

# Ameliorating effect of *Trigonella foenum-graecum* L. (fenugreek) extract tablet on exhaustive exercise-induced fatigue in rats by suppressing mitophagy in skeletal muscle

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**Abstract. – OBJECTIVE:** *Trigonella foenum-graecum* L. (fenugreek) is widely used as a leafy vegetable and spice in China and North Africa. Recent studies have reported that fenugreek can reduce fatigue; however, its antifatigue mechanism remains unclear. Therefore, this study aimed to investigate the potential antifatigue effects of fenugreek extract (FE) on mitophagy and the underlying mechanisms.

**MATERIALS AND METHODS:** We evaluated the potential effects of FE tablet on an exhaustive exercise-induced fatigue (EEF) rat model. Oxidative stress indicators and fatigue biomarkers in the serum and skeletal muscle were detected. Mitophagy and mitochondrial morphology were observed using transmission electron microscopy. The expression levels of mitochondrial autophagy-related proteins were detected using western blot and immunofluorescence.

**RESULTS:** Compared with the model group, FE enhanced the activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase as well as total antioxidant capacity; however, it decreased the level of malondialdehyde in the serum and skeletal muscle after a 7-day treatment. Moreover, certain indicators of mitochondrial function, such as reactive oxygen species levels, ATP levels, cellular and mitochondrial Ca<sup>2+</sup> levels, and ATPase activity, were significantly improved in the FE group compared with the model group. Finally, we found that mitophagy was induced by exhaustive exercise and inhibited by FE. Regarding mitochondrial autophagy-related proteins, the expression levels of LC3B, FUNDC1, PGAM5, PARKIN, and PINK1 in the skeletal muscle tissue were increased in the EEF group compared with the control group. After administration of FE and a positive control drug, a significant reversal in the expression of the above-mentioned proteins was noted.

**CONCLUSIONS:** Our findings demonstrate that FE exerted antifatigue effects in the EEF rat model by regulating the mitophagy-related

FUNDC1/LC3B signaling pathway rather than the PINK1/PARKIN signaling pathway.

*Key Words:*

Fenugreek, Fatigue, Mitophagy, Oxidative stress, Mitochondrial function.

## Introduction

Muscle fatigue, which is experienced daily by most people, refers to the declining ability of a muscle to produce strength, and it has been reported to occur in various pathological conditions and as a result of the normal aging process<sup>1,2</sup>. Proper exercise can eliminate and relieve fatigue-related symptoms; however, muscle fatigue can adversely affect the quality of life of individuals and reduce their well-being and social productivity<sup>3</sup>.

Oxidative stress refers to the damage caused by the imbalance between reactive oxygen species (ROS) and the endogenous antioxidant defense in body tissues or cells<sup>4</sup>. Optimal ROS levels are required to support force production in the skeletal muscle during muscular exercise. In contrast, high ROS levels result in muscle contractile dysfunction and fatigue<sup>5</sup>. Exhaustive exercise produces excess free radicals, ROS, and reactive nitrogen species, thereby oxidative stress-related damage to the muscle tissue and impairing contractility<sup>6</sup>. An increase in ROS levels increases the permeability of the mitochondrial membrane, thereby activating the mitochondrial permeability transition pore (mPTP), which induces ROS release into the cytoplasm, resulting in mitochondrial damage<sup>7</sup>. Mitophagy occurs when the dam-

aged mitochondria are specifically encapsulated by autophagosomes, which fuse with lysosomes, thereby completing the degradation of damaged mitochondria. Stability in the number and quality of mitochondria is maintained via mitophagy, which preserves the homeostasis of the intracellular environment<sup>8</sup>. Furthermore, mitophagy plays an important role in mitochondrial functions that affect skeletal muscle metabolic health<sup>9</sup>.

Skeletal muscle health depends on the number and optimal functioning of mitochondria. Exhaustive exercise leads to excessive mitophagy in the skeletal muscle and damage to the mitochondria, which are then cleared by autophagic lysosomal complexes. A reduction in the number of mitochondria can lead to skeletal muscle weakness<sup>10</sup>. Therefore, a better understanding of the mechanism of exercise-induced mitochondrial dysfunction and mitophagy will provide novel treatment options for fatigue.

*Trigonella foenum-graecum* L. – commonly known as fenugreek – is an edible plant that belongs to the Leguminosae family and is widely cultivated as a food crop in China and North Africa<sup>11</sup>. Fenugreek is traditionally used as a medical ingredient for the treatment of diabetes, hyperlipidemia, ulcers, anti-inflammatory edema, paralysis, gout, weakness, hemorrhoids, and wounds. Moreover, some studies have reported that fenugreek – an active ingredient used in traditional Chinese antifatigue medicine – can reduce fatigue<sup>12,13</sup>. Furthermore, previous studies have reported that a fenugreek seed extract could regulate the indicators of oxidative stress levels, such as total antioxidant capacity (T-AOC) as well as malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), and nitric oxide levels, with the ability to alter the mRNA expression levels of NRF2 and OH-1<sup>14,15</sup>. Meanwhile, according to a recent study, fenugreek contains antioxidants, which can delay the process of skeletal muscle fatigue<sup>5</sup>. In addition, fenugreek has been shown to have significant anti-inflammatory properties<sup>16</sup>. The major active chemical constituents of fenugreek include nicotinic acid, phytic acid, scopoletin, trigonelline, diosgenin, gitogenin, homoorientin, saponaretin, and tigenin (Figure 1). In our previous research<sup>17</sup>, we determined trigonelline and diosgenin contents in the alcohol extract of fenugreek using high-performance liquid chromatography. However, little is known about the potential effects of fenugreek on exhaustive exercise-induced fatigue (EEF) in skeletal muscles. In the present study, an estab-

lished *in vivo* rat model of muscle fatigue was used to investigate the potential antifatigue effects of fenugreek administration on mitophagy.

