

Ellagic acid attenuates muscle atrophy in STZ-induced diabetic mice

Short title: Ellagic acid attenuates muscle atrophy

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Summary

Diabetes is closely connected with skeletal muscle dysfunction. Ellagic acid (EA) possesses a variety of bio-effects and is applied to the improvement of diabetes. The purpose of this study was to explore the potential improvement effect and mechanisms of EA in streptozotocin (STZ)-induced diabetic muscle atrophy. The model of diabetic mice was established by intra-peritoneal STZ to evaluate treatment effect of EA (100 mg/kg/d for 8 weeks) on muscle atrophy. Our data exhibited that EA enhanced fiber size and weight of gastrocnemius, and promoted grip strength to relieve STZ-induced muscle lesions. In serum, the levels of Creatine kinase (CK), lactate dehydrogenase (LDH), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL) were inhibited, while high-density lipoprotein cholesterol (HDL) level was enhanced by EA treatment in diabetic mice. In gastrocnemius, EA decreased Atrogin-1 and MuRF-1 expressions to relieve STZ-induced muscle atrophy. Moreover, EA increased NRF-1 and PGC-1 α expressions to alleviate mitochondrial disorder. Meanwhile, EA suppressed CHOP and GRP-87 levels to relieve ER stress. Lastly, EA inhibited BAX expressions and enhanced BCL-2 expressions to mitigate apoptosis. In conclusion, EA is preventing the event of STZ-induced gastrocnemia by amelioration of mitochondrial dysfunction, ER stress and apoptosis, and could be used in the protection and therapeutic of muscle atrophy in diabetes.

Key words: Ellagic acid, muscle atrophy, mitochondrion,
endoplasmic reticulum stress, apoptosis

Introduction

The leading cause of diabetes is the loss of functional beta-cell mass in the pancreas, which threatens insensitivity and deficiency of insulin. Insulin, as a vital regulator of glucose homeostasis, is closely relevant to the regulation of hyperglycemia and incidence of diabetes [1, 2]. In other words, the primary clinical manifestation of diabetes is uncontrollable high glucose. A large number of previous studies have shown that altered glucose homeostasis causes multiple tissues and organs injury to evoke diabetic complications [3]. Nowadays, diabetic complication has become a crucial research topic owing to its growing number of patients [4]. Many drugs used in the treatment of diabetic complications are becoming more and more widespread, but its side effects are also increasing correspondingly. Hence, the case for developing an effective agent with low side effects is imperative.

Skeletal muscle is an essential component of our body and is necessary for physical capacity. In addition, skeletal muscle is involved in glucose uptake [5]. However, diabetes has been proved to affect skeletal muscle health via reducing muscle mass, cross-sectional area and strength [6]. The main molecular mechanism for development of diabetic muscle atrophy is connected with ubiquitin-proteasome system UPS dysfunction as represented by accumulating ubiquitinated proteins and increasing proteasomal activity [7]. It also disturbs other biological functions, such as endoplasmic reticulum stress, mitochondrial disorder and apoptosis, which are closely involved in muscle wasting [8-10]. Therefore, improvement of ubiquitin-proteasome

system is considered as a critical way to prevent diabetic muscle atrophy.

Ellagic acid is a natural polyphenol widespread in fruits and vegetables. EA has a variety of therapeutic effects both on diabetes and muscle injury due to its multiple biological and pharmacological properties [11, 12]. Previous studies showed that EA could stimulate insulin production and reduce glucose to improve diabetic complications [13]. In diabetes-induced testicular injury, EA was proved to inhibit apoptosis [14]. In addition, EA decreased MDA level and increased SOD activity in ischemia/reperfusion-induced skeletal muscle damage [15]. In cuprizone-induced muscular dysfunction, EA improved motor coordination and enhanced ATP production via regulation of mitochondrial respiratory chain activity [16]. However, there is no report on the efficacy of EA on STZ-induced muscle injury, and its molecular pathogenesis is not illuminated. We hypothesized that the improvement of EA on diabetic muscle atrophy was attributed to its regulations on ER stress, mitochondrial function, and apoptosis. In this study, we aimed to address the protective functions of EA on diabetic muscle atrophy.

Materials and methods

Animals

Male ICR mice (n = 60, 20 ± 2 g) were acquired from Hunan SJA Laboratory animal (Changsha, China). The age of mice was about 8 weeks old. Mice were housed in normal light and dark (12 : 12 h) cycles with applicable humidity and temperature. During the experimental period, mice were ad libitum fed standard food and water. In this study, animal experiments were inspected by the Ethics Committee of Hunan University of Arts and Science (No. HUAS-2021-TY-158).

