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Melatonin protects against sarcopenia in middle-aged mice

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Summary. Background. Sarcopenia is a common age-related disease. Melatonin (MEL) is an age-related endocrine hormone, which displays a crucial role in resisting oxidative stress during aging. Importantly, the antioxidant properties of MEL can be mediated by mitochondria.

Objective. Therefore, we wondered whether MEL could mitigate oxidative stress caused by mitochondria in sarcopenia.

Methods. The middle-aged mice were administered 5 mg/kg/d and 10 mg/kg/d of MEL for 2 months. Young mice were used as the control group.

Results. After treatment with MEL, the grip strength of the fore/hind limbs, running time, and distance were elevated, and the weights of the gastrocnemius (GA), tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (SOL) were enhanced in middle-aged mice. Additionally, MEL was observed to alleviate histological damage and increase the cross-sectional area of muscle fibers in GA tissues of middle-aged mice. Furthermore, following MEL treatment, there was an increase in the percentage and size of normal mitochondria as well as mtDNA copy number but a reduction in the levels of malondialdehyde (MDA), protein carbonyl, and reactive oxygen species (ROS) in the GA tissues of middle-aged mice. At the molecular level, MEL repressed the levels of ATROGIN-1, muscle RING-finger protein-1 (MURF-1), and the ratio of p-P38/P38, but elevated the expression of cytochrome c oxidase subunit 4 (COX4), cystatin C (CYTC), nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (TFAM), and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) in the GA tissues of middle-aged mice. Importantly, 10 mg/kg MEL was more efficacious in the treatment of sarcopenia than 5 mg/kg MEL.

Conclusion. MEL attenuates sarcopenia in middle-aged mice, and the mechanism may relate to mitochondria-induced oxidative stress and the PGC-1α/TFAM pathway.

Key words: Sarcopenia, Melatonin, Mitochondria, PGC-1α/TFAM pathway, Aging

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Introduction

Sarcopenia is an age-related syndrome, which is characterized by a loss of skeletal muscle mass and a reduction in skeletal muscle quality (Di Cesare Mannelli et al., 2020). With the increase in human age, there is a notable decline in skeletal muscle mass, with a reduction of 0.5% to 1% per year from the age of 30, this decline accelerates significantly after the age of 65, leading to a range of adverse consequences including falls, fractures, physical disabilities, decreased quality of life, and even death (Ying et al., 2022). Recent research has indicated that sarcopenia is also associated with other diseases, such as obesity (Gómez-Uranga et al., 2022), chronic heart failure (Qaisar et al., 2021), chronic obstructive pulmonary disease (Pishgar et al., 2021), type 2 diabetes (Pechmann et al., 2020), cognitive impairment (Scisciola et al., 2021), and a variety of cancers (Kim et al., 2022). The causes of sarcopenia involve lifestyle and age-dependent biological changes, including chronic inflammation, decreased antioxidant defense, mitochondrial dysfunction, and decreased regeneration and repair ability (Marzetti et al., 2012; Martone et al., 2017). Although the clinical importance of sarcopenia has been increasingly recognized, a definite therapeutic intervention to effectively treat sarcopenia is still lacking.

The existing research has indicated that mitochondrial dysfunction plays a pivotal role in the development of sarcopenia (Ferri et al., 2020). Meanwhile, an increasing number of studies have

emphasized the crucial role of mitochondrial homeostasis imbalance and oxidative stress in the pathogenesis of muscle atrophy during aging (Cai et al., 2022; Liu et al., 2022). Mitochondria produce a large number of oxygen free radicals while providing energy, and the dynamic balance between the generation of reactive oxygen species (ROS) and antioxidants is closely related to mitochondrial functional homeostasis (Luo and Joiner, 2014). Research has pointed out that impaired mitochondria and oxidative stress may be the underlying causes of obesity, aging, and fatty acid-induced muscle atrophy (Tournadre et al., 2019; Dowling et al., 2022; Li et al., 2022). Therefore, identifying the effects of oxidative stress caused by impaired mitochondria in sarcopenia and developing strategies to alleviate oxidative stress resulting from mitochondrial homeostasis imbalance may be beneficial for the treatment of sarcopenia.

Endocrine changes also exert a pivotal influence on the progression of sarcopenia. Melatonin (MEL) is an age-related endocrine hormone, mainly secreted by the pineal gland (Xu et al., 2020). Sarcopenia, one of the most prevalent age-related diseases in humans, has been closely associated with decreased MEL secretion (Jin et al., 2021; Fernández-Martínez et al., 2023; Altaf et al., 2024). Studies on MEL have demonstrated that it is a natural antioxidant, which displays a crucial role in resisting oxidative stress and free radical generation during aging (Remigante et al., 2024). A study involving cells and animals suggested that the antioxidant properties of MEL can be mediated by mitochondria (Ge et al., 2024). In the process of aging, MEL can also maintain the integrity of muscle mitochondrial function and improve the redox state of resistance training athletes (Leonardo-Mendonça et al., 2017). These results lead us to wonder whether MEL has a reverse effect on oxidative stress resulting from mitochondria in sarcopenia.

To verify this hypothesis, this study intended to select young (10 weeks) and middle-aged C57BL/6J mice (18 months) to examine whether MEL can counteract oxidative stress resulting from mitochondria in sarcopenia, and explore its potential effects on aging-induced sarcopenia. The findings of this study will offer a new idea and direction for the treatment of sarcopenia.

Materials and methods

Animals

Six male C57BL/6J mice of specific pathogen-free (SPF) grade (10 weeks) and eighteen male C57BL/6J mice of SPF grade (18 months) were acquired from the Shanghai Jihui Laboratory Animal Care Co., Ltd. and were labeled as the young and middle-aged groups, respectively (Han et al., 2024). The animals were given seven days to acclimatize to the environment (25±2°C, humidity 55±5%, and a light/dark cycle for 12h). All animal studies were conducted in accordance with the guidelines of the China Animal Care and Use Committee. Study procedures were approved by the Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center (SYXK (Zhe) 2021-0033).