## Materials and Methods

### Chemicals and Reagents

American ginseng tablet (AGT) was purchased from Fujian Shangpu Pharmaceutical Co., Ltd. (Fujian, China). Rabbit anti-FUNDC1 antibody (Merck, ABC506), anti-AMPK alpha 1 + AMPK alpha 2 antibody, anti-AMPK alpha 1 (phospho T183) + AMPK alpha 2 (phospho T172) antibody, anti-LC3B antibody, anti-PARKIN antibody, anti-PINK1 antibody, anti-COX-IV antibody, goat antirabbit IgG H&L (HRP), and goat antimouse IgG H&L (HRP) were purchased from Abcam (Cambridge, UK). Moreover, anti-PGAM5 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Furthermore, GSH peroxidase (GSH-PX), MDA, SOD, T-AOC, ATP assay kit, ROS assay kit, calcium test kit, citrate synthase (CS), Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPase activity, and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### Plant Material and Preparation of the FE Tablet

*Trigonella foenum-graecum* L. (fenugreek) plant was collected from Balikun, Xinjiang (China), and it was identified by Xinxia Li, Professor, Xinjiang Medical University. Pilot production of FE tablets was performed by Xinjiang Huashidan Pharmaceutical Co., Ltd. (lot no. 20180302, Xinjiang, China) according to the extraction and purification methods described in a recent study<sup>18</sup>. The final product was quantified to contain 7.5 mg saponins per tablet in accordance with the method described in “determination of total saponins in health food” published in “Technical Standards for Testing & Assessment of Health Food (2003)” and 0.5 mg trigonelline per tablet in accordance with the method described in “Pharmacopoeia of the People’s Republic of China (2015)”.

### Animals and Ethics Statement

Six-week-old male Wistar rats (body weight, 180–220 g) were provided by the Laboratory Animal Center of Xinjiang Medical University (SYXK, xin: 2018-0003 and SCXK, xin: 2018-0002). They were maintained under standard conditions (temperature, 20°C ± 2°C; humidity, 60%

± 5%; 12-h light/dark cycle) and were provided with free access to standard diet and sterilized water. All animal experiments were approved by the Animal Care Committee of Xinjiang Medical University.

### **EEF and Intervention**

After 3 days of adaptive feeding, 60 rats were randomly classified into the following 6 experimental groups – each consisting of 10 rats: (1) control group: oral gavage was performed using an equal volume of saline for all rats once a day for 1 week; (2) EEF group: EEF experiments and oral gavage were performed using an equal volume of saline for all rats once a day for 1 week; (3) FE-L group: EEF experiments and low-dose FE (58.0 mg/kg) gavage were performed once a day for 1 week; (4) FE-M group: EEF experiments and medium-dose FE (116 mg/kg) gavage were performed once a day for 1 week; (5) FE-H group: EEF experiments and high-dose (230 mg/kg) FE gavage were performed once a day for 1 week; and (6) AGT (positive control drug) group: EEF experiments and AGT (436 mg/kg) gavage were performed once a day for 1 week. A sheet lead corresponding to 5% body weight of rats was attached to the tail of all rats, except for the ones in the control group, and the rats were forced to swim for 15 min in an independent box (120 cm × 80 cm × 60 cm). The rats were allowed to eat normally, but they were not allowed to sleep. After 7 days, the rats were anesthetized using pentobarbital sodium, and blood samples and skeletal muscle tissue were collected from the celiac artery and gastrocnemius, respectively.

### **Measurement of Oxidative Stress and Fatigue Biomarkers in the Serum and Skeletal Muscles**

The blood samples were centrifuged at a centrifugation force of  $3000 \times g$  for 10 min at 4°C in order to obtain the serum. Liquid nitrogen was used to grind the skeletal muscle, and 10% saline-prepared tissue homogenates were prepared. The levels of oxidative stress biomarkers (MDA, SOD, GSH-PX, and T-AOC) were measured using an xMark™ microplate reader (Bio-Rad, Hercules, CA, USA) in the serum or skeletal muscle tissue homogenates. In addition, ROS levels of skeletal muscle tissues were measured using a VLB000D2 fluorescence microplate (Thermo Fisher, Waltham, MA, USA), and ATP levels of skeletal muscle tissues were measured using an xMark™ microplate reader (Bio-Rad). All mea-

surement procedures were performed strictly in accordance with the manufacturer's protocols.

### **Measurement of $Ca^{2+}$ Concentration and ATPase Activity in Skeletal Muscles**

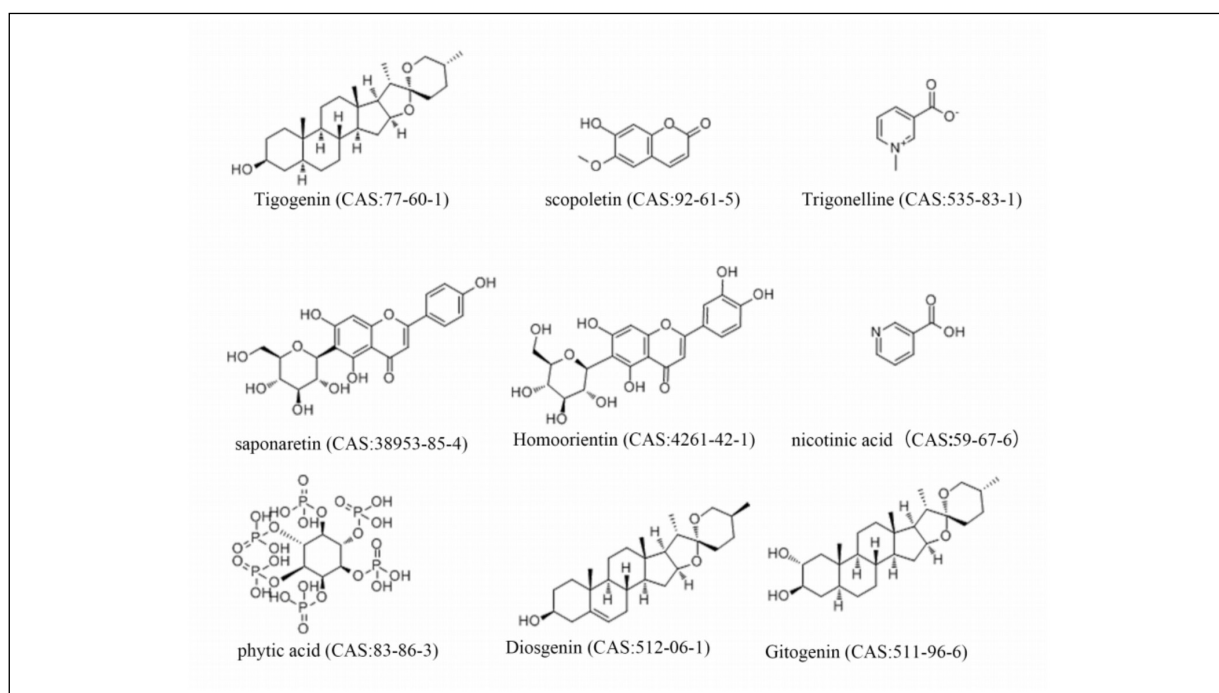
To measure cellular  $Ca^{2+}$  concentration, a single-cell suspension was prepared from a fresh skeletal muscle tissue, and the cells were incubated with Fluo-3/AM (5 μm) at 37°C for 15 min in dark. Fluorescence intensity was measured using LSRFortessa flow cytometry (BD, Franklin Lakes, NJ, USA). Mitochondria were isolated from fresh skeletal muscle tissues using the Cell Mitochondria Isolation Kit (Article No. 89874, Thermo Fisher) in accordance with the manufacturer's protocols. The mitochondrial  $Ca^{2+}$  concentration was measured using the MTB colorimetric kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Additionally,  $Na^+$ - $K^+$ -ATPase and CS activities in skeletal muscle mitochondrial samples were analyzed using an xMark™ microplate reader. All measurements were conducted in accordance with the manufacturer's protocols.