Chemicals and reagents

Ellagic acid (purity: ≥ 98%) and streptozotocin were obtained from Sangon Biotech (Shanghai, China). The antibodies of BAX, BCL-2 and CHOP were obtained from Proteintech (Wuhan, China). The antibodies of Atrogin-1, GRP-78, MuRF-1, NRF-1 and PGC-1 α were obtained from Sangon Biotech (Shanghai, China). The assay kits of HDL, LDL, TC and TG were purchased from Nanjing Jiancheng Biotechnology Institute (Nanjing, China).

Experimental design

Experimental mice were assigned into control group (CON group, n = 20), diabetes mellitus group (DM group, n = 20), and ellagic acid treatment group (DM + EA group, n = 20). STZ was dissolved in citrate buffer (0.1 M, pH 4.5). The model of diabetic mice was established by intra-peritoneal STZ. To confirm experimental

diabetes, blood was taken from caudal vein to test glucose level. Mice with the glucose level of 16.7 mmol/L and above were deemed as a suitable diabetic model and chosen for further experiment [17]. Clinical manifestations of diabetic mice were observed and recorded every day. In diabetic model, mice were intragastrically fed with EA (100 mg/kg/day for 8 weeks), which was considered as DM + EA group. The control (CON) group was accordingly treated with an equivalent amount of saline.

Grip strength

Muscle force was detected by a dynamometer (YLS13, Anhui Zhenghua Bioinstrumentation). During force test, experimental mouse steadily gripped stick and pulled backward [18]. The peak of force was observed and recorded. The grip strength was measured three times to calculate an average value.

Sample preparation

After muscle force test, mice were sacrificed by anesthesia with the injection of pentobarbital. Blood was taken from eyeball for biochemical assessment. Gastrocnemius was excised and weighed. A part of gastrocnemius was preserved at -80 °C for the measurement of protein expression. The remaining tissue was fixed in 4% paraformaldehyde for histopathological analysis.

Biochemical assessment

CK and LDH were examined to estimate muscle injury. TC, TG, LDL and HDL were

examined to estimate lipid metabolism in blood. CK, LDH, TC, TG, LDL and HDL levels were tested by spectrophotometer.

Histological analysis

Haematoxylin and Eosin (HE) staining was utilized to detect the morphological characteristics of gastrocneemia [19]. After fixation, gastrocneemia was dehydrated with alcohol, cleared with xylene, and embedded in paraffin. Then, paraffin block was cut into 5 μm by a rotary microtome. For histological evaluation, sections were stained with haematoxylin and eosin. The staining was observed under a light microscope. Muscle fiber size was statistically assessed and measured by Image J. The results showed the relative myocyte cross-sectional area of gastrocneemia from CON group, DM group, and DM + EA group.

Western blot

Gastrocneemia was homogenized at low temperatures by a homogenizer. Lysis buffer and proteinase inhibitors were added into homogenates, which were centrifuged to obtain supernatants for protein expression analysis [20]. SDS-PAGE was utilized to separate lysate proteins. Wet electroblotting was utilized to transfer individual proteins onto PVDF membrane. After blocking with 5% milk, PVDF membrane was incubated with primary antibodies. PVDF membrane was washed with TBS for 3 times. The corresponding HRP-conjugated antibodies were added onto PVDF membrane. After washing with TBS, the signals were detected by ECL

chemiluminescence. The protein bands were observed under imaging system and its density was recorded. β -actin was considered as loading control. The results were expressed as protein band density relative to β -actin.

Statistics

All data were showed as mean \pm SD. Statistical difference was demonstrated by ANOVA test with Tukey's post hoc test. $p < 0.05$ was deemed statistically significant.

RESULTS

Effects of EA on STZ-induced skeletal muscle atrophy

HE was used to appraise the improvement of EA against STZ-induced morphological characteristics in gastrocnemius. The muscle fiber size was markedly reduced in DM group, while representative myocyte cross-sections were dramatically enhanced by EA treatment (Fig. 1A-B). Moreover, gastrocnemia weight and grip strength were markedly reduced in DM group, while these changes were dramatically reversed by EA treatment (Fig. 1C -D).

Effects of EA on CK and LDH

To appraise the modulation of EA on STZ-induced muscle damage, CK and LDH activities were detected in serum. The activities of CK and LDH were markedly enhanced in DM group, while CK and LDH activities were dramatically reduced by EA treatment (Fig. 2A -B).

Effects of EA on TC, TG, LDL and HDL

To appraise the modulation of EA on STZ-induced lipid metabolic abnormality, TC, TG, LDL and HDL levels were detected in serum. In DM group, the levels of TC, TG and LDL were markedly enhanced (Fig. 3 A-C), while HDL level was markedly reduced (Fig. 3 D). In contrast, these lipid profile alterations were dramatically improved by EA treatment (Fig. 3).