Animal grouping

The middle-aged mice were divided into the middle-aged group, middle-aged+MEL 5 mg/kg (MEL5) group, and middle-aged+MEL 10 mg/kg (MEL10) group, with six mice in each group. The mice in the middle-aged+MEL5 and middle-aged+MEL10 groups were administered MEL 5 mg/kg/day and 10 mg/kg/day by gavage for two months, respectively (Sayed et al., 2019). The mice in the young and middle-aged groups were administered the same volume of saline. The body weight of the mice was monitored weekly.

Grip strength test

Following the final administration, the muscle strength of the mouse limbs was tested using a grip strength instrument (BIO-GS3, Bioseb, USA). Before the grip strength test, mice were acclimated to the grip apparatus for 3 min. Thereafter, the mice were placed on a grid attached to the grip strength instrument and once all four paws had firmly grasped the grid, the mice were slowly pulled backward by the tail until they left the grid. Each mouse was tested five times, with a 10-second interval between each trial. The final measurements for grip strength were assessed by taking the average of the five trials.

Treadmill test

An animal treadmill (LE8710MTS, RWD, China) was applied to measure the running time and distance of mice in each group. Mice were tested on a treadmill at a speed of 10 cm/s for 10 min, after which the speed was increased by 1 cm/s every minute till the speed reached 35 cm/s. The treadmill maintained a speed of 35 cm/s until the mice were fatigued. Fatigue was identified as the animal's failure to operate for 10 s, despite receiving an electrical shock.

Obtaining tissue specimens

At the endpoint, the mice were subjected to euthanasia via inhalation of CO_2. Four muscles of the posterior limbs of each mouse, including the gastrocnemius (GA), tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (SOL), were removed, weighed, and stored at -80°C.

Histological analysis

Hematoxylin and eosin (H&E) kits (G1003, Servicebio, China) were selected to evaluate the

pathological changes in the GA tissues. The GA tissues were fixed using 10% formalin (E672001, Sangon, China). Subsequently, the specimens were then dehydrated before embedding in paraffin. The paraffin blocks were sectioned to a thickness of 10 μm. Following dewaxing, the sections underwent rehydration with decreasing concentrations of alcohol. After that, the sections were stained with hematoxylin for 5 min and differentiated with 1% hydrochloric acid for 30 s. After counterstaining with eosin for 3 min, the sections were dehydrated. Xylene was used as a transparent solvent, and neutral balsam (D054-1-1, Jiancheng, China) was selected to coverslip the sections. The morphology and cross-sectional area of the muscle fibers in the GA tissues were analyzed by two independent experts in a blinded fashion under an optical microscope (Eclipse E100, Nikon, Japan).

Transmission electron microscope (TEM)

In this study, GA tissues of 1×1 mm were fixed at 4°C for 4h with 2.5% glutaraldehyde solution. Following this, the GA tissue blocks were washed and subsequently dehydrated using a series of ethanol solutions, with concentrations ranging from 30% to 100%. The dehydrated tissues were immersed in an acetone solution (67-64-1, PEAKINFO, China) for 20 min. The soaked tissues were then transferred to a solution with a volume ratio of 1:1 between the embedding agent and acetone solution for further soaking for 1 h. After that, the tissues were transferred to a solution with a volume ratio of 3:1 between the embedding agent and acetone solution for soaking for 3h. Following this, the tissues were soaked overnight at room temperature with a pure embedding agent. The permeated samples were transferred to a dry centrifuge tube and then heated overnight at 70°C. The tissues were sliced with an ultrathin microtome (EM_UC7, Leica, Germany) with a section thickness of 50-70 nm. The sections were then stained for 10 min in a lead citrate and uranium dioxyacetate 50% saturated ethanol solution. The sections were scanned randomly at 10 different spots per sample at ×30.0 K magnification. The morphology of mitochondria in the stained sections of each group was monitored under a transmission electron microscope (TEM) (H7650, HITACHI, Japan). At the same time, the total number of mitochondria as well as the proportion and size of normal mitochondria were quantified using ImageJ software. Mitochondria with intact membranes and matrix as well as well-preserved cristae were considered normal (La Barbera et al., 2022; Lee et al., 2022). The proportion of normal mitochondria = the number of normal mitochondria/the total number of mitochondria.

Analysis of mitochondrial DNA (mtDNA) copy number

The total DNA from the GA tissues was isolated using the DNA Quick Extraction Kit (D0065S, Beyotime, China). Quantitative real-time polymerase chain reaction (QPCR) was performed to determine the relative mtDNA copy number utilizing the primer sequences listed in Table 1. The samples were analyzed in a final volume of 25 μL consisting of BeyoFast™ SYBR Green qPCR Mix (D7260, Beyotime, China) 0.2 μM forward and reverse primers and DNA template. The amplification reaction was conducted in an iCycler PCR System (Bio-Rad, Hercules, CA, USA). After the QRT-PCR assay, mtDNA copy numbers were calculated by normalizing to g*DNA* (*β-Globin*).

Detection of malondialdehyde (MDA) and protein carbonyl contents

MDA kits (A003-1-2) and protein carbonyl kits (A087-1-2) were introduced from Jiancheng (China). In this study, a certain mass of GA was weighed, and then nine times the volume of normal saline was added based on the ratio of mass (g)/volume (mL)=1/9. Next, the homogenate was ground with a low-temperature tissue grinder. After centrifugation at 2500 rpm for 10 min, the supernatant was obtained as a tissue homogenate supernatant. The concentrations of MDA and protein carbonyl were measured by enzyme-linked immunosorbent assay (ELISA), per the operating instructions.