### **Ultrastructural Analysis of Mitochondria in Skeletal Muscles**

The skeletal muscle tissue was fixed using a 2.5% Gluta (pH, 7.2-7.4); subsequently, it was fixed using 1% osmium acid and embedded in epoxy resin. Embedding and solidification procedures were then performed using SPI-Pon 812 embedding kit (Structure Probe, Inc., West Chester, PA, USA). Following this, ultrathin (50-60 nm) sections were cut using an ultramicrotome (EM UC7; Leica Microsystems GmbH, Wetzlar, Germany). An electron microscope (magnification × 10,000; JEM1230; JEOL) was used for observing the ultrastructure of the muscle tissue.

### **Immunofluorescence Assay**

The rat skeletal muscle sample, which was frozen in liquid nitrogen, was cut into 8-μm thick sections using a freezing microtome, and the sections were placed on antidropping glass slides. The tissue sections were then treated with 4% paraformaldehyde for 10 min. Thereafter, 5% bovine serum albumin was added, followed by incubation for 30 min at room temperature. The tissue sections were then incubated with primary antibodies (rabbit anti-LC3B antibody, rabbit anti-FUNDC1 antibody, and mouse anti-COX-IV antibody) overnight at 4°C and then with goat antimouse IgG H&L (Alexa Fluor®488) and goat antirabbit IgG H&L (Alexa Fluor®594) second-



**Figure 1.** Active ingredients of fenugreek and chemical structures. A number of active ingredients from fenugreek have been isolated and studied, including nicotinic acid, phytic acid, scopoletin, trigonelline, diosgenin, gitogenin, homoorientin, saponaretin, and tigogenin.

ary antibodies for 1 h. Subsequently, DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to stain the nuclei. The images were captured and analyzed using a laser scanning microscope (Carl Zeiss, Germany).

### Western Blot Analysis

Total protein was isolated from homogenates of the skeletal muscle tissue and mitochondrial samples, and a BCA protein assay kit (TransGen, China) was used to determine the protein concentration of each sample. Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Following this, the protein samples were transferred to polyvinylidene fluoride membranes (IPVH00010, EMD Millipore, Billerica, MA, USA) – blocked with 5% nonfat milk for 1 h – and incubated with primary antibodies against COX-IV, PARKIN, AMPK $\alpha$ , p-AMPK $\alpha$ , PGAM5, FUNDC1, LC3B, PINK1, and  $\beta$ -actin overnight at 4°C. The membranes were then incubated with a secondary antibody [goat antimouse IgG H&L (HRP) and goat antirabbit IgG H&L (HRP)] for 2 h at 25°C. Finally, chromogen solutions A and B were mixed, 2 mL of the mixture was added to the membrane, and SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher) was

used for visualizing the protein bands. The density values of bands were calculated using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., MD, USA). The tissue and mitochondrial protein expression levels were normalized to  $\beta$ -actin and COX-IV, respectively.

### Statistical Analysis

Data were presented as mean  $\pm$  standard error of the mean. Statistical analysis was performed using IBM SPSS Statistics, version 21.0 (IBM Corp., Armonk, NY, USA). Data were analyzed using the one-way analysis of variance method for multiple comparisons. A  $p$ -value of  $<0.05$  was considered statistically significant.

## Results

### Effects of FE on Body Weight

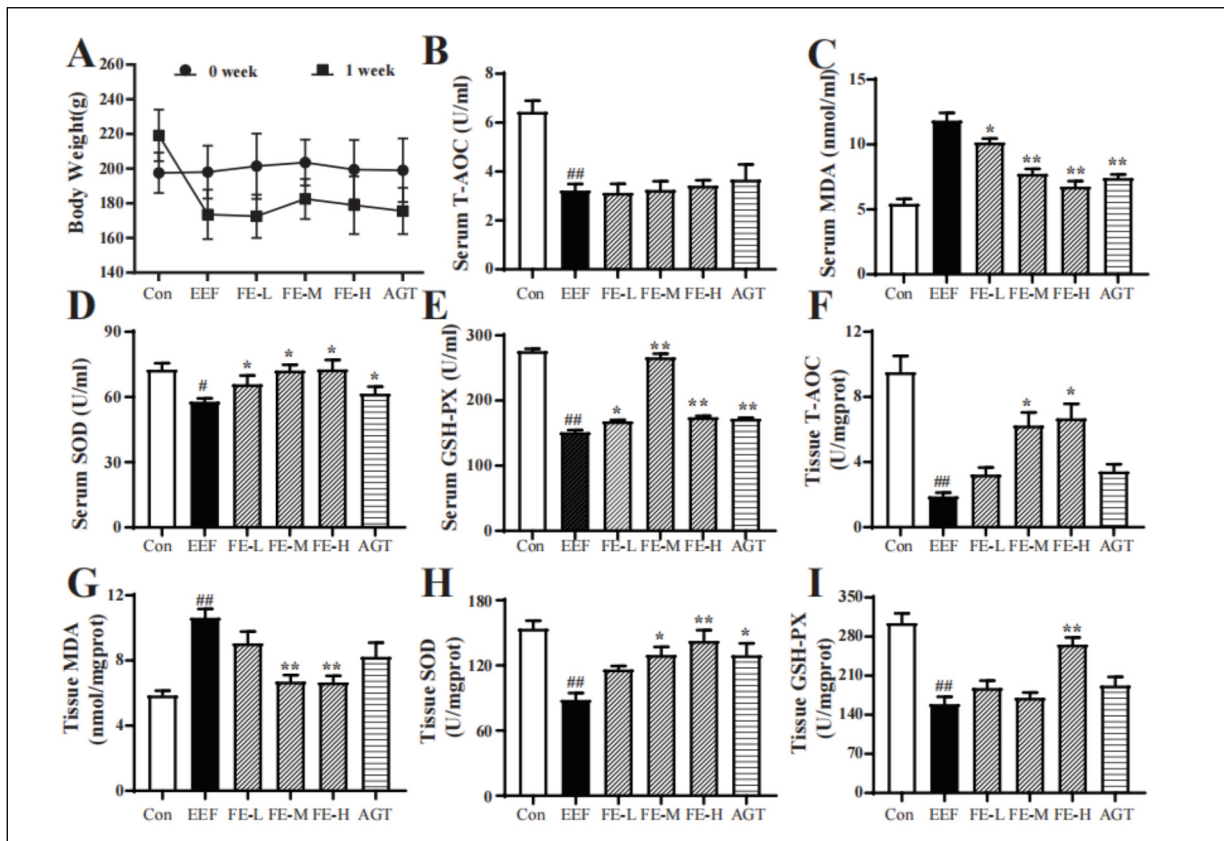
The body weight of rats was recorded before and after the use of FE. As opposed to the natural increase in body weight seen in control rats, the body weight of other test groups decreased significantly compared with that of the control group after 7 days ( $p < 0.01$ ). No difference was found in

the body weight between the FE treatment, EEF model, and AGT treatment groups (Figure 2A).