Effects of EA on protein degradation

To appraise the regulation of EA on STZ-induced UPS dysfunction, the expressions of Atrogin-1 and MuRF-1 were detected in gastrocnemius. Atrogin-1 and MuRF-1 expressions were markedly enhanced in DM group, while EA dramatically reduced these muscle-specific E3 ubiquitin ligases to improve ubiquitin proteasome pathway (Fig. 4).

Effects of EA on mitochondrial disorder

NRF-1 and PGC-1 α are vital and reliable markers of mitochondrial function [21, 22]. NRF-1 and PGC-1 α expressions were markedly reduced in DM group, while EA dramatically enhanced these expressions to moderate mitochondrial disorder in gastrocnemia (Fig. 5).

Effects of EA on ER stress

CHOP and GRP-78 are involved in regulation of ER stress [23]. CHOP and GRP-78 expressions were markedly enhanced in DM group, while EA dramatically reversed STZ-induced increase of ER stress markers in gastrocnemia (Fig. 6).

Effects of EA on apoptosis

BAX and BCL-2 are well-known indicators of apoptotic, which was associated with cellular damage [24]. BAX level was markedly enhanced in DM group, while EA observably suppressed STZ-induced BAX expression in gastrocnemia (Fig. 7A -B).

However, BCL-2 level was markedly inhibited in DM group, while EA observably enhanced BCL-2 expression in gastrocemia (Fig. 7C -D).

Discussion

Skeletal muscle is a locomotive organ in human body. Damage to skeletal muscle can disrupt motor activity and strength. In diabetes, skeletal muscle, as an endocrine organ, plays a vital role in glucose uptake [25]. Previous Studies showed EA might enhance GLUT4 expression in skeletal muscle of diabetic rats to modulate blood glucose levels [26]. Emerging research has demonstrated hyperglycemia is a high-risk factor for muscle injury [27]. In this study, our results showed diabetes led to weakening muscle mass, fiber size and force generation in skeletal muscle, which are the main characteristics of muscular atrophy. However, EA reversed these changes in STZ-induced diabetic mice. Clinically, CK and LDH are common biochemical indexes, excessive levels of which are used to demonstrate muscle injury. In this study, CK and LDH levels were increased in serum of diabetic mice, while EA relieved CK and LDH levels. Therefore, EA was proved to improve muscle morphology and blood biochemical parameters in diabetic mice.

UPS, as a primary component of the proteolytic system, is responsible for protein anabolic and catabolic processes. In diabetes, UPS is elementary to maintaining muscle weight and its disorder results in protein degradation to cause muscle atrophy [28]. Atrogin-1 and MuRF1 are protein ubiquitination-related proteins and involved in regulating protein degradation, excess expressions of which lead to UPS disorder [28]. Hyperglycemia increased Atrogin-1 and MuRF-1 expressions to degrade intracellular proteins in skeletal muscle [30]. In this study, our results showed EA maintained UPS

by inhibiting Atrogin-1 and MuRF-1 expressions in gastrocnemius, suggesting EA improved protein anabolism and catabolism to relieve diabetic muscle atrophy.

Mitochondrial biogenesis is involved in energy generation for skeletal muscle to adapt to exercise. In STZ-induced diabetes, mitochondrial biology, such as mitochondrial fusion, fission and autophagy, was disturbed [31]. NRF-1 and PGC-1 α play an important role in the maintenance of mitochondrial homeostasis. PGC-1 α , as a transcriptional regulator, is also responsible for mitochondrial content and ATP production by modulating NRF-1 [32-34]. Moreover, diabetes inhibits NRF-1 and PGC-1 α expressions to evoke mitochondrial malfunction in various organs [35]. Previous studies showed that EA was involved in the beige remodeling of white adipose tissue by promoting the expression of NRF-1 and PGC-1 α [36]. In this study, our results showed EA enhanced mitochondrial biogenesis in gastrocnemius, as represented by upregulating NRF-1 and PGC-1 α expression, to relieve diabetic muscle atrophy.

ER was responsible for protein modification by folding and processing polypeptide chains into functional proteins [37]. An accumulation of misfolded or unfolded proteins participates in the induction of apoptosis [38]. The activated ER stress is proven to be linked to diabetic muscle atrophy [39]. As a critical modulator of ER stress, CHOP and GRP-78 are up-regulated in diabetic complications. Previous studies showed that hyperglycemia increased the

production of CHOP and GRP-87 to cause muscle atrophy [27]. In addition, EA could inhibit UVA-induced ER stress in human keratinocyte cells [40]. In this study, our results showed EA relieved ER stress by reducing CHOP and GRP-87 expressions in gastrocnemius, suggesting EA was involved in protein modification to alleviate diabetic muscle atrophy.

