. In Figure 2, D-gal obviously induced CK, BUN, ALT, and AST levels ($P<0.01$), while GA visibly decreased above biochemical indicators ($P<0.01$).

Role of GA in oxidation injury in serum

An increase in oxidative stress is a common characteristic during the aging process. Antioxidant enzymes can facilitate

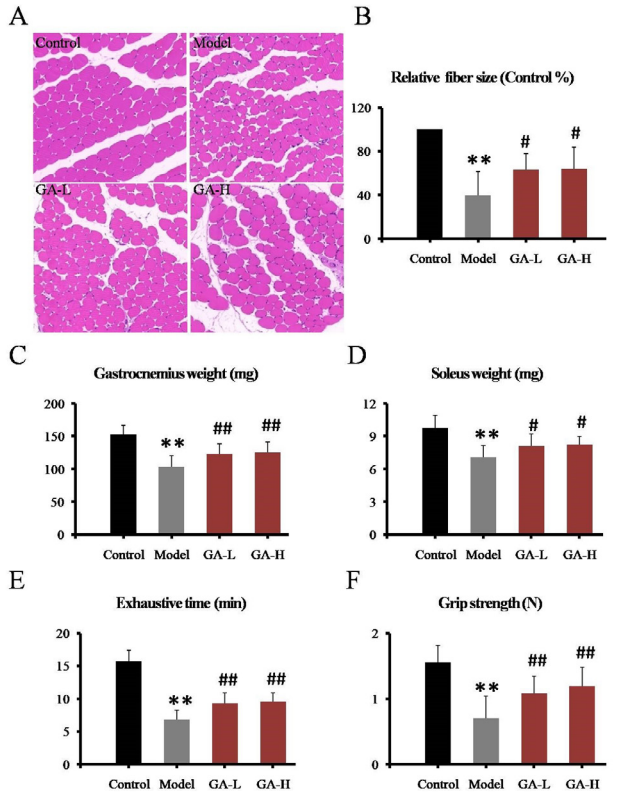

Figure 1. Role of gallic acid (GA) in skeletal muscle dysfunction in senescent mice
(A) Representative images of gastrocnemius stained with H&E (200X magnification), (B) muscle fiber size, (C) gastrocnemius weight, (D) soleus weight, (E) grip strength, and (F) exhaustion time. ** P<0.01 vs control. # P<0.05 vs Model. ## P<0.01 vs model

oxidation resistance to increase cell survival by eliminating reactive oxygen species. Accumulation of lipid peroxidation is demonstrated to aggravate oxidative damage. In Figure 3, D-gal obviously reduced T-AOC, SOD and CAT contents (P<0.01), while MDA concentration was obviously elevated (P<0.01). However, GA possibly alleviated oxidation injury to sustain internal environment homeostasis (P<0.01).

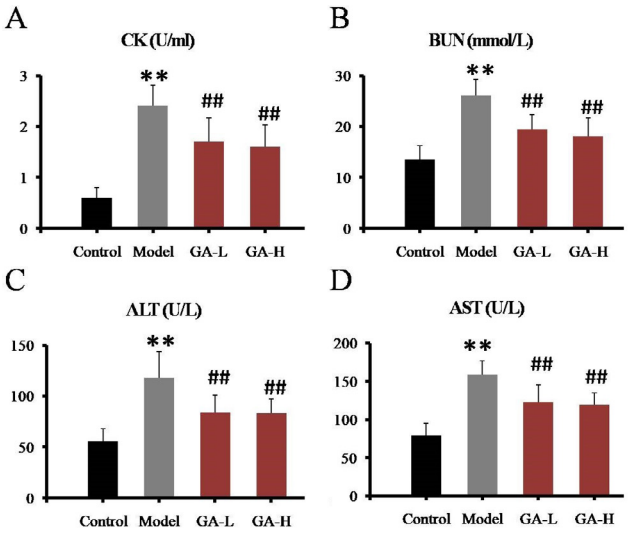

Figure 2. Role of gallic acid (GA) in biochemical indexes in senescent mice
Serum levels of (A) CK, (B) BUN, (C) ALT, and (D) AST. ** P<0.01 vs Control. ## P<0.01 vs Model
CK: Creatine kinase; BUN: Blood urea nitrogen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase

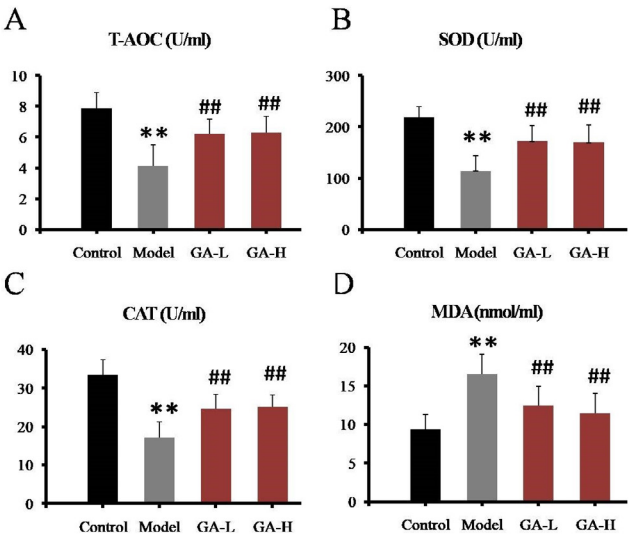

Figure 3. Role of gallic acid (GA) in oxidation injury in senescent mice
Serum contents of (A) T-AOC, (B) SOD, (C) CAT, and (D) MDA. ** P<0.01 vs Control. ## P<0.01 vs Model
T-AOC: Total antioxidant capacity; SOD: Superoxide dismutas; CAT: Catalase; MDA: Malondialdehyde

Role of GA in inflammatory response in serum

Inflammatory response is demonstrated as a prominent activation during the aging process, which is taken for contributing to organism damage. Aging results in the overproductions of TNF-α and IL-6 in the body. In Figure 4, D-gal obviously promoted TNF-α and IL-6 concentrations (P<0.01). However, GA possibly suppressed inflammatory reaction (P<0.01).

Role of GA in atrophy in skeletal muscle

The aged-induced muscle loss is a result of protein aggravation. Skeletal muscle atrophy is a crucial pathological change in sarcopenia. The ubiquitin-proteasome system plays a major role in muscle atrophy. In Figure 5, D-gal obviously strengthened atrogin 1 and Murf-1 expressions (P<0.01). However, GA possibly alleviated D-galactose-evoked increase of protein degeneration factors to improve muscle atrophy (P<0.01).

Role of GA in fibrosis in skeletal muscle

In sarcopenia, aging aggravates myopathy and muscle structure change, as represented by accelerating fibrosis in skeletal muscles. Collagen I is a marker factor to exhibit collagen fibers deposition and fibrotic changes. Tgf-β1 is a potent profibrogenic cytokine and can activate fibrosis process in skeletal muscles. In Figure 6, D-gal obviously enhanced collagen I and Tgf-β1 expressions (P<0.01). However, GA possibly restrained the TGF-β pathway to improve muscle fibrosis (P<0.01).

Role of GA in mitochondrial disorder in skeletal muscle

Mitochondrial disorder is an important inducement during the aging process that aggravates the development of sarcopenia. Mitochondrion is a major site of energy production, which plays a vital role in exercise performance. Pgc-1α as a master regulator of mitochondrion is involved in activating Nrf-1. In Figure 7, D-gal obviously reduced Nrf-1 and Pgc-1α expressions (P<0.01). However, GA possibly mitigated D-galactose-evoked mitochondrial disorder in skeletal muscle (P<0.01).

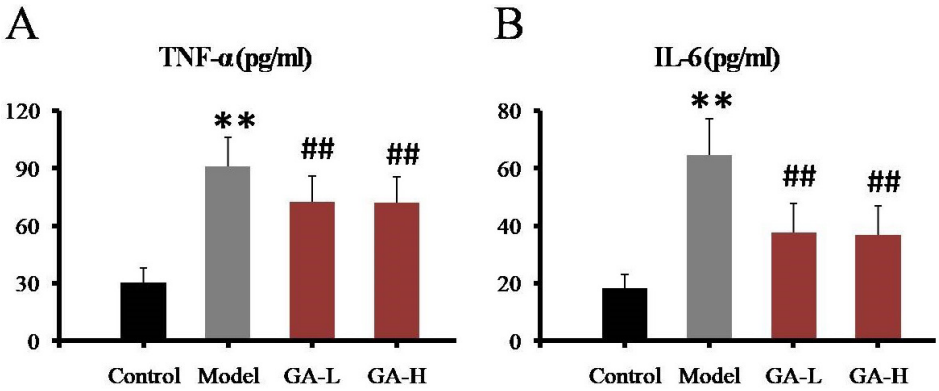

Figure 4. Role of gallic acid (GA) in inflammatory response in senescent mice
Serum concentrations of (A) TNF-α and (B) IL-6. ** $P<0.01$ vs Control. ## $P<0.01$ vs Model