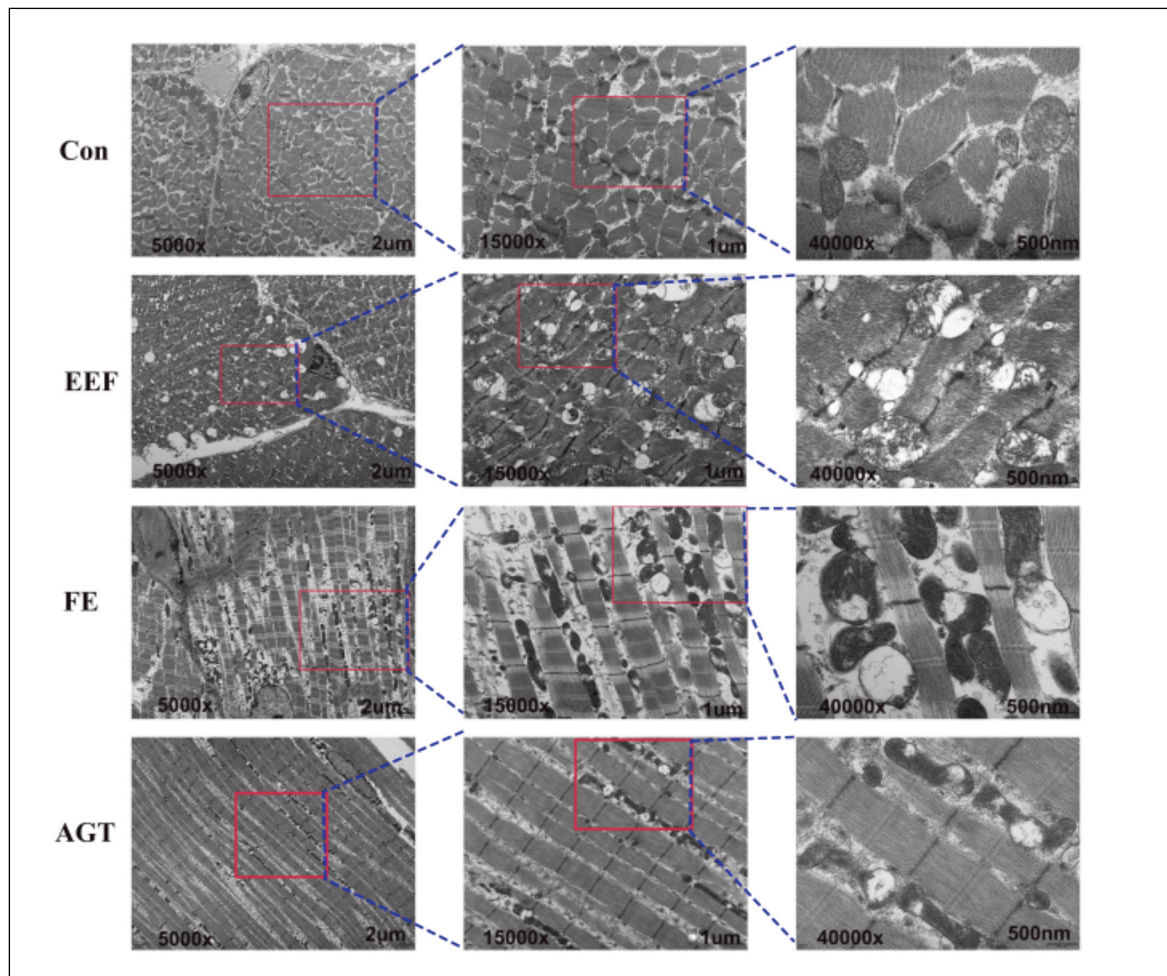
### FE Reduces Oxidative Stress

Subsequently, we attempted to identify indicators associated with oxidation in rat serum and skeletal muscle. Compared with serum and skeletal muscle tissue in the control group, T-AOC, SOD, and GSH-PX were significantly increased in the EEF group, although MDA was significantly increased ( $p < 0.01$ ). Moreover, we found that compared with EEF, the low, moderate, and high doses of AGT and FE did not have any effect on T-AOC in the rat serum (Figure 2B), but they could reduce the content of MDA (Figure 2C). The FE-H group demonstrated significantly higher ef-

fects than the AGT group. Compared with EEF, the low, moderate, and high doses of FE could increase SOD and GSH-PX activities in the serum of rats (Figure 2D-2E), among which the high dose improved SOD activity most significantly. The effect of FE was better than that of AGT, and the moderate dose of FE increased GSH-PX activity most apparently. Subsequently, we also detected the abovementioned indicators in the skeletal muscle and found that T-AOC differed between the serum and skeletal muscle; moreover, compared with EEF, FE and AGT could increase T-AOC in the skeletal muscle. The FE-H group revealed significant increase in the serum T-AOC levels (Figure 2F). Changes in SOD, MDA, and GSH-PX were similar to those found in the se-



**Figure 2.** The effects of FE on body weight and oxidative stress. **A**, The body weight was measured before and after the experiment. **B**, Effects of FE and AGT (positive drug) on T-AOC activity in serum after a 7-day treatment in various rats. **C**, Effects of FE and AGT (positive drug) on MDA level in serum after a 7-day treatment in various rats. **D**, Effects of FE and AGT (positive drug) on SOD activity in serum after a 7-day treatment in various rats. **E**, Effects of FE and AGT (positive drug) on GSH-Px activity in serum after a 7-day treatment in various rats. **F**, Effects of FE and AGT (positive drug) on T-AOC activity in skeletal muscle tissue after a 7-day treatment in various rats. **G**, Effects of FE and AGT (positive drug) on MDA level in skeletal muscle tissue after a 7-day treatment in various rats. **H**, Effects of FE and AGT (positive drug) on SOD activity in skeletal muscle tissue after a 7-day treatment in various rats. **I**, Effects of FE and AGT (positive drug) on GSH-Px activity in skeletal muscle tissue after a 7-day treatment in various rats. The values are expressed as mean  $\pm$  SEM ( $n = 8$ ). \* $p < 0.05$ , \*\* $p < 0.01$  represent statistical significance against the EEF group; # $p < 0.05$ , ### $p < 0.01$  represent statistical significance against the Con group.