Apoptosis is a controllable mechanism for orderly death of cells to maintain a stable internal environment. A disruption of apoptotic signal induces muscle damage in diabetes [41]. BAX and BCL-2 have been suggested to be central to the apoptotic signals. Previous studies showed that hyperglycemia increased BAX expression and decreased BCL-2 expression in diabetic complications [42]. Moreover, hyperglycemia-induced mitochondrial malfunction and excess ER stress activate apoptosis. Previous studies showed that EA possessed anti-apoptotic effects in diabetic cardiomyopathy [43]. In muscle, EA reduced CCl₄-induced muscle tissue injury via regulation of apoptosis pathway [44]. In this study, our results showed EA possessed anti-apoptotic effects by down-regulating BAX expression and up-regulating BCL-2 expression in gastrocnemius, suggesting EA was useful for an orderly process of cell death in the treatment of diabetic muscle atrophy.

Conclusions

Our research revealed that EA attenuated diabetic muscle atrophy which was linked to its accommodation of ER stress, mitochondrial function and apoptosis. These results

demonstrated EA could be developed as a novel and natural medicine to alleviate muscle atrophy in diabetes.

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

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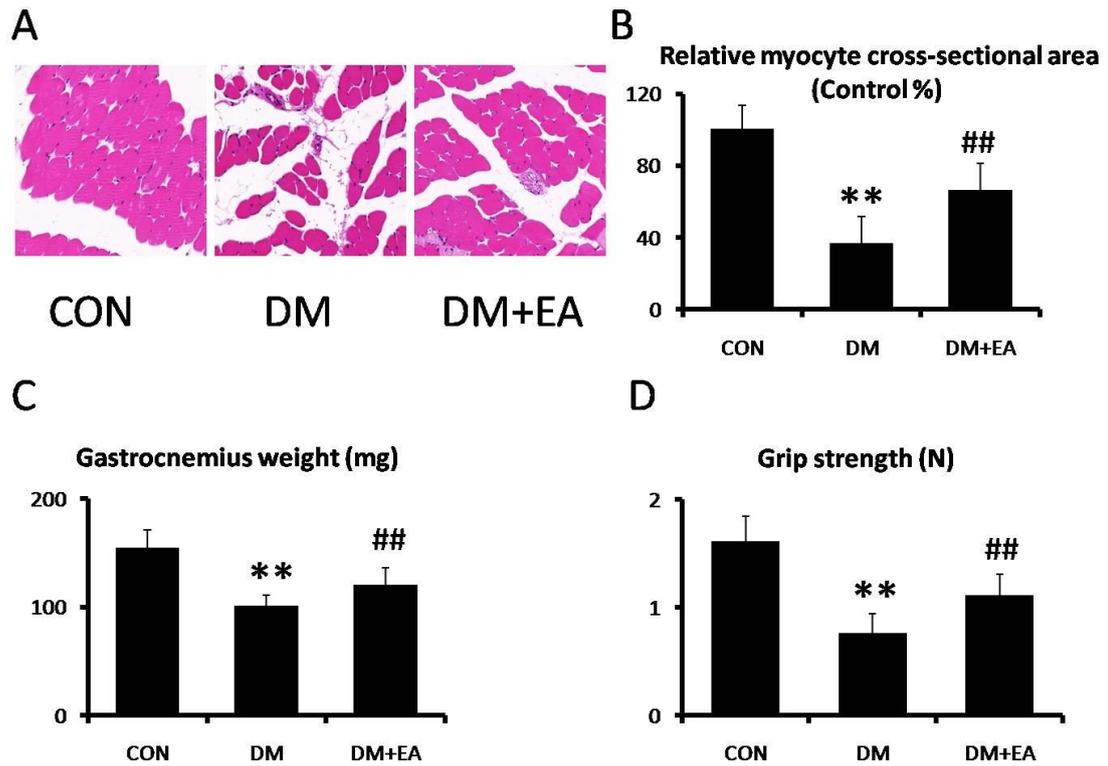


Fig. 1. Effects of EA on STZ-induced skeletal muscle atrophy. (A, B) morphological characteristics were analyzed by HE staining in gastrocnemia (200 ×). (C) Gastrocnemia weight. (D) Grip strength. ** $p < 0.01$ vs CON; ## $p < 0.01$ vs DM.

