Resveratrol treatment reduces the appearance of tubular aggregates and improves the resistance to fatigue in aging mice skeletal muscles

Luana Toniolo[a,c], Pina Fusco[b], Luca Formoso[b], Alessandra Mazzi[b], Marta Canato[a], Carlo Reggiani[a,c], Emiliana Giacomello[b,c,*,1]

[a] Laboratory of Muscle Biophysics, Department of Biomedical Sciences, University of Padova, Padova, Italy
[b] Department of Molecular and Developmental Medicine, University of Siena, Italy
[c] Interuniversity Institute of Myology, Italy

ARTICLE INFO

Section Editor: Christiaan Leeuwenburgh

Keywords:
Resveratrol
Aging
Skeletal muscle
Tubular aggregates

ABSTRACT

Resveratrol (RES) is a polyphenolic compound found in grapes, peanuts, and in some berries. RES has been reported to exhibit antioxidant, anti-inflammatory, anti-proliferative properties, and to target mitochondrial-related pathways in mammalian cells and animal models. Therefore, RES is currently advised as supplement in the diet of elderly individuals. Although it is hypothesized that some of RES beneficial actions likely arise from its action on the skeletal muscle, the investigation of RES effects on this tissue remains still elusive. This study reports the effects of a 0,04% RES-supplemented diet for six months, on the skeletal muscle properties of C57/BL6 aging mice. The analysis of the morphology, protein expression, and functional-mechanical properties of selected skeletal muscles in treated compared to control mice, revealed that treated animals presented less tubular aggregates and a better resistance to fatigue in an ex-vivo contraction test, suggesting RES as a good candidate to reduce age-related alterations in muscle.

1. Introduction

One of the most serious consequences of aging is its effect on skeletal muscle (Rosenberg, 1997). In fact, although aging affects many other tissues and organs, loss of muscle mass together with its impaired metabolic properties (Conley et al., 2000; Hollmann et al., 2007; Chabi et al., 2008) may have a negative impact on whole-body metabolism.

Muscle aging, is a multifactorial phenomenon also referred to as 'sarcopenia', characterized by a significant decline in muscular function and performance. Sarcopenia consists of a slow but progressive loss of muscle mass with advancing age, which results from a variety of both quantitative and qualitative changes, such as the progressive denervation, alterations in the excitation-contraction coupling (Ryall et al., 2008; Boncompagni et al., 2006; Marzetti and Leeuwenburgh, 2006), decrease in myofiber cross-sectional area (Murgia et al., 2017), loss of muscle fibers, and changes in fiber type (Murgia et al., 2017), with a progressive reduction of fast in favour of slow fibers (Murgia et al., 2017; Cristea et al., 2010).

One morphological characteristic that in recent years is attracting attention is the presence of tubular aggregates (TAs), which are accumulations of densely packed tubules arising from sarcoplasmic reticulum of striated muscles (Engel, 1964). In humans, TAs develop mainly in type II fibers (Engel et al., 1970), are associated with a wide variety of muscle disorders (Schiaffino, 2012) but also present in asymptomatic probands (Engel et al., 1970). Contrarily to what observed in humans, TAs in skeletal muscles of inbred strains mice are restricted to type IIB fibers, related to sex and age (Agbulut et al., 2000; Chevessier et al., 2004) and also in a variety of congenital myopathies (Schiaffino, 2012; Giacomello et al., 2015). Although the mechanisms responsible of their onset remain still unclear, it has been recently proposed that they arise from an altered proteostasis mechanism (Schiaffino, 2012), and from mitochondrial dysfunction (Vielhaber et al., 2001).

Overall, during aging skeletal muscle undergoes an exstensive modification of both morphological and biochemical profiles. Associated to these effects, a 'metabolic dysregulation' is also observed, with reduced sensitivity to insulin, impaired oxidative defence, and decreased mitochondrial function (Carter et al., 2015). In humans, insulin resistance has been associated with a misregulation of the ratio of oxidative type I to glycolytic type II muscle fibers, and decreased expression of genes involved in the regulation of mitochondrial activity (Hoeks and Schrauwen, 2012).

* Corresponding author at: Department of Medicine, Surgery and Health Sciences, University of Trieste, Italy.
E-mail address: egiacomello@units.it (E. Giacomello).
[1] Current address: Department of Medicine, Surgery and Health Sciences, University of Trieste, Italy.

Accepted 18 July 2018

In recent years, it has been proposed (Lopez-Lluch and Navas, 2016) that dietary restriction alleviates many of the age-related diseases, improves stress-resistance, and decelerates the functional decline in elderly individuals. As a consequence, the attention has been focused on the study of "dietary-restriction mimetic" compounds able to provide benefits similar to dietary restriction without a reduction of the caloric intake (Chung et al., 2012).

In this context, Resveratrol (3,5,49-trihydroxystilbene), a bioactive phenol found in grapevine, peanuts, and in some berries, has been described to be able to mimic dietary restriction, to extend the lifespan of diverse species from yeast to mammals (Baur et al., 2006; Narkar et al., 2008; Feige and Auwerx, 2008; Pearson et al., 2008), and most importantly, to counteract the effects of physical inactivity and aging by activating the protein deacetylase sirtuin-1 (Sirt1) and increasing PGC 1-alpha activity (Feige et al., 2008; Lagouge et al., 2006; Rodriguez-Bies et al., 2016). Moreover, RES has been described to alter the progression of type II diabetes, by enhancing insulin sensitivity in the skeletal muscle of aging mice (Baur et al., 2006; Lagouge et al., 2006).

Even though muscle specific effects of RES have been proposed as responsible of overall effects on the modulation of insulin sensitivity and resistance to obesity (Pearson et al., 2008; Lagouge et al., 2006), the investigation of its impact on skeletal muscle remains elusive. Recent reports on the effect of this molecule after long-term supplementation in mice show that RES exerts specific actions on the skeletal muscle tissue. Interestingly, RES has been proposed to reprogram muscle gene expression (Lagouge et al., 2006), improve the aerobic capacity of muscle (Lagouge et al., 2006), and counteract the high fat induced Myosin Heavy Chain (MyHC) switch (Hyatt et al., 2016). As a global effect, RES acts positively by improving resistance to fatigue (Rodriguez-Bies et al., 2016; Wu et al., 2013), exercise performance (Wu et al., 2013), biochemical profiles, and pathological responses in aging models (Murase et al., 2009). Although it results difficult to have a full picture of RES effects and potentialities, due to protocols that propose a wide range of doses and are mainly performed in short term, altogether these observations provide to RES the capability to moderate the age-related decline in physical performance.