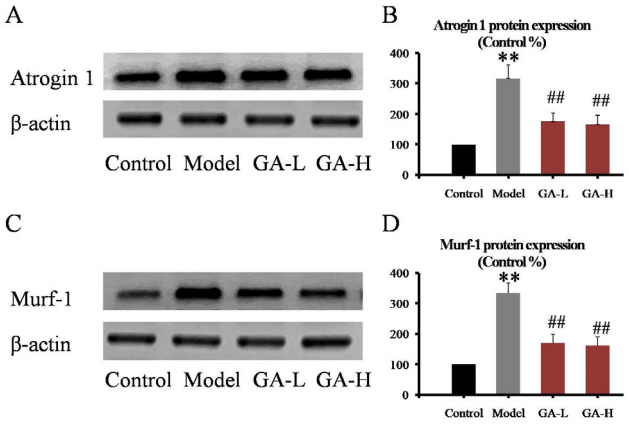

Figure 5. Role of gallic acid (GA) in muscle atrophy in senescent mice
Western blot and semiquantitative analysis of (A, B) atrogin 1 and (C, D) Murf-1. ** $P<0.01$ vs Control. ## $P<0.01$ vs Model

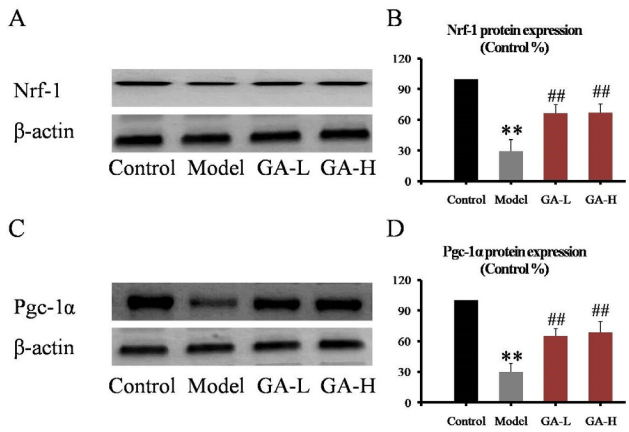

Figure 7. Role of gallic acid (GA) in mitochondrial disorder in senescent mice
Western blot and semiquantitative analysis of (A, B) Nrf-1 and Pgc-1α (C, D). ** $P<0.01$ vs Control. ## $P<0.01$ vs Model

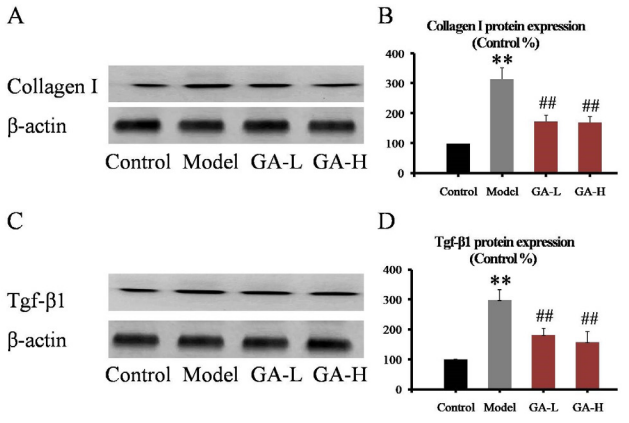

Figure 6. Role of gallic acid (GA) in muscle fibrosis in senescent mice
Western blot and semiquantitative analysis of (A, B) collagen I and (C, D) Tgf-β1. ** $P<0.01$ vs Control. ## $P<0.01$ vs Model