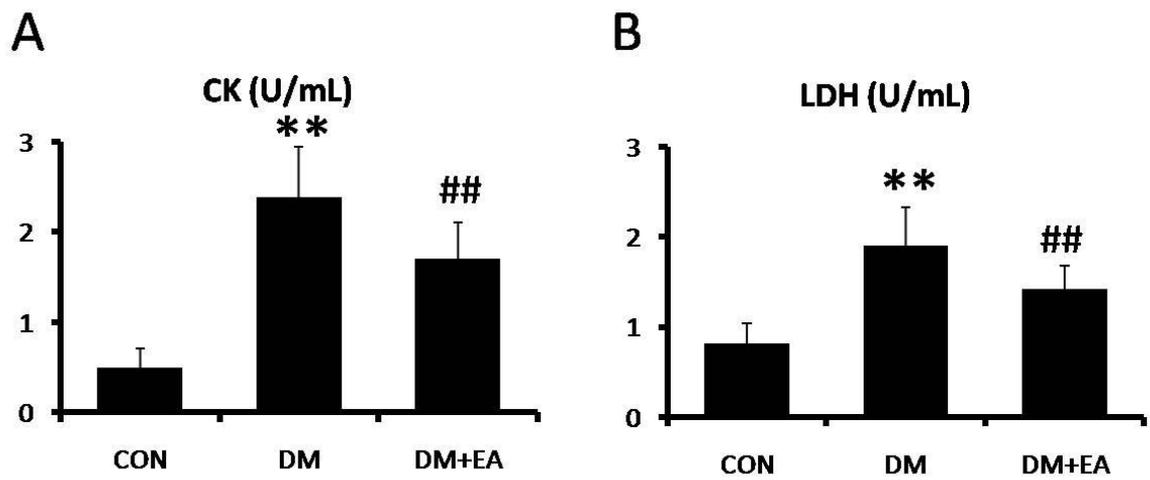


Fig. 2. Effects of EA on CK and LDH in serum. (A) CK and (B) LDH levels were detected by spectrophotometer. ** $p < 0.01$ vs CON; ## $p < 0.01$ vs DM.

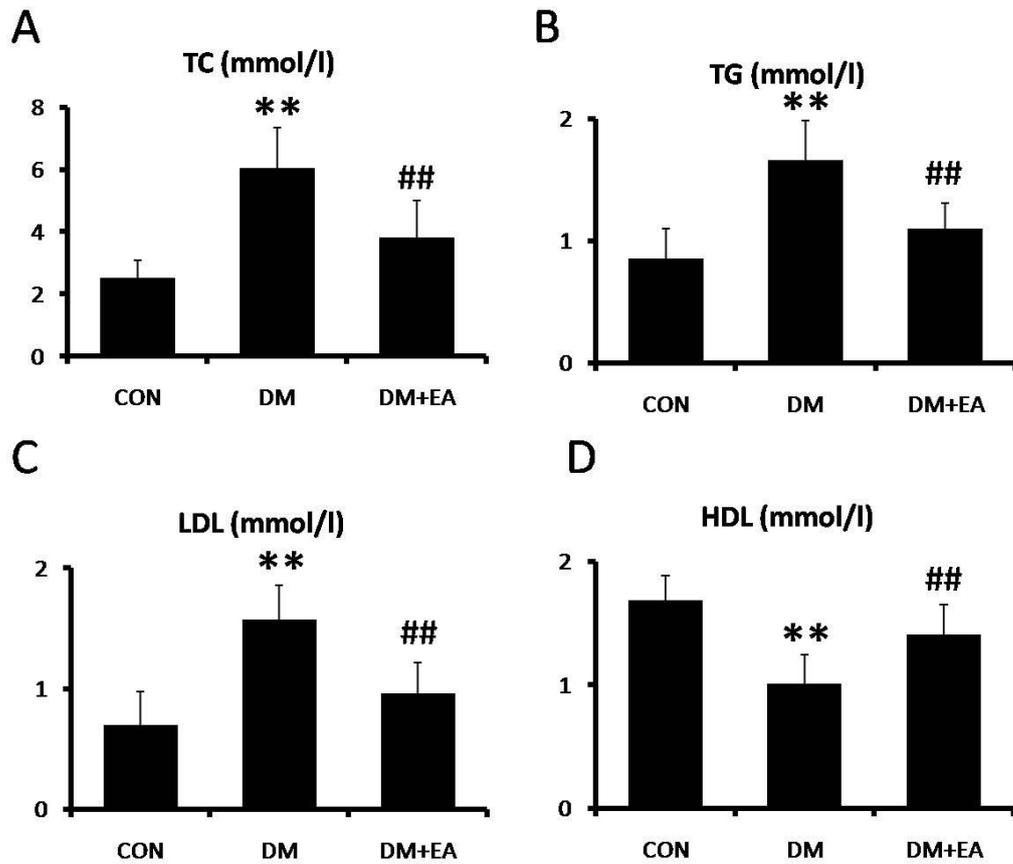


Fig. 3. Effects of EA on lipid metabolic in serum. (A) TC, (B) TG, (C) LDL and (D) HDL levels were detected by spectrophotometer. ** $p < 0.01$ vs CON; ## $p < 0.01$ vs DM.