Aimed at gathering new insights on the effects provided by RES treatment during aging, our experimental plan envisaged a 6-month supplementation of 0,04% RES in the diet of 12 months old C57/BL6 mice. According to previous studies (Dutta and Sengupta, 2016), the mice age at the beginning of treatments corresponds to middle aged individuals, approximately to 55 years old humans, and at the end of the treatment, 18 months, corresponds approximately to 65 years old humans. We analyzed the morphological properties, the modulation of protein expression, and the functional-mechanical characteristics of selected skeletal muscles in treated mice compared to a control group fed with a standard diet. Our data show that RES reduces the appearance of tubular aggregates (TAs), counteracts the MyHC switch and improves the resistance to fatigue in an ex vivo test. Taken together, our results further confirm RES as a supplement to the diet useful for reducing some of the age-related alterations to skeletal muscle tissue.

2. Materials and methods

2.1. Animals and treatment

The experimental procedures used throughout all the experiment were in accordance with the European legislation on the use and care of laboratory animals (EU Directive 2010/63) and were approved by the Ethics Committee of the University of Siena and from Ministero della Salute, Italy. Animals were fed a chow diet and distilled water ad libitum, and were maintained on a regular cycle (12-h light/dark) at room temperature. C57/BL6 mice aged 12 months have been randomly divided in two groups: one group fed for 6 months with a standard diet supplemented with 0,04% RES as previously reported by Baur et al. (2006), and a second group of mice fed with a standard diet. The reported results are the product of three independent experiments involving 15 animals per group for a total of 90 animals. In each experiment, after the 6-month treatment period, control and treated mice were subdivided in 3 groups. One group of mice was sacrificed and the tibialis anterior and gastrocnemius muscles were harvested for protein expression, histological and immunofluorescence analyses. One group was dedicated to the ex vivo analyses of Exstensor Digitorum Longus (EDL) and soleus muscle contractile performance, and a third group was subjected to a treadmill endurance test.

2.2. Antibodies

Primary antibodies used in the present work are listed in Table S1.

2.3. Cryostat sectioning

Tibialis anterior muscles were dissected from mice, directly frozen in isopentane cooled in liquid nitrogen, and cryoprotected with Tissue-Tek II OCT compound (Miles Inc., USA). Transverse sections, 8 μm thick, were cut with a Leica cryostat (CM 1850, Leica Microsystem, Austria).

2.4. Immunofluorescence staining

Sections were fixed with 3% para-formaldehyde, blocked with 0.2% BSA and 5% goat serum in PBS to avoid non-specific binding of the antibodies, and incubated with primary antibodies overnight in a humidified chamber at 4 °C. The sections were extensively washed with PBS-BSA 0.2% and incubated with antimouse and antirabbit Cy2 or Cy3 conjugated secondary antibodies (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature, washed with PBS-BSA 0.2%. Sarcolemma staining was performed with Wheat Germ Agglutinin (WGA) labeled with AlexaFluor-488 (1:500; Life Technologies), in PBS-BSA 0.2% for 30 min at room temperature and extensively washed. Samples were mounted with Mowiol (Sigma Aldrich, Italy) added with 0.025% DABCO (Sigma Aldrich, Italy) as antifading agent. The specimens were analyzed with a confocal microscope (LSM510, Zeiss, Jena, Germany).

2.5. Succinate dehydrogenase (SDH) stain

Slides were directly placed in freshly prepared SDH solution (Cobalt Chloride 2%, Natrium Succinate 0.2 M, MTT Thiazolyl Blue 1 mg/ml in Tris-HCl buffer 0.2 M, pH 7.4) from −80 °C and let to incubate 15–20 min. The enzymatic reaction was stopped by quickly dipping the slides in ddH_2O, the slides where air-dried in dark and mounted. Images were acquired with an Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with a Micro-MAX digital CCD camera (Princeton Instruments, Trenton, USA). SDH activity was quantified with ImageJ free software (http://imagej.nih.gov/ij/) by converting images to greyscale and the grey intensity was calculated for each fiber.

2.6. Toluidine blue stain

Tissue sections were incubated for 2 min in 0.1% toluidine Blue solution, extensively rinsed in distilled water and then dehydrated with 95% and absolute ethanol alcohol, followed with two changes of xylene and mounted. Images were acquired with an Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with a Micro-MAX digital CCD camera (Princeton Instruments, Trenton, USA). Cross sectional area (CSA) of fibers was determined with ImageJ software from the photographs of the sections stained with Toluidine Blue or from images used to determine SDH activity, depending on the experiment.

2.7. SDS PAGE and immunoblotting analyses

The electrophoretic determination of MyHC isoforms composition was performed by solubilizing gastrocnemius muscles in Laemmli solution (62.5 mMTris, pH 6.8, 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol, with 0.1% E-64 and 0.1% leupeptin as anti-proteolytic agents). The soluble protein concentration was measured by Follin-Lowry method. Appropriate amounts of the protein suspension (about 5 µg of total protein/lane) were loaded onto polyacrylamide gels. The separation of MyHC isoforms was achieved on 8% polyacrylamide slab gels with a protocol derived from Talmadge and Roy (1993) with some modifications. Electrophoretic run was performed in Minigel sized apparatuses (Mini Protean III, Bio-Rad Laboratories, Hercules, CA) at 4 °C, for 1 h at 50 V and at 70 V for 40 h in total. Gels were stained with Coomassie Blue for the quantitative determination of the relative amount of the MyHC isoforms. After image acquisition, gels were analyzed using the program Image Studio Lite (LI-COR). The relative proportion of MyHC isoforms was calculated by determining the intensity of each band after automatic subtraction of local background.

For western blot analysis, gastrocnemius samples (about 30–40 µg of total protein/lane) were separated on 12% SDS-PAGE gels, and, were electrophoretically transferred onto nitrocellulose membranes (Amersham Hybond ECL, GE-Healthcare). The transferred proteins were visualized with Ponceau S and membranes were scanned to calculate the relative protein content. Membranes were then blocked for 1 h in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4) supplemented with 5% non-fat dry milk and incubated for 1 h with specific primary antibodies at room temperature. After extensive washings, membranes were incubated for 1 h at room temperature with the secondary antibodies conjugated with horseradish peroxidase (GE-Healthcare, Little Chalfont, UK), and immunodetection was performed with a chemiluminescence kit (ECL kit, Amersham Biosciences, Uppsala, Sweden). Immunoreactivity was analyzed by means of the C-DiGit® Blot Scanner and analyzed using the program Image Studio Lite (LI-COR). For quantifying the intensities of immunoreactive bands at least three independent experiments were analyzed. Sample loading was normalized by staining membranes with Ponceau S (Fortes et al., 2016). Differences between treated and control samples are reported as percentage change.