**Figure 3.** The effects of FE on mitophagy observed by transmission electron microscope (TEM). Exhaustive exercise-induced fatigue experiment and treatment with FE (116 mg/kg) and AGT (436 mg/kg) for seven days. TEM of ultrastructural and autophagic changes of mitochondria in rat skeletal muscle ( $n = 3$ ). The blue dotted line opening directional insets represent magnifications of the boxed regions of the left panel.

rum, and FE and AGT could increase SOD and GSH-PX and decrease MDA levels compared with EEF (Figure 2G-2H).

### **Changes in Mitochondrial Structure**

Differences in morphology and the number of mitochondria were observed in all groups. In the control group, two mitochondria with normal size, complete mitochondrial cristae, and regular morphology were present in almost all intermyofibrillar spaces. Mitochondria in the EEF group were rounder and more massively enlarged than those in the control group, and a significant perinuclear clustering of damaged mitochondria was observed. Additionally, the cavitation and cristae grew shorter and fewer in number as the matrix particles decreased or disappeared. Autophago-

some also proliferated in large numbers and enveloped the mitochondria. As shown in Figure 3, the majority of the mitochondria in the FE and AGT groups grew into strips, the number of autophagosomes decreased, and the mitochondrial morphology returned to normal.

### **FE Improves Mitochondrial Function**

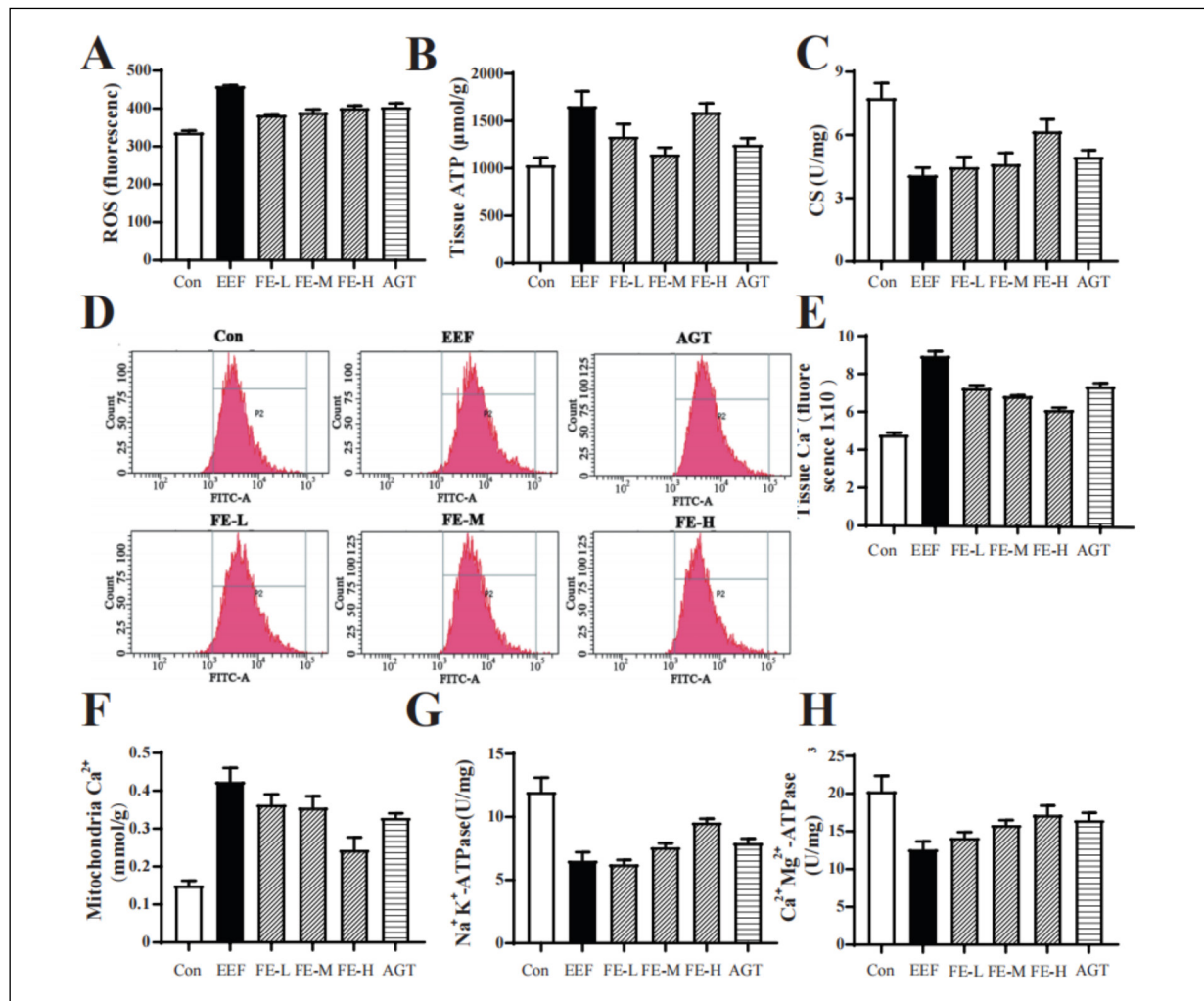
We, then, evaluated mitochondrial function by measuring ROS level, ATP level, cellular and mitochondrial  $\text{Ca}^{2+}$  levels, and ATPase activity. Compared with the control group, ROS, tissue ATP, and mitochondrial  $\text{Ca}^{2+}$  levels were increased in the EEF group as opposed to decreased CS,  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity, and  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -ATPase levels. Moreover, compared with the EEF group, the FE and AGT groups could reduce the ROS levels

(Figure 4A). The ATP levels in the FE and AGT groups were lower than those in the EEf group (Figure 4B). In addition, the CS and levels of other administrative groups were comparable to those of the EEf group, except for the FE-H group's CS, which increased compared with the AGT group's CS (Figure 4C). We also detected the  $Ca^{2+}$  levels in tissues and mitochondria. Compared with the EEf group, both the FE and AGT groups could reduce the tissue  $Ca^{2+}$  levels, among which the FE-H group had the most significant effect (Figure 4D-F). In mitochondria, FE-H and AGT increased  $Ca^{2+}$