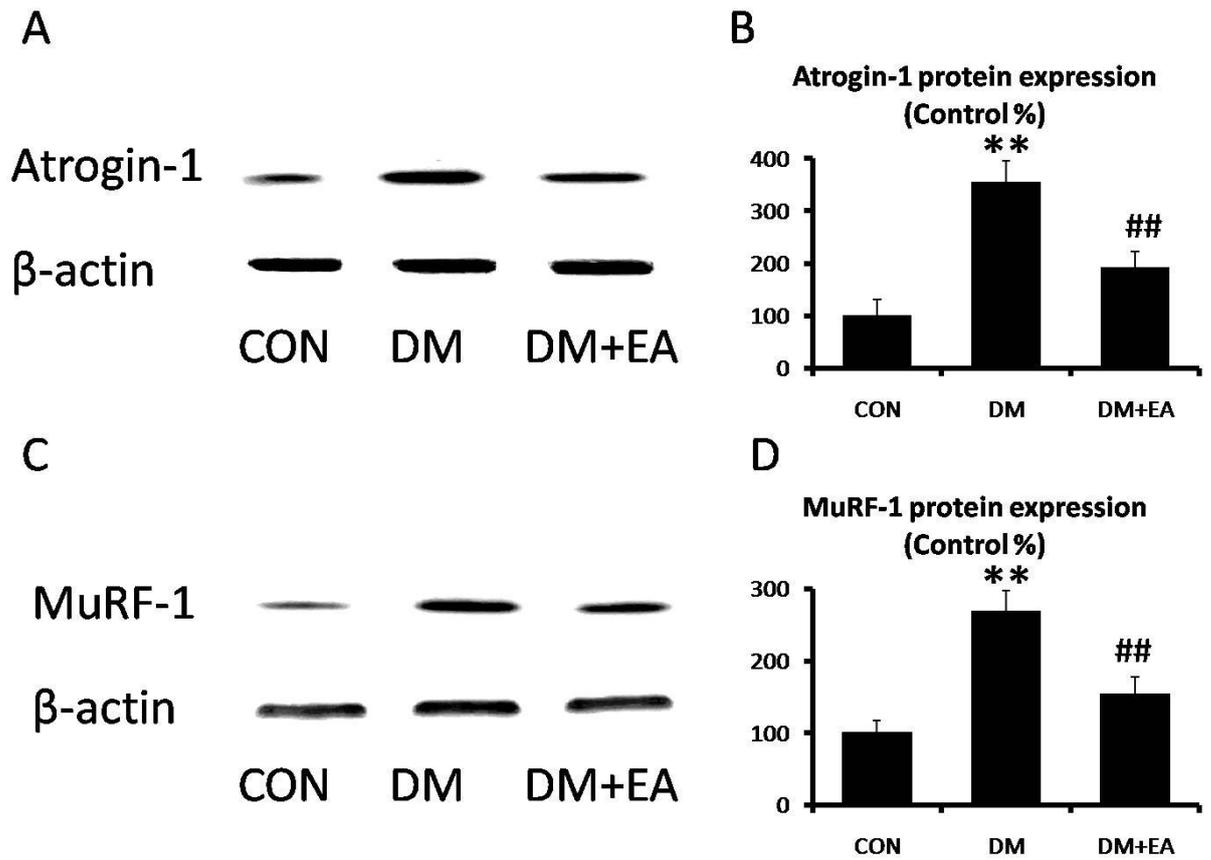


Fig. 4. Effects of EA on protein degradation in gastrocnemius. (A) Atrogin-1 and (B) MuRF-1 levels were detected by Western blot. (C) Atrogin-1 and (D) MuRF-1 relative expressions were quantized. ** $p < 0.01$ vs CON; ## $p < 0.01$ vs DM.