Western blot analysis of Slow and Fast myosin immunoreactivity was performed by using photographic films. ImageJ software was used for quantifying the intensities of immunoreactive bands from three independent experiments. Sample loading was normalized with the immunoreactive band detected with the anti-alpha-Actinin antibody. The differences between treated and control samples are reported as percentage change.

2.8. Analysis of ex vivo muscle contractile performance

Exstensor digitorum longus (EDL) and Soleus muscles were dissected from treated and non-treated mice in warm oxygenated Krebs solution (Sigma Aldrich, Italy) and were securely tied at the tendon insertion. Muscles were mounted in a myograph (Muscle Tester System, SI, Heidelberg, Germany) equipped with a force transducer (SI H KG7B, SI, Heidelberg, Germany) and a micro-manipulator-controlled little rod in a small chamber filled with a circulating oxygenated Krebs solution. Temperature was kept constant at 25 °C. For optimizing the stimulation conditions muscle length was increased until force development during tetanus was maximal. The responses to a single stimulus (twitch) or to a series of stimuli at various rates producing unfused or fused tetani were recorded. Time-to-peak tension, time-to-half relaxation, twitch tension, maximum tetanic tension values were measured.

Fatigue was recorded with 2-min trains (100-Hz tetani for EDL and 80 Hz for soleus) under isometric conditions and expressed as the percentage of force remaining after 120 s compared with the first pulse. Data reported are the means obtained from three to four mice per group for each of the three distinct treatments performed.

2.9. Treadmill endurance test

Endurance was measured with a test to exhaustion on the treadmill (Treadmill Control LE8700, Panlab s.l., Spain) equipped with a shock source. Control and treated mice were treadmill exercised with a flat rolling belt instrument for 3 days. In the first day, animals were placed into the testing chamber and the treadmill was turned on at the initial speed of 5 cm/s and then gradually increased to 40 cm/s the third day when time to exhaustion was recorded. Data reported are the means obtained from eight mice per group.

2.10. Statistical analyses

Student's *t*-test and F-test were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com). Data are expressed as means ± SD.

3. Results

3.1. General health conditions upon RES supplementation

The present study reports data from three independent experiments where, C57/BL6 mice aged 12 months have been randomly divided in two groups: one group fed ad libitum for 6 months with RES 0,04% supplied in the diet, and a second group fed with a standard diet. During the treatment mice were weighted monthly, and the final body weight difference was calculated and plotted in graph of Fig. 1A. No significant changes in the body weight variation have been observed between treated and non-treated groups.

Exstensor digitorum longus (EDL), representative of fast muscles, and soleus representative of slow muscles, were dissected from treated and non-treated mice and were weighted in order to understand whether RES supplementation could induce muscle mass changes. As shown in Fig. 1B, no significant difference was observed in treated compared to untreated animals.

Aiming at verifying whether the food intake of animals was similar in the two groups, we measured the amount of food eaten by each animal for one week, repeatedly during the treatments. Control and treated animals did not eat significantly different amounts of food. The food intake was 3,5 ± 1,2 and 4 ± 1,4 per mouse per day respectively (Fig. 1C). This amount corresponds to approximately 1,6 mg of RES per mouse per day. Considering that the mean weight was around 30 g, the dose corresponded to 50–55 mg/kg RES per day.

During the three experiments 5 control mice and 3 treated mice died, and the mean survival rate in the two groups was not different (Fig. 1D) in the time interval considered (18 months total). It can be therefore concluded that the treatment with RES does not cause major differences in the considered parameters. However, we cannot exclude that further differences could be detected in a more prolonged experimental plan.

3.2. RES treatment and skeletal muscle performance

To verify the impact of RES on skeletal muscle performance, we investigated the contractile properties of EDL and soleus muscles in an ex-vivo test from 12 control and 12 treated mice. Twitch and tetanus tensions, time to peak, half-relaxation time, and resistance to fatigue were measured. No significant difference was found in EDL and soleus muscles in twitch (Fig. 2A) and tetanus tensions (Fig. 2B), time to peak (Fig. 2C), and half-relaxation time (Fig. 2D) parameters. Interestingly, the resistance to fatigue was significantly increased in RES treated EDL and soleus muscles (Fig. 2E). When the endurance of mice was analyzed in a treadmill test, where animals were left running until exhaustion, RES treated mice did not show significant differences compared to