Measurement of ROS

Fresh GA tissues from each group were collected and digested into single-cell suspensions. Then, an appropriate volume of diluted 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was added to the cell suspension to achieve a final concentration of 10 μmol/L. Following a 20-minute incubation at 37°C in a cell culture incubator, the cells were washed and the level of ROS was quantified using flow cytometry (NovoCyte, Agilent, USA).

qRT-PCR

Total RNA was extracted from GA tissues using Trizol reagent (R0016, Beyotime, China), and subsequently converted to cDNA using the cDNA First

Table 1. Primer sequences for analysis of mitochondrial DNA copy number.

Gene	Forward Primer	Reverse Primer
mtDNA	AAAGCCACCTTGACCCGATT	GATTCGTAGGGCCGCGATA
g*DNA* (*β-Globin*)	GTGCACCTGACTGATGCTGA	ACGATCATATTGCCCAGGAGC

Strand Synthesis Kit (D7168S, Beyotime, China). For quantification of mRNA expression, the cDNA was amplified with the use of BeyoFast™ SYBR Green qPCR Mix under the iCycler PCR System. The results were normalized to glyceraldehyde -3-phosphate dehydrogenase (*Gapdh*). The $2^{-\Delta\Delta Ct}$ was taken to calculate the mRNA level. The primers employed in this experiment are listed in Table 2.

Western blot

The GA tissues were homogenized and lysed in radioimmunoprecipitation assay (RIPA) buffer (P0013D, Beyotime, China) with phenylmethanesulfonyl fluoride (PMSF) (ST506, Beyotime, China). After centrifugation, the acquired supernatant was quantified using bicinchoninic acid (BCA) kits (pc0020, Solarbio, China). After denaturation, the protein was separated by electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes. Next, 5% bovine serum albumin (BSA, 4240GR100, BioFRoxx, Germany) was applied to seal the membranes at 37°C for 60 min. Subsequently, the sealed membranes were incubated with primary antibodies (4°C, overnight). The next day, the membranes were immersed in Anti-Rabbit IgG H&L (HRP) antibody (1:5000, ab7090, Abcam, UK) at 37°C for 1h. Visualization of protein was performed with enhanced chemiluminescence (ECL) reagents (GK10008, GlpBio, USA) under a gel imaging system (610020-9Q, Clinx, China). The primary antibodies of ATROGIN-1 (1:1000, DF7075), muscle RING-finger protein-1 (MURF-1, 1:2000, DF7187), cytochrome c oxidase subunit 4 (COX4, 1:2000, AF5468), cystatin C (CYTC, 1:2000, DF6457), nuclear respiratory factor 1 (NRF-1, 1:2000, AF5298), mitochondrial transcription factor A (TFAM, 1:1000, DF3232), p-P38 (1:2000, Ab178867), P38 (1:2000, Ab31828), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α, 1:1000, AF5395), and β-ACTIN (1:10000, 81115-1-RR) were introduced from Affinity (China).

Statistics

The data are reported as mean±standard deviation. The statistical analysis was implemented with Statistical Product and Service Solutions (SPSS) software (23.0, IBM, USA). One-way analysis of variance (ANOVA) with Tukey's test was exploited for multiple comparisons. The Kruskal-Wallis H test was employed for the measurement of data that did not conform to a normal distribution. $p<0.05$ was considered statistically significant.

Results

The effects of MEL on grip strength, treadmill tests, body weight, and muscle weight in middle-aged mice

The grip strength and treadmill tests exhibited that, relative to the young group, the grip strength of the fore/hind limbs, running time, and distance for the middle-aged group were lower ($p<0.01$). While the situations were partly reversed after MEL5 and MEL10 mediation (Table 3, $p<0.01$). Then, this study monitored the changes in body weight and muscle weight. The initial and final weights of the middle-aged group were higher, while the weights of the GA, TA, EDL, and SOL were lower than those of the young group ($p<0.01$). The

Table 2. Primer sequences for Quantitative real-time polymerase chain reaction.

Gene	Forward Primer	Reverse Primer
Mouse *Cox4*	ATTGGCAAGAGAGCCATTTCTAC	TGGGGAAAGCATAGTCTTCACT
Mouse *Cytc*	ACCCTGATGGAGTATTTG	GCTATTAGTCTGCCCTTTC
Mouse *Atrogin-1*	CAGCTTCGTGAGCGACCTC	GGCAGTCGAGAAGTCCAGTC
Mouse *Murf-1*	CCAGGCTGCGAATCCCTAC	ATTTTCTCGTCTTCGTGTTCCTT
Mouse *Nrf-1*	AGCACGGAGTGACCCAAAC	AGGATGTCCGAGTCATCATAAGA
Mouse *Tfam*	AACACCCAGATGCAAAACTTTCA	GACTTGGAGTTAGCTGCTCTTT
Mouse *P38*	TGACCCTTATGACCAGTCCTTT	GTCAGGCTCTTCCACTCATCTAT
Mouse *Pgc-1α*	TATGGAGTGACATAGAGTGTGCT	GTCGCTACACCACTTCAATCC
Mouse *Gapdh*	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

Table 3. The grip strength and treadmill tests of aged mice with melatonin treatment.

Groups	Grip strength of fore limbs (g/body weight)	Grip strength of hind limbs (g/body weight)	Distance (m)	Time (Second)
Young	3.58±0.37	9.05±0.52	522.64±29.26	1981.76±84.23
Middle aged	2.13±0.2**	5±0.35**	306.84±22.31**	1487.36±87.65**
Middle aged+MEL5	2.55±0.15++	5.93±0.4++	448.9±33.98++	1750.85±220.37++
Middle aged+MEL10	2.68±0.25++	6.12±0.39++	428.24±28.89++	1890.52±308.27++

Data are expressed as mean ±SD, $n=6$; Compared to the Young group, *$p<0.05$, **$p<0.01$; compared to the middle aged group, +$p<0.05$, ++$P<0.01$.

introduction of MEL5 and MEL10 effectively enhanced the weights of the GA, TA, EDL, and SOL of middle-aged mice (Table 4, $p<0.05$).