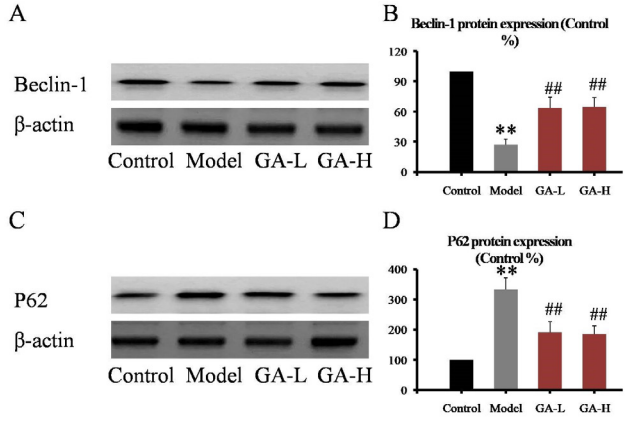

Figure 8. Role of gallic acid (GA) in autophagy in senescent mice
Western blot and semiquantitative analysis of (A, B) Beclin-1 and (C, D) P62. ** $P<0.01$ vs Control. ## $P<0.01$ vs Model

Role of GA in autophagy in skeletal muscle

Autophagy plays a key role in aging and muscular disorders which is closely associated with sarcopenia. Autophagy is part of catabolic pathways and can clear away damaged organelles via lysosomal machinery to improve physiology. In Figure 8, D-gal obviously restrained Beclin-1 expression and heightened P62 expression ($P<0.01$). However, GA possibly relieved D-galactose-evoked abnormal autophagy in skeletal muscles ($P<0.01$).

Role of GA in apoptosis in skeletal muscles

Apoptosis pathway is visibility activated during the aging process, which is believed to results in sarcopenia. The ratio of Bax/Bcl-2, as integral parts in apoptotic cascade, is credibly involved in playing an important role in moderation of apoptotic machinery. In Figure 9, D-gal obviously heightened Bax expression and restrained Bcl-2 expression ($P<0.01$). However, GA possibly alleviated D-galactose-evoked excessive apoptosis in skeletal muscle ($P<0.01$).

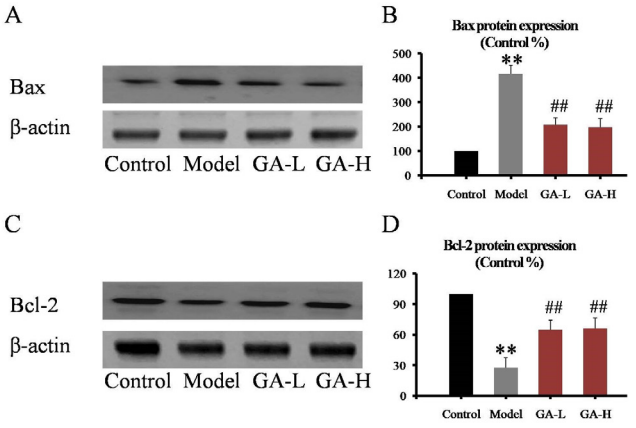

Figure 9. Role of gallic acid (GA) in apoptosis in senescent mice
Western blot and semiquantitative analysis of (A, B) Bax and (C, D) Bcl-2. ** $P<0.01$ vs Control, ## $P<0.01$ vs model

Discussion

Sarcopenia is associated with aging. The symptom of sarcopenia is muscle loss (21). Increased with age, less muscle mass affects skeletal muscle function (22). Previous research showed GA possessed a variety of physiological effects on skeletal muscle. GA could be used as a skeletal muscle relaxant in animal experiment (23). In high-fat-fed mice, GA partially reduced plasma FFA and intramuscular lipid deposition (24). In streptozotocin-treated rats, GA partially stimulated insulin secretion and enhanced GLUT4 expression in soleus muscle tissue to improve diabetes mellitus (25). In type 2 diabetic rats, GA promoted glucose uptake via PI3K/p-Akt signaling pathway and slightly enhanced PPARγ expressions in skeletal muscles (26). D-galactose is an inducer of sarcopenia. During aging, skeletal muscle weigh is reduced and athletic ability is weakened. In this study, GA alleviated D-galactose-induced skeletal muscle loss, muscle force decline and dyskinesia, suggesting GA might be involved in ameliorating sarcopenia in aged mice.