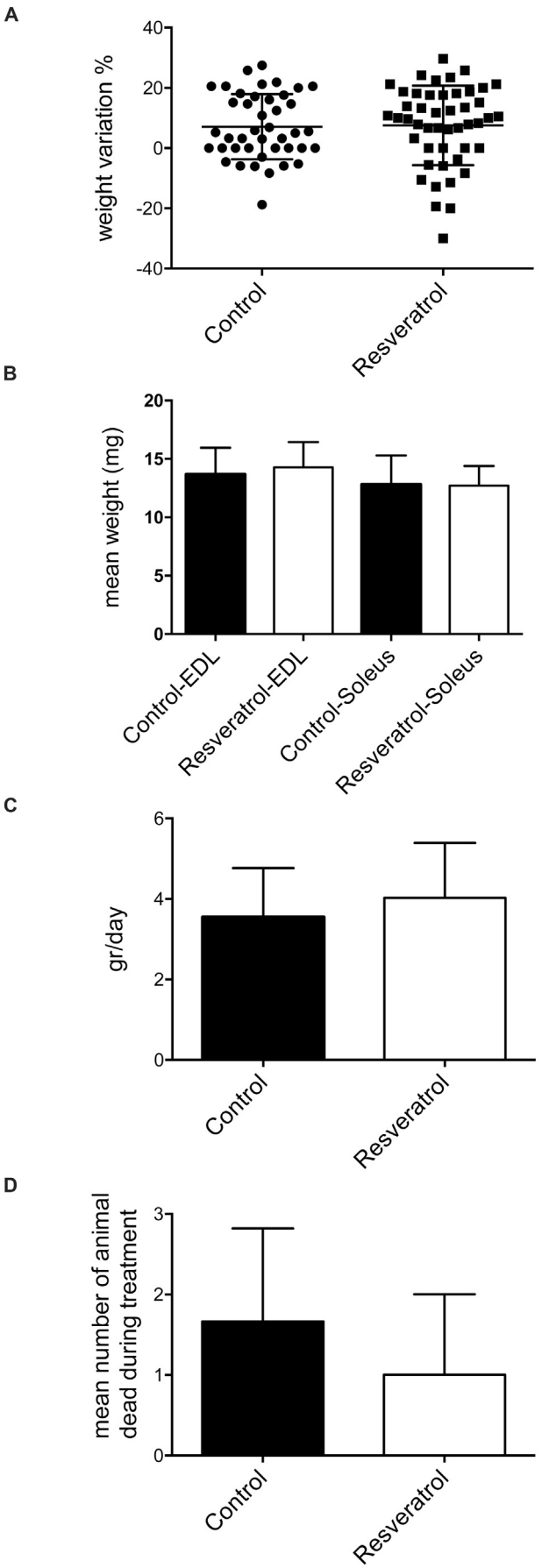

Fig. 1. Weight variation, exstensor digitorum longus (EDL) and soleus muscles weight, food intake, and survival rate comparison following RES supplementation. (A) The body weight difference was plotted as percentage change, data show that RES supplementation does not induce significant difference compared to control mice. (B) EDL and soleus muscles from treated (black columns) and control (white columns) mice do not display significantly different weights. (C) Food intake in treated and control mice is not significantly different. (D) The mean number of deaths is not significantly different in treated and control mice. The reported results are the product of three independent experiments involving 45 RES treated and 45 control mice, body mass variation and number of deaths were measured in 45 RES treated and 45 control mice, muscles weight mean values were calculated from 12 treated and 12 control mice, food intake was measured in 5 treated and 5 control mice.

controls (Fig. 2F).

3.3. Analysis of skeletal muscle histological properties upon RES supplementation

Skeletal muscle histo-pathological conditions following RES treatment were determined by histological observation of cross-sectioned tibialis anterior muscles. We investigated the presence of centralized myonuclei, measured the cross sectional area (CSA), and the presence of tubular aggregates (TAs).

Cross sections of tibialis anterior muscles from treated and non-treated mice did not present fibers with centralized nuclei as shown in Fig. 3A. We measured the CSA of fibers in tibialis anterior muscles sections from 9 treated and 9 untreated mice ($n > 2000$). The mean CSA was significantly reduced by 8,6% in treated compared to control muscles as reported in Fig. 3B ($P < 0,0001$). Moreover, the frequency test to compare variance of CSA showed a significant difference with $P < 0,05$. In Fig. 3C we plotted the relative percentage of fibers in different CSA classes: in treated mice small and medium-small CSA fibers resulted more frequent, while large CSA fibers resulted less frequent compared to control mice.

The observation of tibialis anterior cross sections evidenced several fibers presenting stained internal structures ascribable to TAs, as highlighted by arrowheads in Fig. 3A. In humans, TAs develop mainly in type II fibers (Engel et al., 1970; Schiaffino, 2012), while in C57BL/6 mice they are restricted to type IIB fibers, related to sex and age (Agbulut et al., 2000; Chevessier et al., 2004). We immunostained tibialis anterior sections from 19 control and 17 RES treated mice with an anti-Triadin antibody to specifically detect TAs (Chevessier et al., 2004) and we observed that the number of TAs was significantly reduced by 26% in treated mice compared to control ($P < 0,05$), as shown in Fig. 3D and quantified in graph in Fig. 3E. Interestingly, the quantitation of the expression levels of the inflammation marker Cyclooxygenase II (Cox II) revealed a significant reduction in RES supplemented mice (Fig. 3F and G), proposing that RES can modulate inflammatory processes also in vivo, as previously shown in vitro (Subbaramaiah et al., 1998; Nakata et al., 2012) and this effect can be responsible of the reduction of TAs formation.

3.4. RES and myosin heavy chain expression

The classification of contractile properties of single fibers is related mainly with MyHCs expression (Schiaffino and Reggiani, 2011), therefore we investigated their expression in treated animals compared to control. Preliminary western blot analysis on gastrocnemius lysates, with general antibodies recognizing the slow MyHCs and the fast MyHCs showed no significant variation in their relative quantity (Fig. 4A and B). Interestingly, the analysis of MyHCs expression by electrophoresis (Talmadge and Roy, 1993) confirmed that MyHCI expression was not affected by RES treatment, while the distribution of the relative amount of fast MyHC isoforms was changed. MyHCIIB levels where significantly higher in treated muscles compared to controls