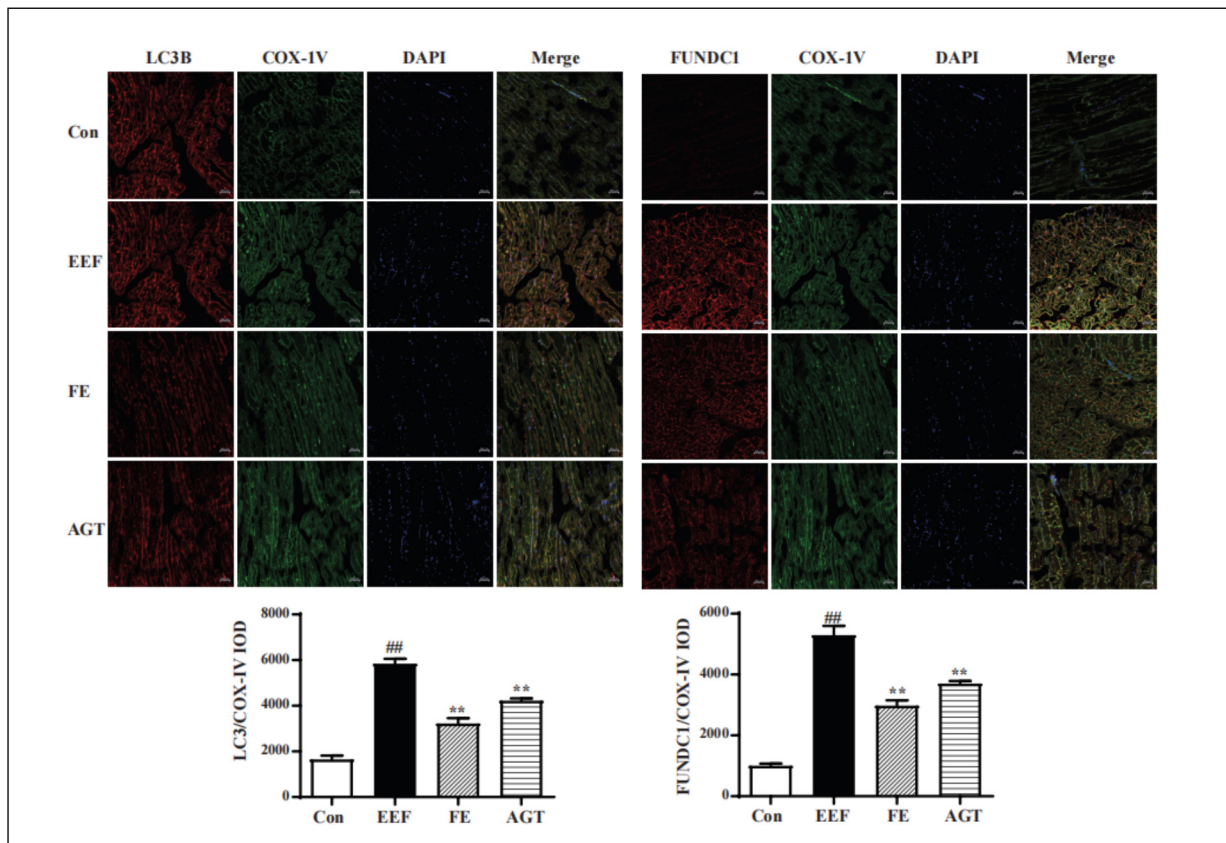
levels (Figure 4G). Finally, compared with the EEf group, the  $Na^{+}$ - $K^{+}$ -ATPase activity of the FE-H and AGT groups increased (Figure 4H), and both the groups could increase the  $Ca^{2+}$ - $Mg^{2+}$ -ATPase activity (Figure 4H), among which the FE-H group had the most significant effect.

### FE Suppresses Mitophagy in Skeletal Muscles

As mentioned above, we identified changes in the morphological structure and function of skeletal muscle mitochondria in the FE group. Sub-



**Figure 4.** Effects of FE on mitochondrial function. **A**, Effects of FE and AGT on ROS level in skeletal muscle tissue after a 7-day treatment in various rats (n = 3). **B**, Effects of FE and AGT on ATP level in skeletal muscle tissue after a 7-day treatment in various rats (n = 8). **C**, Effects of FE and AGT on CS activity in skeletal muscle tissue after a 7-day treatment in various rats (n = 8). **D-E**, Effects of FE and AGT on Cellular  $Ca^{2+}$  level in skeletal muscle tissue after a 7-day treatment in various rats (n = 8). **F**, Effects of FE and AGT on mitochondrial  $Ca^{2+}$  concentration in skeletal muscle tissue after a 7-day treatment in various rats (n = 8). **G**, Effects of FE and AGT on  $Na^{+}$ - $K^{+}$ -ATPase activity in skeletal muscle tissue after a 7-day treatment in various rats (n = 8). **H**, Effects of FE and AGT on  $Ca^{2+}$ - $Mg^{2+}$ -ATPase activity in skeletal muscle tissue after a 7-day treatment in various rats (n = 8). The values are expressed as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  represent statistical significance against the EEf group; # $p < 0.05$ , ## $p < 0.01$  represent statistical significance against the Con group.



**Figure 5.** We used immunofluorescence to observe the effects of FE on mitophagy. It was performed exhaustive exercise-induced fatigue experiment and treatment with FE (116 mg/kg) and AGT (436 mg/kg) for seven days (scale bar: 50  $\mu$ m). LC3B and FUNDC1 showed red fluorescence, whereas COX-IV showed green. The nuclei were stained with DAPI (blue-fluorescence). Integrated optical density (IOD) was measured, and LC3B/COX-IV IOD and FUNDC1/COX-IV IOD was calculated. The values are expressed as mean  $\pm$  SEM (n = 3). \* $p$  < 0.05, \*\* $p$  < 0.01 represent statistical significance against the EEF group; # $p$  < 0.05, ### $p$  < 0.01 represent statistical significance against the Con group.

sequently, double immunofluorescence staining of LC3B and FUNDC1 with COX-IV was used to measure mitophagy flux in skeletal muscle tissue (Figure 5). The results showed that both the red and yellow fluorescence caused by FUNDC1 and the combination of COX-IV and FUNDC1, respectively, were enhanced in the EEF group compared with those in the control group. These results indicated that FUNDC1 was expressed in mitochondria and that the expression level of FUNDC1 in mitochondria was significantly increased after strenuous exercise. Compared with the EEF group, the red fluorescence intensity caused by FUNDC1 was weaker in the FE and AGT groups, whereas the yellow fluorescence intensity caused by the combination of COX-IV and FUNDC1 was weaker in the FE and AGT groups than in the EEF group. These results suggest that vigorous exercise can induce mitophagy and FE can inhibit mitophagy.