Aging is a harm factor of healthy longevity and causes many organs senescent, such as muscle, kidney, and liver. Previous research showed GA had an extensive protective effect on muscles, live and kidney injury. In snake envenoming explored rats, GA partially reduced CK activity to lessen muscle damage and increase myogenin to accelerate muscle repair (27). In sodium arsenite-induced renal toxicity, GA decreased BUN levels to relieve kidney damage (28). In carbon tetrachloride-induced liver toxicity, GA decreased ALT and AST levels to relieve hepatic damage (29). D-galactose can induce increased CK, BUN, ALT, and AST levels during aging. In this study, GA reversed above adverse biochemical indicators levels in aged mice, suggesting GA might ameliorate muscle, kidney, and hepatic damage against D-galactose-induced aging.

The mechanism of aging is complex. Oxidative imbalance is a vital incentive of aging, which plays a primary role in oxidative stress-induced geriatric syndromes. During aging, oxidative products are produced in large quantities, while oxidation resistance is reduced in the living organism. Previous research showed GA protected living organisms from peroxidation. GA affected carbohydrate metabolism and enhanced T-AOC level to alleviate oxidative stress (30). In cyclophosphamide-treated mice, GA enhanced CAT activity to retard renal toxicity (31). In ethanol exposed zebrafish, GA degraded extracellular nucleotides and enhanced SOD activity to suppress brain damage (32). In hyperuricemic mice, GA regulated urate transporters and relieved MDA to suppress kidney damage (33). The old people are susceptible to be impaired by ROS because of the reduced capacity of anti-oxidization. During aging, D-galactose induced decrease of T-AOC and increase of MDA levels in serum. In addition, antioxidant enzymes levels might be decreased in senescent mice. In this study, GA recovered oxidation resistance and reduced oxidative products in aged mice, suggesting GA might ameliorate internal oxidation against D-galactose-induced aging.

Aging aggravates inflammatory response to cause organism damage. Previous research showed GA possessed anti-inflammatory effects in many disease models. GA decreased inflammatory cytokine levels in serum to mitigate pain and depression (34). In non-alcoholic fatty mice, GA repressed inflammatory pathways to protect liver functions (35). In cigarette smoke-treated mice, GA blunted TNF-α expression to protect pulmonary inflammation (36). Up-regulation of TNF-α and IL-6 caused an imbalance in inflammatory response. In addition, D-galactose induced TNF-α and IL-6 levels. In this study, GA reduced TNF-α and IL-6 levels in aged mice, suggesting GA might ameliorate inflammatory response against senility.

Muscle atrophy is the main cause of sarcopenia. UPS is involved in proteolysis to regulate protein homeostasis and muscle mass. In other words, loss of proteostasis leads to muscle atrophy. Up-regulation of ubiquitin ligases caused a disorder in ubiquitin-proteasome system. In STZ-diabetes, GA inhibited atrogin 1 expression in skeletal muscle to prevent myopathy (37). In C2C12 cells, GA partially inhibited Murf-1 expression to promote skeletal muscle development (38). In addition, D-galactose increased atrogin 1 and Murf-1 expressions in skeletal muscles to induce sarcopenia. In this study, GA reduced atrogin 1 and Murf-1 expressions in aged mice, suggesting GA might sustain the ubiquitin-proteasome system against D-galactose-induced sarcopenia.

Fibrosis is a crucial pathological alteration in musculoskeletal system disease. D-galactose aggravated skeletal muscle injury, which was involved in fibrosis. In transverse aortic constriction-treated mice, GA suppressed connective tissue growth factor and deposition of collagen I to mitigate cardiac fibroblasts (39). In type I diabetes rats, GA possibly inhibited fibronectin expression to positively affect testicular fibroblasts (40). In glyoxal–treated rats, GA suppressed collagen deposition and collagen III mRNA expression to mitigate renal fibrosis (41). In thioacetamide-treated rats, GA was involved in regulating the Tgf-β1/Smad3 pathway to exhibit hepatoprotective and mitigate liver fibrosis (42). In bleomyci–treated mice, GA modulated the Tgf-β1/Smad2 pathway and balanced NOX4/Nrf2 to attenuate pulmonary fibrosis (43). In addition, D-galactose increased collagen I and Tgf-β1 expressions to induce skeletal muscle fibrosis. In this study, GA might reduce TGF-β pathway against senility.