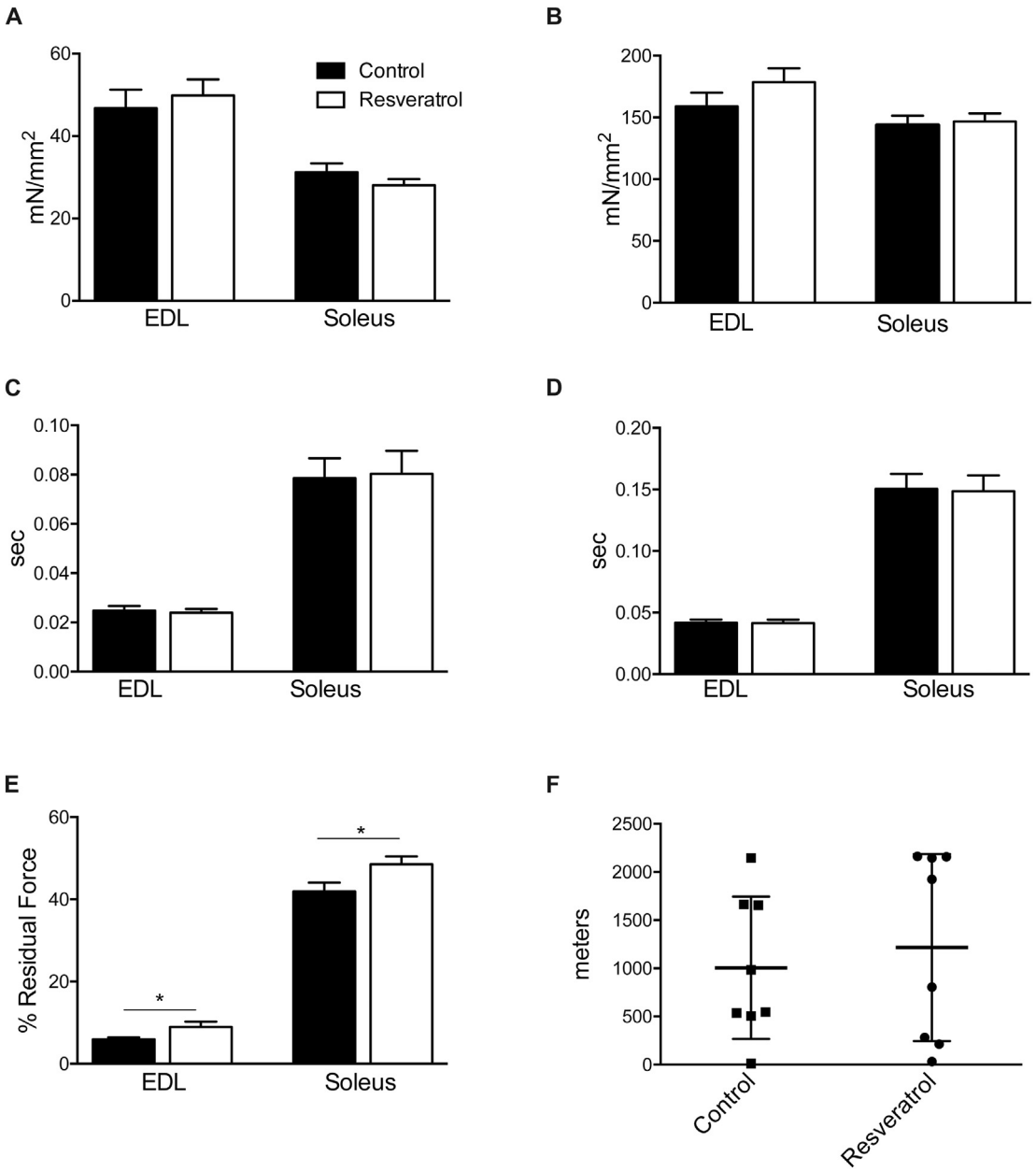

Fig. 2. Contractile response parameters of EDL and soleus muscles, and performance in a treadmill test in RES treated and control mice. (A) Twitch tension, (B) Tetanus, (C) Time to peak and (D) Half relaxation time were not different in control (black columns) and treated mice (white columns). (E) Resistance to fatigue was significantly higher (*P < 0,05) in RES treated mice compared to control (n = 12 control and n = 12 RES treated mice). (F) RES treated mice performance in a treadmill exhaustion test is not significantly different from control mice (n = 8 control and n = 8 RES treated mice).

and, accordingly, MyHCIIA/IIX levels were decreased (Fig. 4C). Furthermore, the immunostaining of cryosections from tibialis anterior from 4 randomly chosen control and treated mice with the anti MyHCIIA antibody SC-71, showed a decreasing trend for type IIA fibers (Fig. 4D and E). As a further confirmation of the capability of RES to modify the properties of muscle fibers, the quantification of the levels of the fast isoform of the sarco/endoplasmic reticulum Ca^{2+}-ATPase, (Serca 1), showed a significant increase in treated muscles (*P < 0,05, Fig. 4F and G).

3.5. RES effects on metabolic properties

Previous reports have provided evidence for the effective role of RES on modulating the mitochondrial pathway (Lagouge et al., 2006). We analyzed the mitochondrial activity in tibialis anterior with the Succynate Dehydrogenase activity assay (SDH). As reported in Fig. 5A and B, the mean specific activity was 10% significantly higher (P < 0,001) in treated muscles compared to control. We also measured the mitochondrial 20 kDa outer membrane protein (Tom 20), as marker of mitochondria volume, and Cyclooxigenase IV (Cox IV), which reveals their specific activity. As shown in Fig. 5C and D, quantitation of Tom 20 and Cox IV proteins did not show any significant change. We can conclude that treated muscle fibers display a more oxidative metabolism without variation of the mitochondria total number.

4. Discussion

Sarcopenia is a multifactorial syndrome that, in humans, displays its first consequences in the middle age phase (Rosenberg, 1997; Marzetti et al., 2018). Recent research activities are focused on preventive and