MEL alleviated the histological damage and increased the cross-sectional area of the muscle fibers in GA tissues in middle-aged mice

In this study, it was observed that mice in the young group exhibited neatly arranged myofibrils, clear nuclei, uniform cytoplasmic staining, and moderate muscle fiber space. In contrast, mice in the middle-aged group displayed a large number of GA muscle fibers that were broken and the sarcomere structure was disordered. The muscle fibers in the middle-aged+MEL5 group and middle-aged+MEL10 group were broken and fragmented, however, the pathological damage was clearly improved and the muscle fibers appeared fuller

Table 4. The body weight and muscle weight changes of aged mice with melatonin treatment.

Groups	Young	Middle aged	Middle aged+MEL5	Middle aged+MEL10
Body weight (g)				
Initial	21.91±1.37	33.62±0.94**	32.42±1.44	33.34±0.96
Final	22.62±1.47	35.07±1.68**	33.52±1.59	34.29±1.39
Muscle weight (mg/g)				
Gastrocnemus	10.07±0.48	6.87±0.58**	7.68±0.52+	7.98±0.50+
Tibialis antenor	4.35±0.24	2.77±0.20**	3.01±0.17+	3.2±0.20++
Extensor digtorum longus	1.81±0.13	1.03±0.06**	1.34±0.12++	1.49±0.18++
Soleus	0.79±0.07	0.51±0.07**	0.69±0.06++	0.72±0.05++

Data are expressed as mean ±SD, n=6; Compared to the Young group, *$p<0.05$, **$p<0.01$; compared to the middle aged group, +$p<0.05$, ++$p<0.01$.

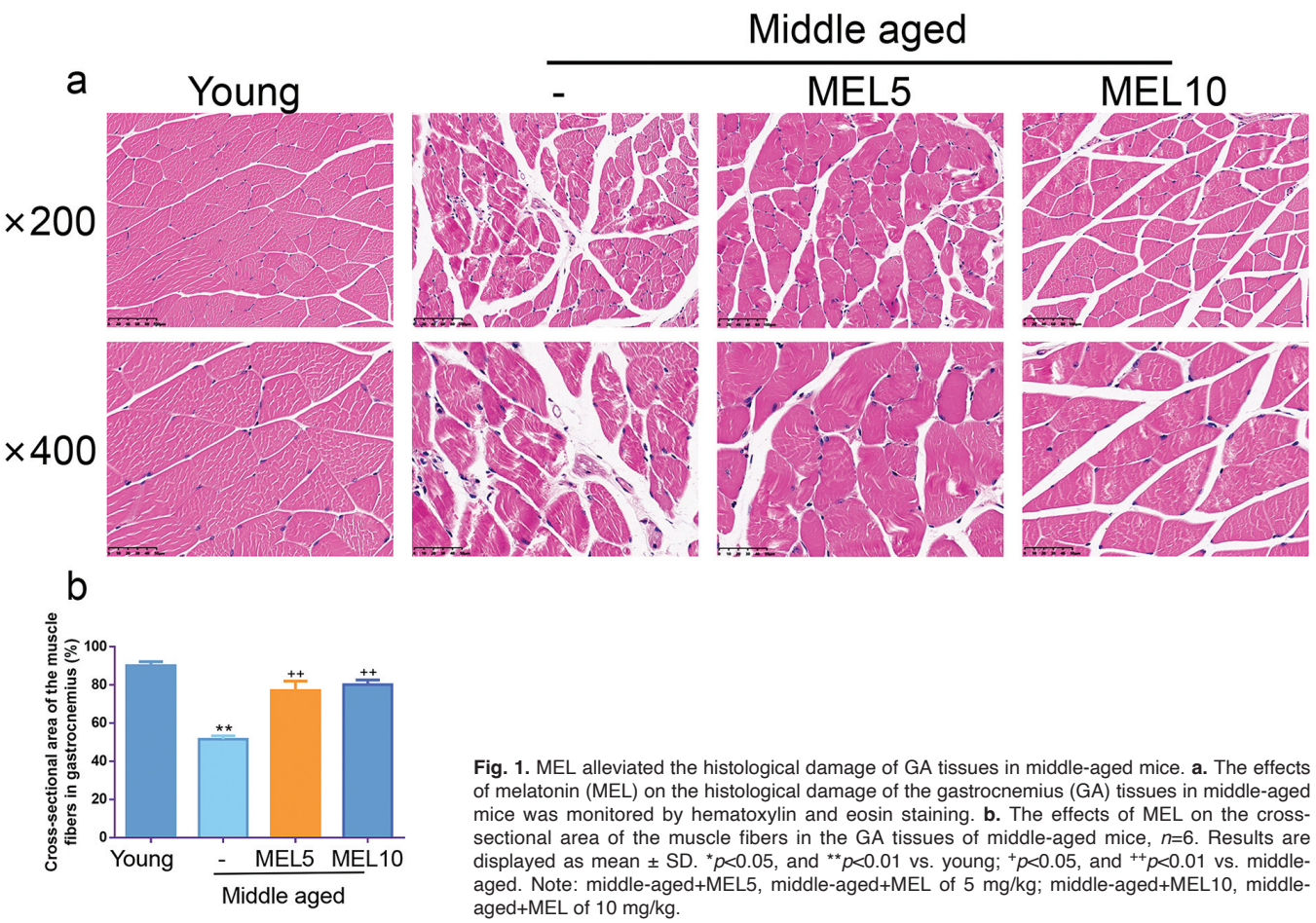

Fig. 1. MEL alleviated the histological damage of GA tissues in middle-aged mice. **a.** The effects of melatonin (MEL) on the histological damage of the gastrocnemius (GA) tissues in middle-aged mice was monitored by hematoxylin and eosin staining. **b.** The effects of MEL on the cross-sectional area of the muscle fibers in the GA tissues of middle-aged mice, $n=6$. Results are displayed as mean ± SD. *$p<0.05$, and **$p<0.01$ vs. young; +$p<0.05$, and ++$p<0.01$ vs. middle-aged. Note: middle-aged+MEL5, middle-aged+MEL of 5 mg/kg; middle-aged+MEL10, middle-aged+MEL of 10 mg/kg.