### ***Effects of FE Administration on the Expressions of Mitophagy-associated Proteins***

Compared with the skeletal muscle tissue of the control group, the expressions of LC3B, FUNDC1, PGAM5, PARKIN, and PINK1 in the EEF group were increased ( $p$  < 0.05). Compared with the skeletal muscle tissue of the EEF group, the FE and AGT groups could significantly reduce the expressions of LC3B, FUNDC1, and PGAM5 ( $p$  < 0.05), but no significant change was found in the expressions of PARKIN and PINK1 ( $p$  > 0.05) (Figure 6A-F). Subsequently, we tested AMPK and found that the expression level of phosphorylated AMPK $\alpha$  in the EEF group was significantly increased compared with that in the control group, whereas the expression level of AMPK expression was not increased.

After administration of FE and AGT, a significant reversal in the expressions of the above-mentioned proteins was found (Figure 6G-I).



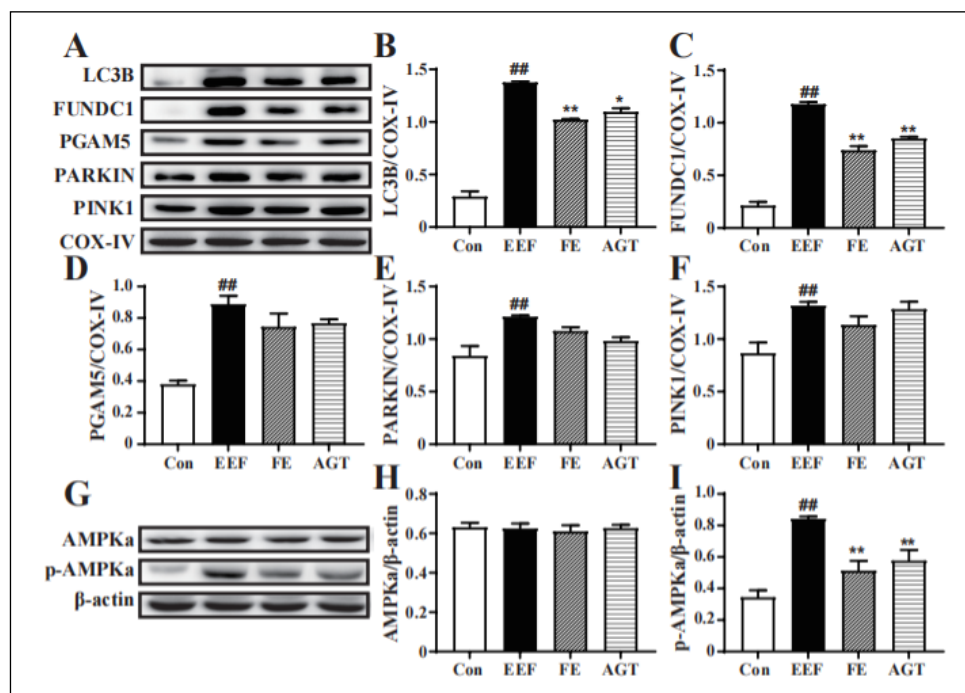
## Discussion

*Trigonella foenum-graecum* L. (fenugreek) is an edible plant that is commonly consumed as a leafy vegetable or spice<sup>11</sup>. A previous study revealed that fenugreek tended to have prominent antioxidant and antitumor properties<sup>11</sup>; however, only a few studies have investigated its antifatigue effect. The results of the present study indicate that FE has antifatigue effects, which may be effective in improving EEf skeletal muscle and antioxidant capacity. It can also regulate mitochondrial function by modulating FUNDC1/LC3B signaling pathway rather than PINK1/PARKIN signaling pathway to inhibit mitophagy.

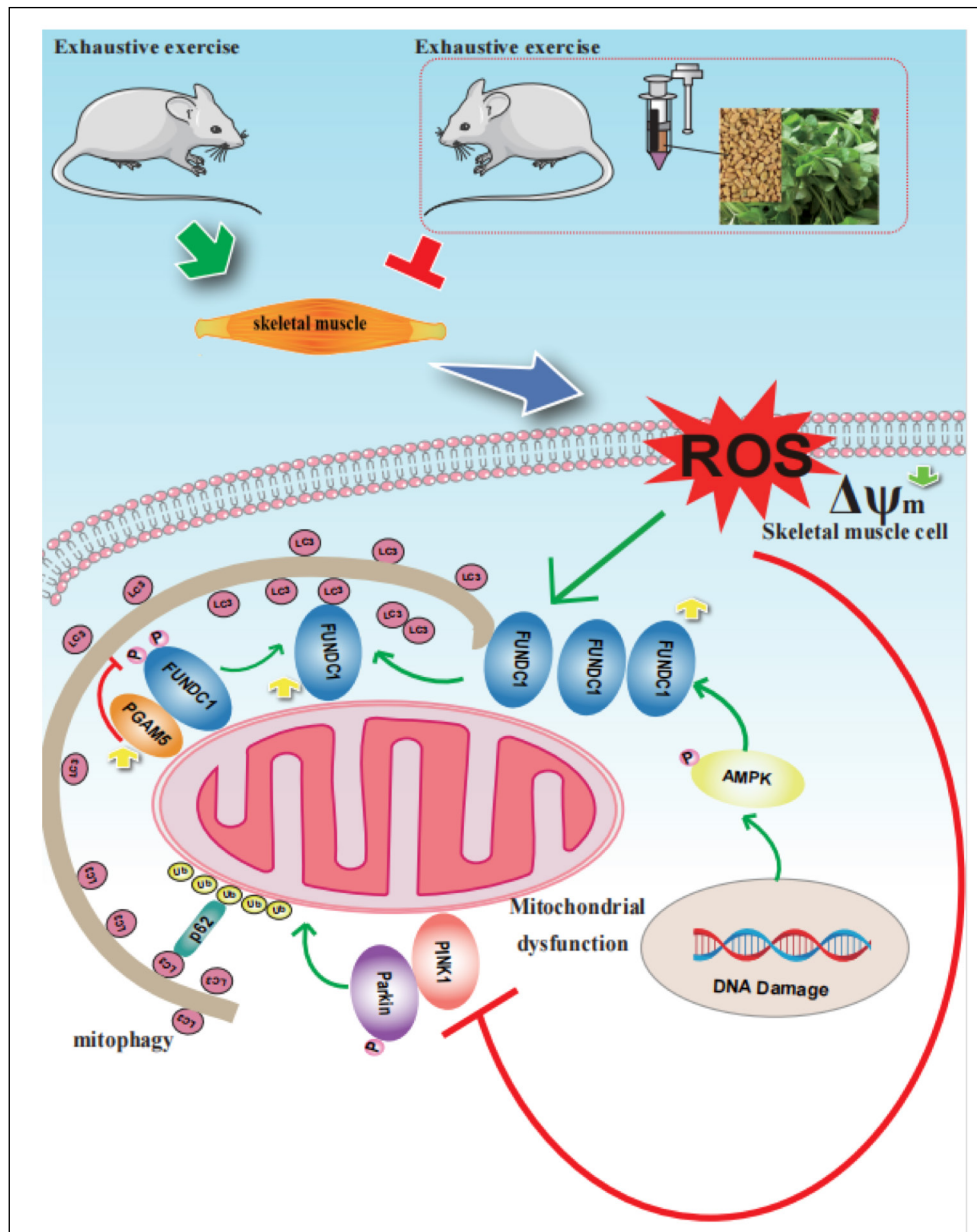
A previous study<sup>6</sup> reported that strenuous or active exercise may promote oxidative damage in skeletal muscles. In general, MDA content is a direct quantitative indicator of tissue lipid peroxidation and is related to the tissue quality<sup>18,19</sup>, and its level can indicate the level of ROS and degree of lipid peroxidation<sup>20</sup>. Our study found that FE could effectively reduce