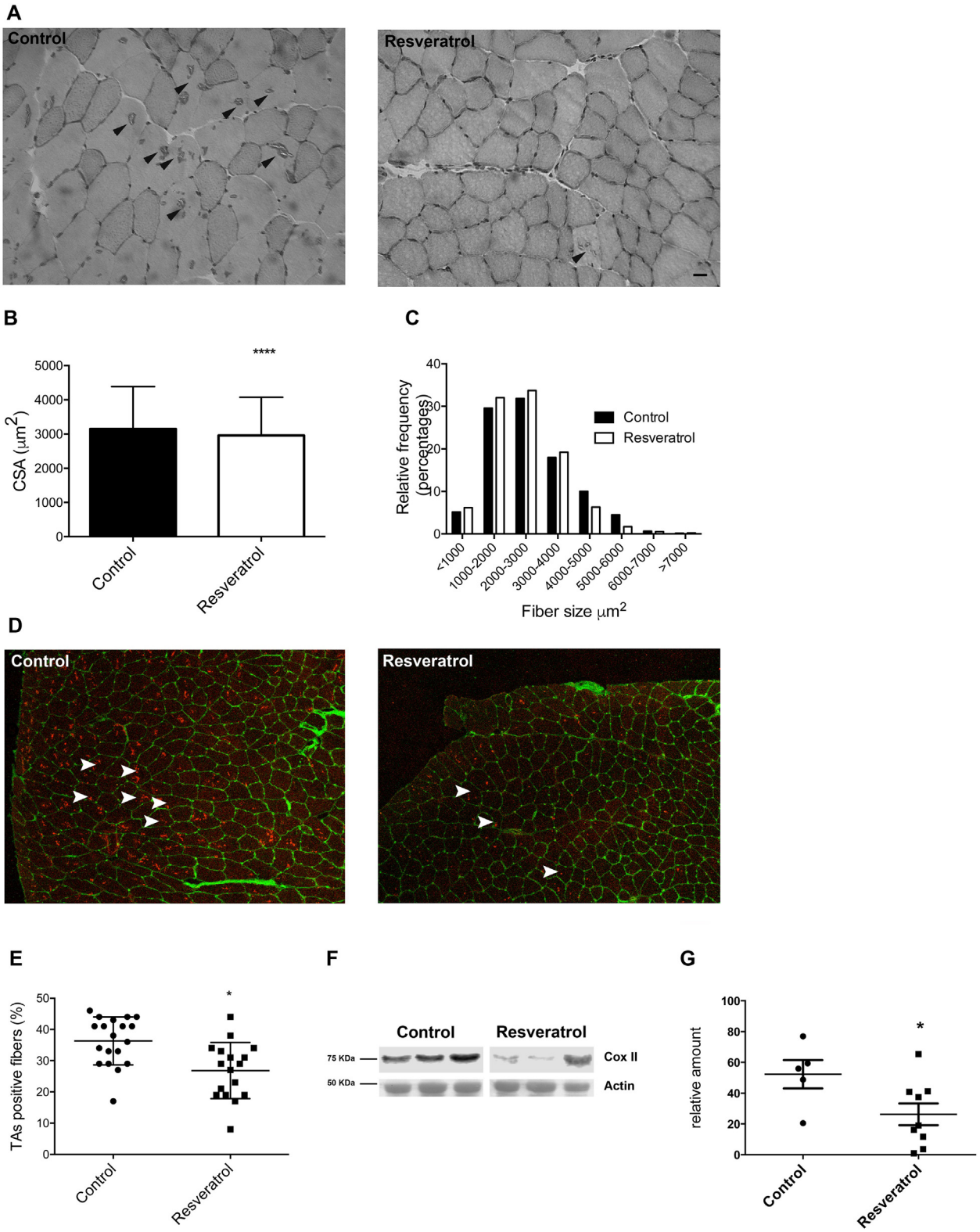

Fig. 3. Histological analysis of tibialis anterior cross sections in RES treated and control mice. (A) Toluidine blue stained cross section from tibialis anterior do not indicate any centralized nuclei, but reveal the presence of several TAs in control compared to RES supplemented mice. Arrowheads point towards TAs. Bar 5 μm. (B) The mean CSA is significantly reduced in RES treated (white columns) compared to control mice (black columns) ****$P < 0{,}0001$; data are obtained from > 2000 fibers from 9 treated and 9 untreated tibialis anterior muscles. (C) The CSA distribution is significantly different in RES treated mice as calculated by an F-test ($P < 0{,}05$). (D) Tibialis anterior cross sections were immunostained with anti-Triadin antibody (in red), which detects also TAs. WGA-AlexaFluor488 (green) was used to detect membranes. Arrowheads point towards TAs. Bar 10 μm. (E) The number of TAs is significantly lower (*$P < 0{,}05$) in RES treated ($n = 17$) compared to control ($n = 19$) mice. (F) Western blot image of gastrocnemius lysates analyzed with anti Cox II antibody. (G) Cox II expression in RES treated mice results significantly decreased. (*$P < 0{,}05$, $n = 5$ control and $n = 9$ RES treated mice). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

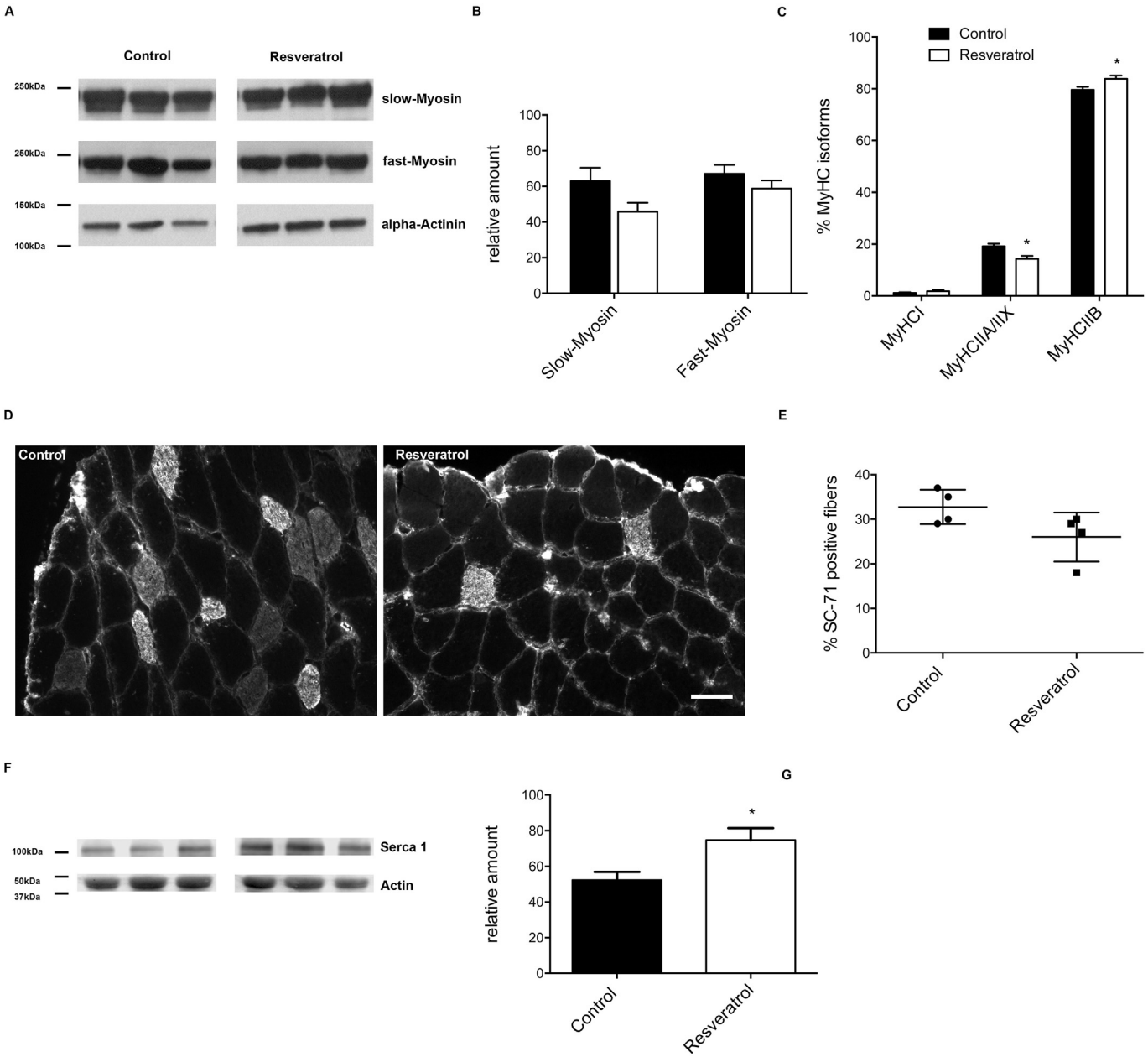

Fig. 4. MyHCs and Serca 1 expression in RES treated compared to control mice. (A) Western blot analysis on gastrocnemius lysates with general antibodies recognizing the slow MyHC and the fast MyHCs. (B) The analysis of slow and fast MyHCs does not show significant changes, in treated (white columns) compared to control mice (black columns) (C) The electrophoretic analysis of MyHC isoforms expression in gastrocnemius muscle shows that, in treated animals there is a significant higher MyHCIIB content and a lower MyHCIIA/X (*P < 0,05; n = 15 for treated and untreated mice), while the relative amount of MyHCI does not change. (D) Representative pictures of tibialis anterior cross sections immunostained with SC-71 antibody. Bar 10 μm. (E) Quantitation of fibers positive for SC-71 antibody shows a not significant decrease of the percentage of positive fibers in treated animals (P = 0.0906, n = 4 control and n = 4 RES treated mice). (F) Western blot analysis on gastrocnemius lysates, with anti Serca 1 antibody. (G) Serca 1 expression in RES treated mice results significantly increased (*P < 0,05; n = 5 control and n = 5 RES treated mice) upon RES supplementation.

therapeutic strategies (Yoshimura et al., 2017) aimed at reducing the impact of sarcopenia on the health of elderly individual and, as a consequence, on the public-health systems.