than those observed in the middle-aged group (Fig. 1a). In addition, in comparison with the young group, the cross-sectional area of the muscle fibers in the GA tissues of middle-aged mice was significantly reduced. However, after treatment with 5 mg/kg and 10 mg/kg of MEL, the cross-sectional area of the muscle fibers in the GA tissues was effectively increased (p<0.01, Fig. 1b).

The effects of MEL on the number and size of normal mitochondria of the GA tissues of middle-aged mice

The TEM analysis showed that the myofibrils of the GA tissues in the young group were clear and mitochondria were arranged into a beaded shape. In contrast, the myofibrils of the GA tissues of the middle-aged group were blurred, and disordered, the stripes were not clear, and the mitochondria were deformed and broken. The pathological damage of mitochondria in the GA tissues of the middle-aged+MEL5 and middle-aged+MEL10 groups was found to be less severe than that observed in the middle-aged group (Fig. 2a). Furthermore, we also demonstrated that the total number of mitochondria as well as the proportion and size of normal mitochondria in the middle-aged group was largely lessened when compared with the young group (p<0.01). Nevertheless, MEL at both 5 mg/kg and 10 mg/kg prominently increased the total number of mitochondria as well as the proportion and size of the normal mitochondria in middle-aged mice (Fig. 2b-2d, p<0.01). Furthermore, the mtDNA copy number in the GA tissues of middle-aged mice was lower than that of the young group, while MEL5 and MEL10 were observed to significantly increase the mtDNA copy number in the GA tissues of middle-aged mice (Fig. 2e, p<0.01).

The effects of MEL on oxidative stress and muscle function-related protein expression in GA tissues of middle-aged mice

The results of Figures 3a,b illuminated that the contents of MDA, protein carbonyl, and ROS in the GA tissues of middle-aged mice were significantly increased, while MEL at 5 mg/kg and 10 mg/kg