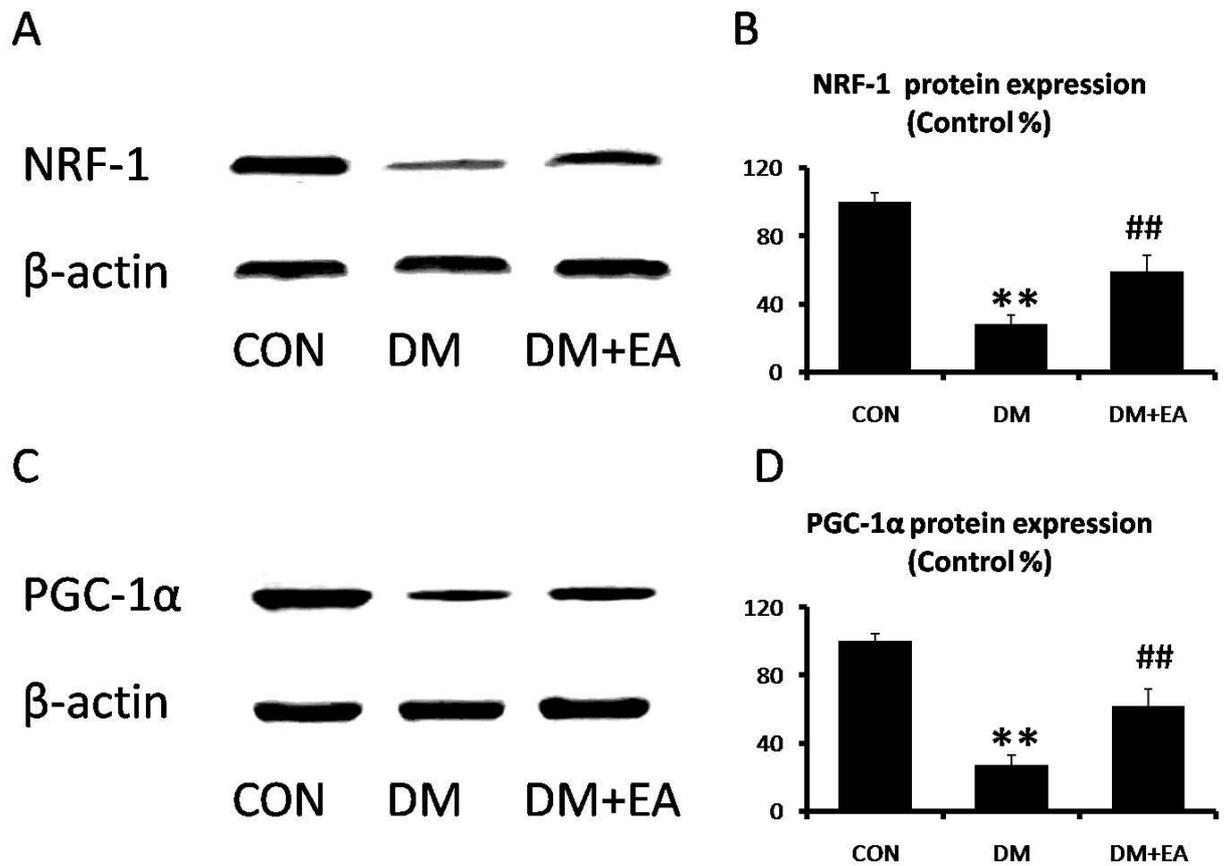


Fig. 5. Effects of EA on mitochondrial function in gastrocnemius. (A) NRF-1 and (B) PGC-1α levels were detected by Western blot. (C) NRF-1 and (D) PGC-1α relative expressions were quantized. ** $p < 0.01$ vs CON; ## $p < 0.01$ vs DM.