Mitochondrial dysfunction is nearly related to all geriatric diseases. Mitochondria, as primary sites of energy production, play an important role in athletic ability. During the aging process, mitochondrial dysfunction is more and more prominent. In obese mice, GA enhanced glucose

tolerance and increased PGC1-α mRNA expression in brown adipose tissue (44). In bisphenol A-exposed rats, GA was involved in improving enzymes of respiratory chain and intactness of mitochondria and pyruvate dehydrogenase to sustain number and structure of mitochondria against liver injury (45). In Na2S2O4-exposed SH-SY5Y cells, GA improved mitochondrial membrane potential, oxygen consumption, and ATP synthesis levels in hypoxia/reoxygenation-induced mitochondrial discords (46). In aging rats, GA regulated Pgc-1α, TFAM and Nrf2 mRNA expressions to protect mitochondria against D-galactose-induced heart injury (17). Pgc-1α plays an important role in mitochondrial oxidative metabolism. Nrf-1 is involved in modifying mitochondrial function. In skeletal muscles, D-galactose decreased Nrf-1 and Pgc-1α in aged mice. In this study, GA prevents these changes induced by D-galactose in aged mice, suggesting GA might regulate mitochondrial biology against aged-relevant sarcopenia.

Autophagy pathway in skeletal muscles is associated with sarcopenia. During the aging process, autophagy was inhibited to cause many kinds of organ injury, including skeletal muscles. In addition, autophagy, as a vital proteolytic cascade, is involved in cleaning injured mitochondria to maintain skeletal muscle structure and function. Regulating autophagy might be a viable therapeutic approach to improve geriatric diseases. GA enhanced autophagic flux, as indicated by decreasing p62 protein levels in liver (47). In TAC-induced mice, GA activated the autophagy-dependent mechanism via the activation of Beclin-1 protein levels to improve cardiac hypertrophy and myocardial fibrosis (48). In ischemia-reperfusion rats, GA regulated Beclin-1 expression to protect the intestinal tissue (49). In skeletal muscles, D-galactose increased Beclin-1 and decreased P62 expressions in aged mice. In this study, GA prevents these changes induced by D-galactose in aged micee, suggesting GA might regulate autophagy pathway against aged-relevant sarcopenia.

Apoptosis dysbiosis is closely related to muscle damage, which leads to muscle force decline and dyskinesia. During the aging process, aging induces apoptosis dysbiosis to affect normal physiological functions in the body. In skeletal muscles, aging aggravates apoptosis to cause muscle loss. In addition, apoptosis is linked to mitochondrial function and autophagic flux. In spontaneously hypertensive rats, GA prevented S mRNA levels to relieve CaMKII δ-induced apoptosis (50). In hypothyroid rats, GA suppressed oxidative, endoplasmic reticulum stresses and Bax/Bcl-2 ratio to attenuate adult-onset hippocampal damage (51). In type 2 diabetic rats, GA possibly ameliorated glucose tolerance and enhanced BCl-2 expression in hippocampus to improve neurodegeneration (52). In aging rats, GA modulated apoptosis against D-galactose-induced cardiac hypertrophy (17). In skeletal muscles, D-galactose mitigated Bax and accelerated Bcl-2 expressions in aged mice. In this study, GA prevents these changes induced by D-galactose in aged mice, suggesting GA might regulate apoptosis pathway against aged-relevant sarcopenia.

Conclusion

In this study, GA was proved to ameliorate muscle mass and weight to enhance grip strength and athletic ability in aged mice induced by D-galactose. In serum, GA lowered oxidative stress, enhanced antioxidant status, and diminished inflammation, which contribute to its possible anti-aging function induced by D-galactose. In skeletal muscles, GA improved atrophy and fibrosis, making it possibly benefitial to preventing D-galactose-induced sarcopenia. Mechanistically, the roles of GA were related to its functions in amelioration of mitochondrial biology, enhancement of autophagy, and restraint of apoptosis. Therefore, GA could be used to improve sarcopenia because of its theoretical basis in anti-aging and muscular protection.

Acknowledgment

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Authors' Contributions

L X contributed to study design. L X, Z Z, M Z, and L M carried out experiments. L X and M Z discussed the results. L X and L M drafted the manuscript and revised the final manuscript critically.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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