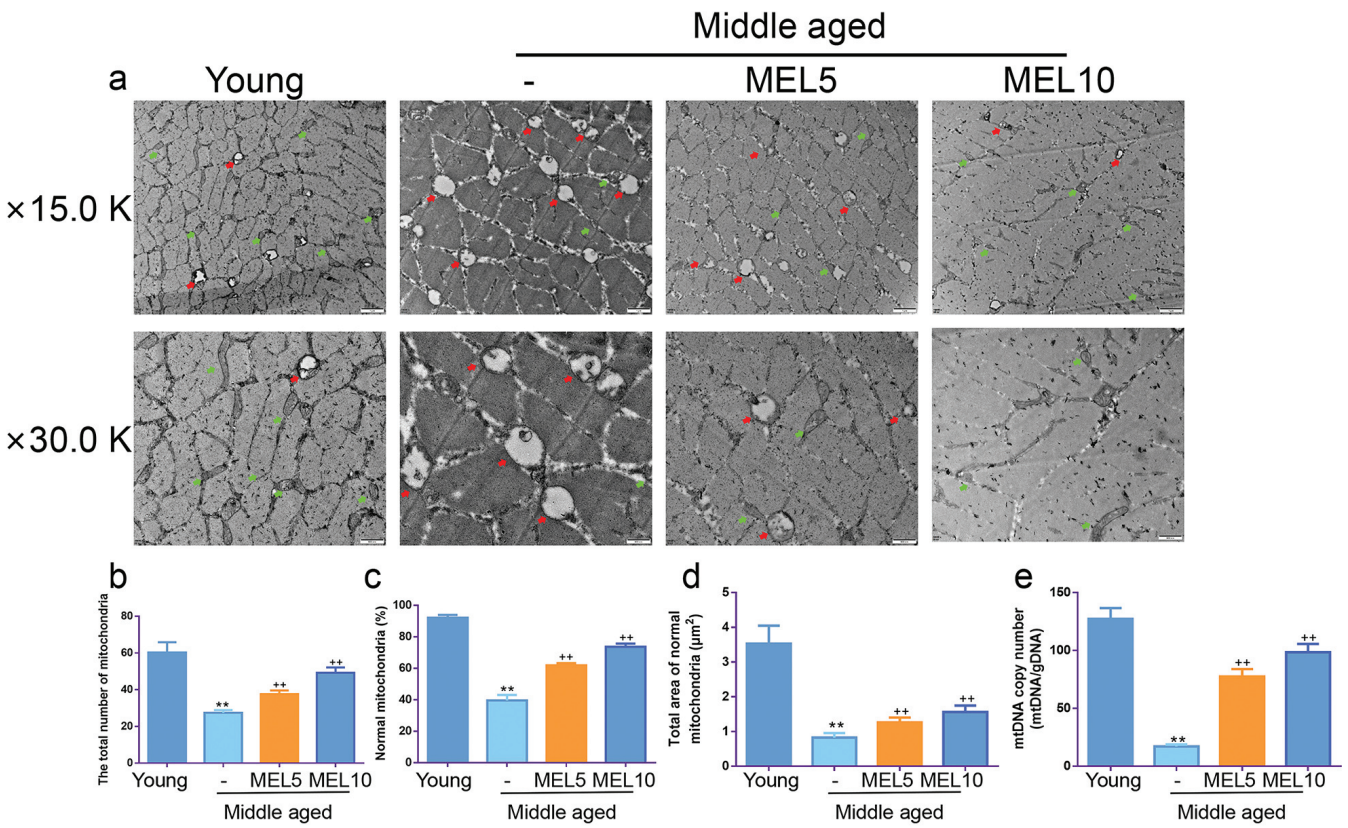

Fig. 2. The effect of MEL on the number of mitochondria of the GA tissues in middle-aged mice. **a.** Transmission electron microscope (TEM) images of GA tissues. **b.** The measure of the total number of mitochondria in the TEM image, n=6. **c.** Quantitative results of percentage of normal mitochondria in the TEM image, n=6. **d.** Analysis of normal mitochondrial size in the GA tissues, n=6. **e.** Detection of mitochondrial DNA copy number in the GA tissues, n=3. Green arrows indicate normal mitochondria and red arrows indicate damaged mitochondria. Results are manifested as mean ± SD. $^{*}p$<0.05, and $^{**}p$<0.01 vs. young; ^{+}p<0.05, and ^{++}p<0.01 vs. middle-aged. Note: middle-aged+MEL5, middle-aged+MEL of 5 mg/kg; middle-aged+MEL10, middle-aged+MEL of 10 mg/kg.

effectively decreased MDA, protein carbonyl and ROS levels in the GA tissues of middle-aged mice ($p<0.05$). The expression of ATROGIN-1 and MURF-1, markers of skeletal muscle atrophy, were notably upregulated in the GA tissues of middle-aged mice ($p<0.01$). The addition of MEL (5 and 10 mg/kg) strongly repressed the expression of ATROGIN-1 and MURF-1 in the GA tissues of middle-aged mice (Fig. 3c,d, $p<0.05$).

The effects of MEL on the PGC-1α/TFAM pathway in GA tissues of middle-aged mice

Next, western blot and qRT-PCR analysis were performed to detect PGC-1α/TFAM pathway-related protein and mRNA levels, including COX4, CYTC, NRF-1, TFAM, p-P38, P38, and PGC-1α. A decrease in the expression of COX4, CYTC, NRF-1, TFAM, and PGC-1α was observed in middle-aged mice, while the ratio of p-P38/P38 presented an increase ($p<0.01$). MEL (5 and 10 mg/kg) was found to elevate the expression of COX4, CYTC, NRF-1, TFAM, and PGC-1α but weaken the ratio of p-P38/P38 of middle-aged mice at both the protein and mRNA levels (Fig. 4a-c, $p<0.05$).

Discussion

The maintenance of skeletal muscle quality is important in the prevention of age-related metabolic