MDA and ROS levels, and the results of the FE group were superior to those of the AGT group. SOD and GSH-PX are required to prevent oxidative damage. SOD reduces high levels of ROS and protects cells from damage, whereas GSH-PX enables cells to protect themselves against ROS-induced damage<sup>21</sup>. FE could reduce SOD and GSH-PX caused by fatigue and resist fatigue. Interestingly, we found that both the FE and AGT groups were effective at enhancing the decrease in T-AOC caused by skeletal muscle fatigue. However, this finding was not confirmed by serum tests; hence, it requires further investigations.

Mitochondria play a crucial role in muscle contraction and regulation of  $Ca^{2+}$  levels<sup>22</sup>. Our study found a significant increase in the number of mitochondria and ATP levels in the model group. Moreover, our study indicated that muscle work must be supported by the readily available ATP energy supply, which is consistent with the findings of previous reports suggesting that aerobic exercise increases skeletal muscle mitochondrial content<sup>23</sup>.  $Na^+K^+$ - and  $Ca^{2+}Mg^{2+}$ -AT-



**Figure 6.** The effects of FE administration on the expression of mitophagy-associated proteins. Representative western blotting images showing the protein expression of PINK1, PARKIN, LC3B, PGAM5, and FUNDC1 in rat skeletal muscle mitochondria (A). Semiquantitative densitometric analysis of LC3B, (B) FUNDC1, (C), PGAM5 (D), PARKIN (E), and LC3B and PINK1 (F). Representative western blotting images showing the protein expression of AMPKa and p-AMPKa in rat skeletal muscle (G). Semiquantitative densitometric analysis of AMPKa (H) and p-AMPKa (I). The values are expressed as mean  $\pm$  SEM (n = 3). \* $p$  < 0.05, \*\* $p$  < 0.01 represent statistical significance against the EEf group; # $p$  < 0.05, ## $p$  < 0.01 represent statistical significance against the Con group.



**Figure 7.** FE exerted antifatigue effects in the EEF rat model through regulation of the mitophagy-related FUNDC1/LC3B signaling pathway, rather than the PINK1/PARKIN signaling pathway.

Pase are the major ATPases, which require ATP for muscle activity<sup>5</sup>, and CS is an enzyme that regulates the tricarboxylic acid cycle<sup>24</sup>. In the present study, the Na<sup>+</sup>-K<sup>+</sup>-ATPase, Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPase, and CS activities<sup>25</sup> were significantly reduced in the model group, but this effect could be reversed by FE administration on ATPase. Furthermore, the literature shows that the increase in mitochondrial Ca<sup>2+</sup> levels can result in disruption of mitochondrial membrane poten-

tial and opening of MPTP<sup>26</sup>. Mitophagy is triggered when mitochondria cannot maintain their membrane potential<sup>27,28</sup>, but it is also induced by ROS<sup>29</sup>. Skeletal muscle mitophagy results were validated in the EEF model<sup>10</sup>.

Mitophagy is an important mechanism for maintaining mitochondrial quality and achieving self-control by removing damaged or excessive mitochondria. Any disturbance in this process can cause certain harm<sup>30,31</sup>. A recent study<sup>32</sup>

demonstrated that AMPK activation could be induced by ROS and can be regarded as a regulator of autophagy and mitophagy. The function of FUNDC1 during mitophagy has been extensively explored. FUNDC1 acts as a mitophagy receptor that recruits MAP1LC3B/LC3B (LC3) through its LC3-interacting region motif to initiate mitophagy in cells<sup>33</sup>. The PINK1/PARKIN signaling pathway, which mediates mitochondrial damage, has been extensively studied in mitophagy<sup>34</sup>, and dysregulation of this PINK1/PARKIN signaling pathway in Alzheimer's disease (AD)<sup>35</sup> and Parkinson's disease<sup>36</sup> has been reported<sup>37</sup>. Finally, we explored the mechanism of FE-induced mitophagy. PGAM5, a mitochondrial protein phosphatase genetically and biochemically linked to PINK1, reduces PINK1 levels in cells at the onset of mitophagy and promotes impaired autophagosome phagocytosis by dephosphorylation of the cleaved mitochondrial receptor FUNDC1<sup>38,39</sup>. Interestingly, only FUNDC1, LC3B, and phosphorylated AMPK expression levels were significantly downregulated as opposed to PGAM5, PINK1, and PARKIN.

## Conclusions

FE regulates mitophagy by altering the PGAM5 and FUNDC1/LC3B pathways rather than the PINK1/PARKIN signaling pathway. FE administration exerts antifatigue effects and improves exercise performance in rats by regulating the mitophagy-related FUNDC1/LC3B signaling pathway rather than the PINK1/PARKIN signaling pathway (Figure 7). However, the precise molecular mechanisms underlying the antifatigue properties of FE require further investigations.

## Conflict of Interest

The authors declare no conflicts of interest.

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## Authors' Contributions

Dong Yan: Conceptualization, Writing – Original Draft, Investigation, and Visualization; Yao Yan: Conceptualization, Writing – Review & Editing, and Data Curation; Jun Lian: Methodology, Validation, and Formal Analysis; Xiaomin Lu: Investigation, Formal Analysis, and Visualization; Linlin Li: Investigation, Formal Analysis, and Validation.

## Ethics Statement

This study was approved by the Animal Research Ethics Committee of Xinjiang Medical University (Ethical Approval No. IACUC20180905-16).

## Data Availability Statement

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

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