Aiming at simulating the treatment of middle-aged subjects (Dutta and Sengupta, 2016), the present study has been designed to analyze the impact of RES long-term supplementation on the skeletal muscle during aging. C57/BL6 mice, aged 12 months, were treated with 50–55 mg/kg/day RES for 6 months, skeletal muscle were harvested and analyzed for the morphological properties, the expression of MyHCs, and the physiological-contractile parameters. Present data propose that in our experimental conditions, RES is capable to modulate histological and contractile features in aging skeletal muscles and protect from some age-dependent impairment.

The overall observation of muscles from RES treated mice compared to controls revealed specific differences. Treated animals presented myofibers with a reduced CSA and a reduced number of TAs. Fiber size has been described to be correlated with MyHC expression, in fact, in the mouse tibialis anterior muscle, among fast fibers, IIA fibers are the smallest followed by IIX, and the largest IIB that, in turn show a broad distribution in their CSA (Hamalainen and Pette, 1993). In our experiment, we observed an increase of the percentage of small and medium-small size fibers. In parallel, electrophoresis analysis indicated

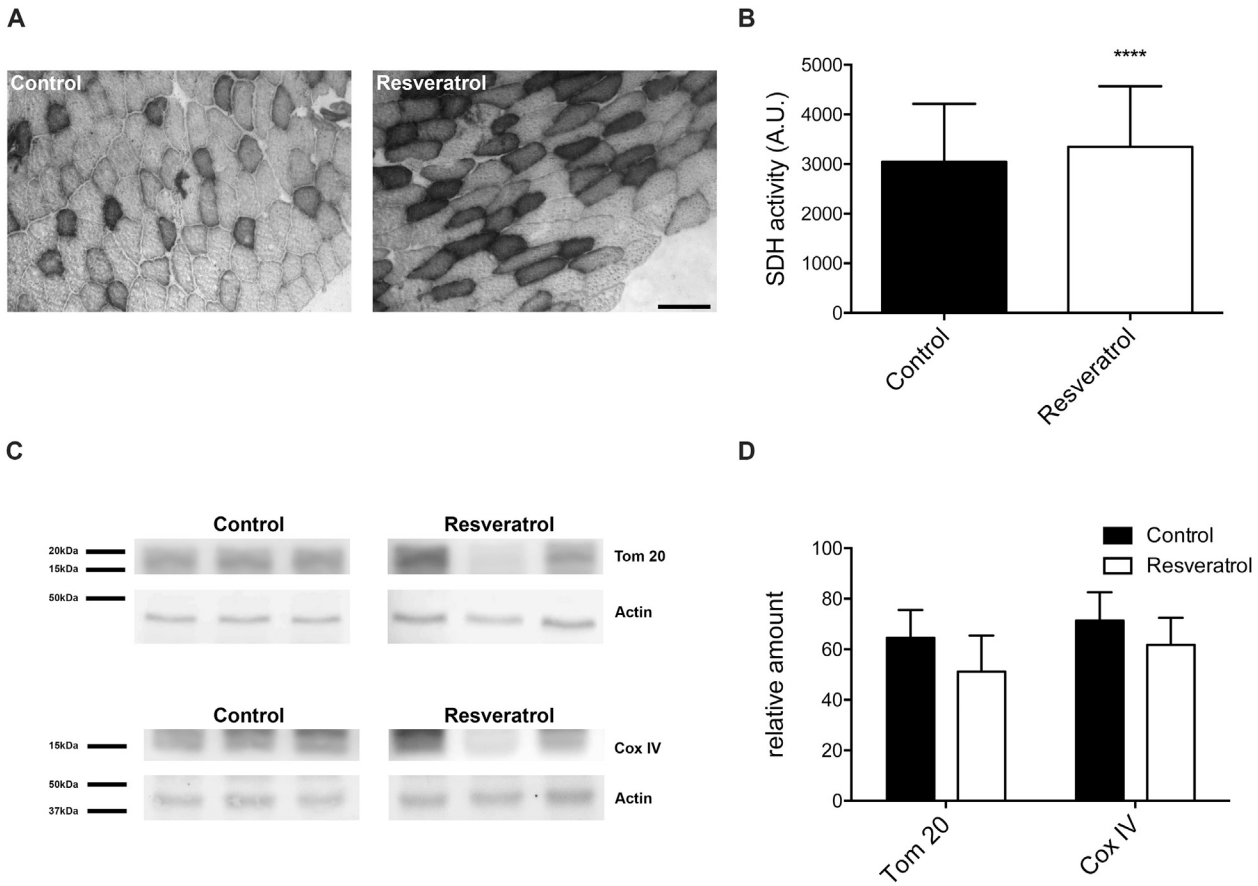

Fig. 5. Mitochondrial activity in RES treated mice compared to controls. (A) Representative picture of tibialis anterior cross sections subjected to the SDH assay. Bar 10 μm. (B) The SDH activity was measured for each fiber, the plot shows that the mean SDH activity is significantly increased in treated muscles (****P < 0,0001, n = 4 control and n = 5 RES treated mice). (C) Western blot analysis on gastrocnemius lysates, with anti-Tom 20 and Cox IV antibodies. (D) Tom 20 and Cox IV levels were not significantly modified buy RES supplementation (n = 5 control and n = 5 RES treated mice).

that the ratio of slow and fast MyHC does not change, however, the percentage distribution of MyHCII resulted different in treated animals compared to control with a relative increase of MyHCIIB and relative decrease of IIA. This observation can be explained by a general decrease of the CSA regardless the different MyHC expressed. In this context, the CSA decrease was recently reported as an effect of a caloric restriction regimen (Elashry et al., 2017). It can be proposed that RES, as a caloric restriction mimetic, most probably reduces the CSA maintaining higher levels of MyHCIIB.