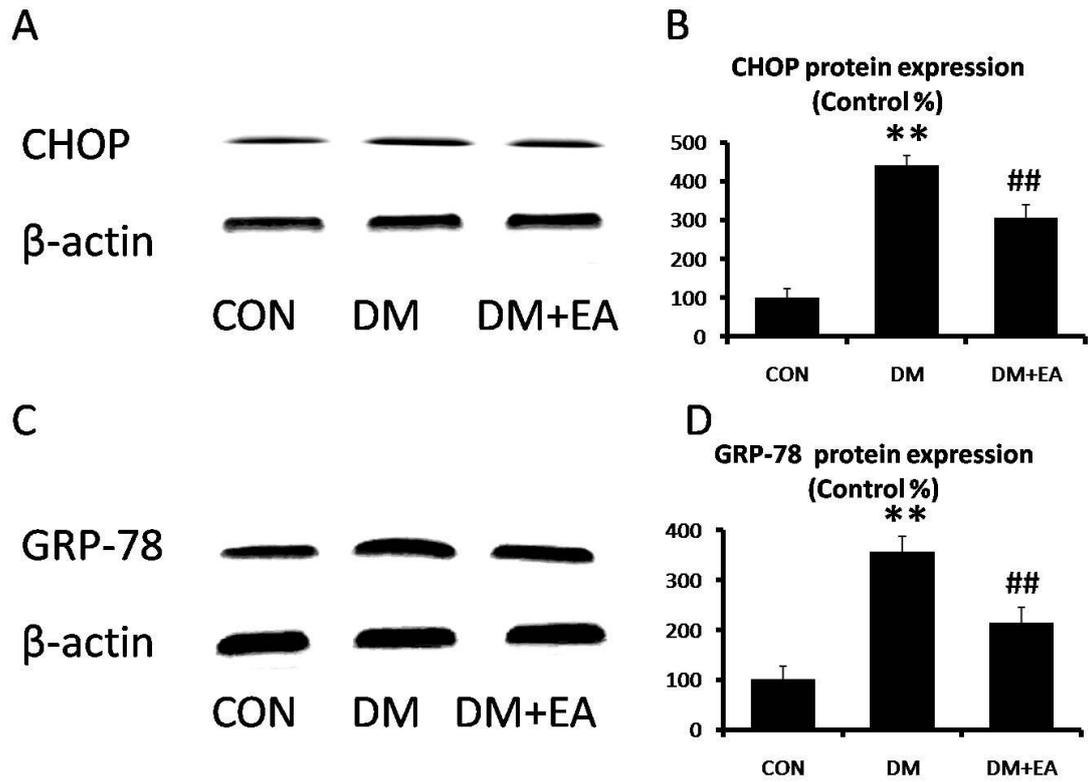


Fig. 6. Effects of EA on ER stress in gastrocnemius. (A) CHOP and (B) GRP-78 levels were detected by Western blot. (C) CHOP and (D) GRP-78 relative expressions were quantized. ** $p < 0.01$ vs CON; ## $p < 0.01$ vs DM.

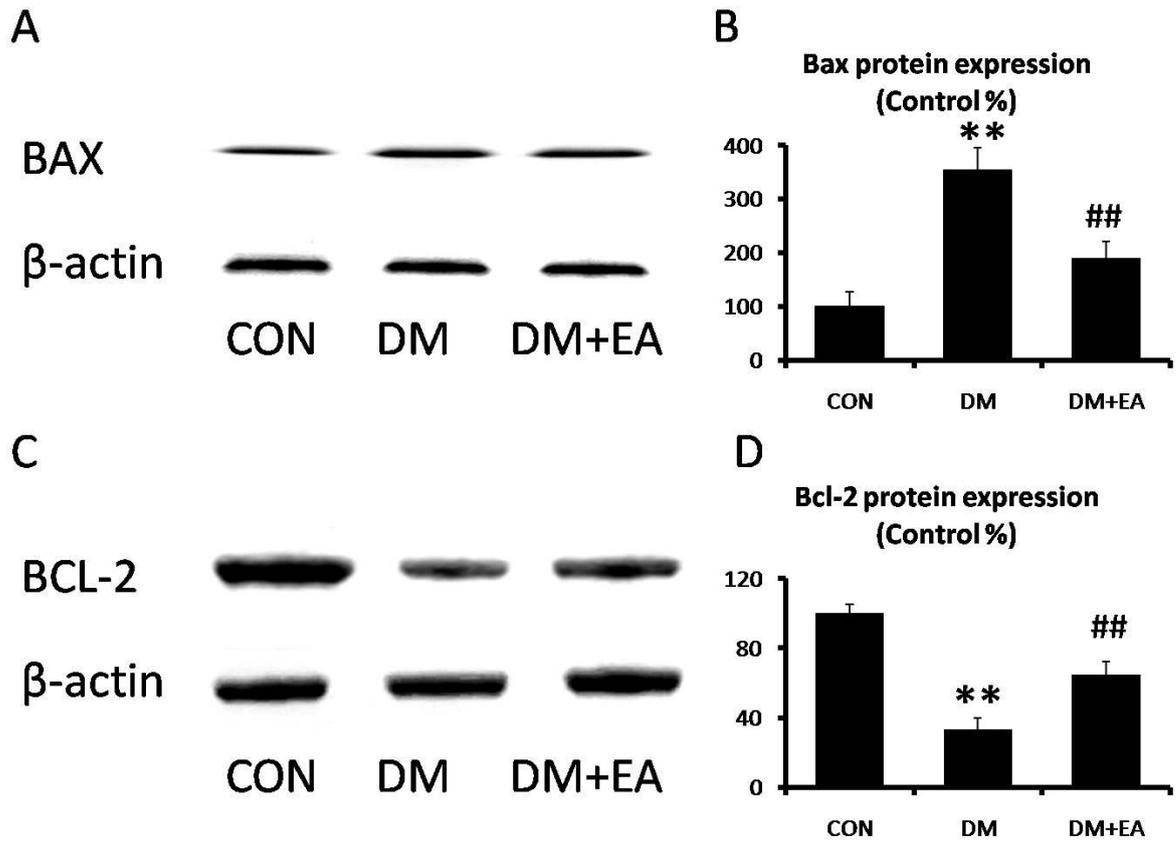


Fig. 7. Effects of EA on apoptosis in gastrocnemius. (A) BAX and (B) BCL-2 levels were detected by Western blot. (C) Bax and (D) Bcl-2 relative expressions were quantized. ** $p < 0.01$ vs CON; ## $p < 0.01$ vs DM.