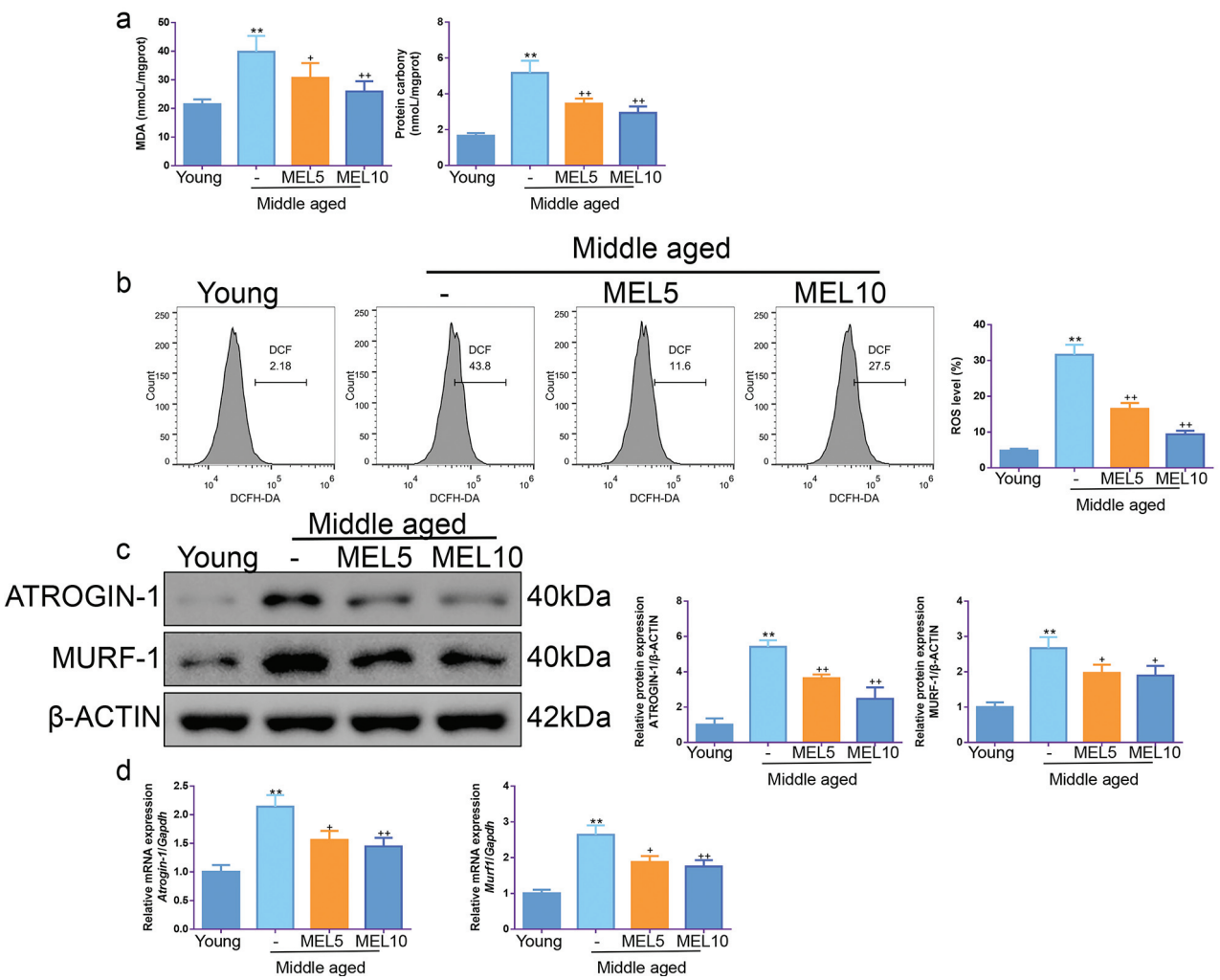

Fig. 3. The effect of MEL on oxidative stress and muscle function-related protein expression in the GA tissues of middle-aged mice. **a.** The effects of MEL on malondialdehyde (MDA) and protein carbonyl in the GA tissues of middle-aged mice were assessed by ELISA, $n=6$. **b.** The effects of MEL on reactive oxygen species (ROS) in the GA tissues of middle-aged mice were assessed by flow cytometry, $n=3$. **c, d.** The effects of MEL on the protein and mRNA levels of ATROGIN-1 and muscle RING-finger protein-1 (MURF-1), markers of skeletal muscle atrophy, in the GA tissues of middle-aged mice, were assessed by western blot and quantitative real-time polymerase chain reaction (qRT-PCR), $n=3$. Results are presented as mean ± SD. $^{*}p<0.05$, and $^{**}p<0.01$ vs. young; $^{+}p<0.05$, and $^{++}p<0.01$ vs. middle-aged. Note: middle-aged+MEL5, middle-aged+MEL of 5 mg/kg; middle-aged+MEL10, middle-aged+MEL of 10 mg/kg.

decline and physical weakness. Endocrine system changes are a major contributing factor to sarcopenia. MEL is an endocrine substance secreted by the pineal gland, which shows no harmful adverse effects in humans (Kleszczyński et al., 2020). MEL secretion levels tend to decline with age, which will influence muscle mass and strength (Jin et al., 2021). Furthermore, MEL has been described as a potential therapeutic agent for sarcopenia, with the ability to improve circadian rhythm, which is disrupted with age, and influence the expressions of peripheral clock genes (Fernández-Martínez et al., 2023). MEL has been successfully applied in preclinical mouse models and Duchenne muscle dystrophy (DMD) patients, improving muscle metabolism and strength (Hibaoui et al., 2011; McCormick and Vasilaki, 2018). In our research, by conducting grip strength and treadmill tests, MEL was found to enhance the grip strength, running time, and distance of middle-aged mice. Also, MEL effectively elevated the weights of the GA, TA, EDL, and SOL of middle-aged mice. The above results demonstrated that MEL could alleviate the loss of skeletal muscle mass.

The present evidence indicates that mitochondrial dysfunction is the central mechanism of sarcopenia (Buch et al., 2020). Enlarged mitochondria are frequently observed in aged skeletal muscle, which is characterized by highly interconnected networks, morphological abnormalities, and decreased bioenergy synthesis efficiency. This may be attributed to increased mitochondrial fusion and/or decreased mitochondrial division and decreased mitochondrial autophagy energy (Tandler and Hoppel, 1986; Yoon et al., 2006). A research report revealed that aging reduces mitochondrial size and results in poor connections between mitochondria (Vue et al., 2023). The data obtained from TEM confirmed that the myofibrils of the GA tissues in middle-aged mice were blurred, and disordered, and the stripes were not clear. Additionally, the mitochondria were observed to be deformed and broken in the GA tissues of middle-aged mice. These findings were indicators of aging and supported other reports that normal mitochondrial function is lost with age (Shigenaga et al., 1994; Rodríguez et al., 2007, 2008). After MEL mediation, the total number of mitochondria, the percentage and size of normal mitochondria as well as mtDNA copy number in middle-aged mice were increased, indicating that MEL improved the age-dependent mitochondrial changes in middle-aged mice.

The increase in oxidative stress that occurs during

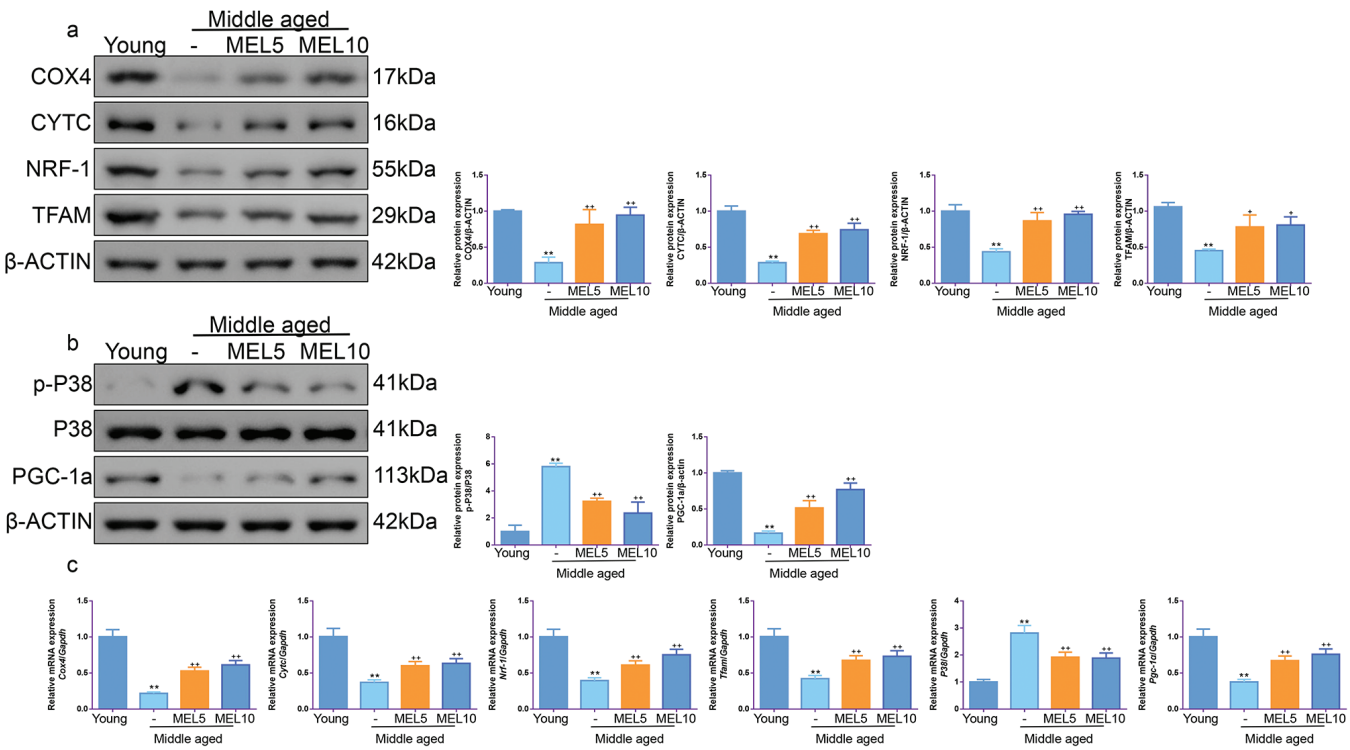

Fig. 4. The effects of MEL on the PGC-1α/TFAM pathway in GA tissues of middle-aged mice. **a-c.** Western blot and qRT-PCR were applied to detect the effects of MEL on PGC-1α/TFAM pathway-related protein and mRNA levels, including cytochrome c oxidase subunit 4 (COX4), cystatin C (CYTC), nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (TFAM), p-P38, P38, and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), n=3. Data are manifested as mean ± SD $^{*}p<0.05$, and $^{**}p<0.01$ vs. young; $^{+}p<0.05$, and $^{++}p<0.01$ vs. middle-aged. Note: middle-aged+MEL5, middle-aged+MEL of 5 mg/kg; middle-aged+MEL10, middle-aged+MEL of 10 mg/kg.

aging directly or indirectly leads to muscle fiber atrophy (Hur et al., 2024). The relationship between oxidative stress and sarcopenia has been experimentally identified (Cai and Dong, 2024). The increase of oxidative stress in sarcopenia is primarily attributable to mitochondrial homeostasis imbalance (Song et al., 2024). The strong antioxidant effect of MEL effectively protects mtDNA from oxidative damage (Gu et al., 2024). A substantial body of evidence has unveiled that MEL can improve muscle function in athletes, restrain oxidative stress and inflammatory response, and restore some damaged mitochondria (Escames et al., 2003, 2006; Leonardo-Mendonça et al., 2015, 2017). By detecting oxidative stress-related factors, we found that MEL effectively reduced the contents of MDA, protein carbonyl, and ROS in the GA tissues of middle-aged mice. Adenosine 5‘-monophosphate (AMP)-activated protein kinase (AMPK) is a sensor of the cellular energy state and a key regulator of skeletal muscle mitochondrial function and oxidative stress (Lin et al., 2022). PGC-1α and AMPK play an important role in maintaining intracellular redox balance (Wu et al., 2021). PGC-1α modulates mitochondrial biogenesis and is associated with several processes related to energy metabolism (Kuang et al., 2024). As part of this relationship, PGC-1α cooperates with NRF-1 to activate TFAM protein expression (Lou et al., 2024). TFAM is essential for the maintenance, expression, and delivery of mtDNA and can regulate the replication and transcription of mtDNA (Song et al., 2023). In the skeletal muscle of rats, PGC-1α protein expression is highly correlated with the content of mitochondria in the muscle and its aerobic metabolic capacity, and the expression of PGC-1α protein is higher in muscles with more mitochondria and stronger aerobic metabolic capacity (Scarpulla, 2011). PGC-1α overexpression has been demonstrated to counteract muscle atrophy resulting from denervation and fasting by reducing the mRNA expression levels of atrophy-related factors, *Atrogin-1* and *Murf-1* (Geng et al., 2010). In skeletal muscle atrophy caused by various diseases, ATROGIN-1 and MURF-1 are specifically expressed in skeletal muscle, and play a role in connecting muscle cells through ubiquitin, which is crucial for protein degradation in muscle cells (Doucet et al., 2007; Frost et al., 2007). Yu et al. reported that MEL protects mitochondrial function via the repression of mitochondrial oxidative stress, thereby alleviating myocardial ischemia/reperfusion damage in type 1 diabetic rats, and its mechanism is associated with the modulation of the AMPK-PGC1α-Sirtuin 3 (SIRT3) signal (Yu et al., 2017). In dental papilla cells, MEL enhances the expression of PGC-1α, NRF-1, and TFAM (Jiang et al., 2019). Our study examined the effects of MEL on the expression of mitochondrial function- and muscle atrophy-related factors and pathways mentioned above in sarcopenia. MEL was found to elevate the expression of COX4, CYTC, NRF-1, TFAM, and PGC-1α but weaken the that of ATROGIN-1 and MURF-1 and the p-P38/P38 ratio of middle-aged mice, both at protein and mRNA levels. This indicates that MEL prevented sarcopenia in middle-aged mice, and the mechanism may relate to mitochondria-induced oxidative stress and the PGC-1α/TFAM pathway. In the future, these pivotal findings of the study will be further supported through clinical trials, thereby providing novel insights into the treatment of age-related sarcopenia.

Conclusion

To sum up, our findings suggested that MEL protected against sarcopenia in middle-aged mice, and the underlying mechanism may relate to mitochondria-induced oxidative stress and the PGC-1α/TFAM pathway. These results highlighted that MEL might have potential for the prevention and treatment of sarcopenia.

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