It has been demonstrated that aging induces a loss of fast fibers in human muscles (Ciciliot et al., 2013), and this effect is accompanied by a functional and biochemical re-adaptation (Murgia et al., 2017). Interestingly, our observation that RES treated muscles display a maintenance of fast type IIB myosin, can attribute to RES the capability to preserve muscles from aging, providing a myosin composition more similar to younger muscles.

Fiber size, MyHC expression and mitochondrial activity are generally interlinked influencing skeletal muscle activity. It has been recently reported (Murgia et al., 2017) that aging modifies this equilibrium by inducing a loss of fast fibers together with a reduction of the oxidative capacity of slow fibers. Here, we report that RES treatment induced a 10% increase of SDH specific activity, and no significant changes in Tom 20 or Cox IV levels, indicating that in our experimental conditions, RES induces a shift of muscle fibers metabolism towards a more oxidative behavior maintaining the mitochondria total number. Our observation showing that in treated muscles there is a higher SDH activity despite the increased expression of fast MyHCIIB could result contradictory. Interestingly, Ikeda et al. (2006) reported that, during run training, fast fibers are the target of the metabolic rearrangement ascribable to PGC1-alpha, one of the most important mitochondrial modulator, which is positively regulated by RES (Feige et al., 2008; Lagouge et al., 2006; Rodriguez-Bies et al., 2016). Our experiments showing an increase of MyHCIIB together with the shift towards a more oxidative metabolism could be ascribed to the predominant effect of RES on PGC1-alpha pathway in fast fibers.

The more oxidative metabolism of muscle fibers explains, at least partially, the significant increase of the resistance to fatigue. In fact, resistance to fatigue can be influenced by several factors (Allen et al., 2008), as the oxidative capacity, the ATP regeneration rate and the calcium handling. In this regard, our finding showing the more oxidative metabolism together with the increase of Serca 1 expression, consistent with a different calcium handling, could account for the better resistance to fatigue as measured by an ex-vivo test. However, the modification of this parameter can be the result of other observations like the presence of tubular aggregates and the presence of different inflammatory responses.

In contrast with the ex-vivo resistance test, the treadmill performance test did not show significant difference. Accordingly, a previous study from Ringholm and collaborators reported that lifelong RES supplementation had no effect on several parameters including the endurance capacity (Ringholm et al., 2013). We can argue that other age-related factors could have influenced the outcome of the performance that probably RES supplemented alone is not able to overcome. In the mentioned work the Authors do not find any significant change in the oxidative capacity, in the role of PGC1-alpha and the angiogenic properties in Wilde Type female mice (Ringholm et al., 2013), while these parameters are affected when PGC-1alpha deficient mice are subject to a RES supplemented diet accompanied by exercise. We

suggest that the difference between our and Ringholm et al. (2013) observations can be interpreted as the result of different stress statuses: in Ringholm work, female mice are treated for 12 months starting at the age of 3 months and sacrificed when 15 months old, while we treated 12 months old male mice for 6 months and sacrificed them when 18 months old. The sex-dependent hormones and the different time frame of analysis could be source of important differences in the levels of "stress". Therefore, as proposed by Ringholm et al. (2013), the present work can represent a further confirmation that RES improves metabolic parameters when these are initially impaired. Although using different doses, a recent work from Rodriguez-Bies et al. (2016) further supports this hypothesis. Studies aimed at understanding the difference between male and female muscle aging and further investigation to find the best time interval to start RES supplementation, able to positively impact selected symptoms of muscle aging, are needed to clarify these doubts.

In skeletal muscle fibers, a further marker of the defective mitochondrial activity is provided by the presence of tubular aggregates (Schiaffino, 2012; Agbulut et al., 2000; Chevessier et al., 2004; Giacomello et al., 2015; Vielhaber et al., 2001). TAs have been proposed to originate from sarcoplasmic reticulum, in age and sex- dependent manner, in prevalence in type II fibers (Engel et al., 1970; Agbulut et al., 2000). Our data show that RES treatment induces a significant reduction of TAs. We can hypothesize that the reduction of TAs, despite the increase of MyHCIIB, is the results of a direct action of RES on the mechanisms that contribute to their formation. Considering that TAs have been proposed to originate from sarcoplasmic reticulum in presence of an impaired mitochondrial activity, it can be argued that RES creates a more favorable metabolic condition in aging muscles, with a reduced inflammatory response, where the formation of TAs is arrested or delayed. Our data give further support to the idea that the impairment of energy metabolism that could depend on aging but also on pathological conditions (Boncompagni et al., 2006; Schiaffino, 2012; Giacomello et al., 2015; Vielhaber et al., 2001) is one determinant of TAs formation. Further experiments are required to reveal the mechanisms involved in this action.

In summary, our data show that RES treatment at the dose of 50–55 mg/kg/day for six months induces a shift to a more oxidative metabolism, an increase of MyHCIIB expression, improves the resistance to fatigue, and reduces the number of TAs, confirming RES as a good candidate for counteract age related skeletal muscle modification, and opening a perspective on its specific activity in the prevention of TAs in muscles. Therefore, RES could be considered a good candidate not only for the attenuation of some sarcopenia-related alterations, but also in the treatment of muscle pathologies with TAs as already proposed for different myopathies (Bastin and Djouadi, 2016), Duchenne dystrophy models (Hori et al., 2011), and heart failure (Sung and Dyck, 2015).

Whether RES action in sarcopenia attenuation and on the reduction of TAs in mice models depends on the duration or the doses provided is still not clear. Taking in account that, by definition, sarcopenia consists in a continuous decline in muscle mass and strength and alteration of multiple health parameters (Rosenberg, 1997), we are incline to think that aging individuals should be controlled and treated chronically. However, it remains crucial the time interval when the subjects should act (either with a pharmacological intervention, diet control or exercise) in order to prevent and control sarcopenia. Therefore, further experiments will be aimed at finding the best time interval to start RES supplementation in mice models to reduce some of the age-dependent alterations to skeletal muscle. In parallel, considered the wide range of doses used in different protocols, and the reported relationship between dose and effects reported in the literature (for a review see (Madreiter-Sokolowski et al., 2017)), it will be of interest to investigate the minimal dose responsible of the mentioned effects and applicable to human individuals.

Acknowledgments

This work was supported by the Regione Toscana Grant "Bando Salute 2009" N° 132, to E. Giacomello and from MIUR-Finanziamento delle attività base di ricerca (FFARB) to L. Toniolo.

Author contributions

E.G. and L.T. conceived and designed the experiments; E.G., L.T., L.F., P.F., A.M., M.C. performed the experiments; E.G. and L.T. analyzed the data; C.R. contributed with helpful advice, instrumentation, some reagents and materials; E.G. and L.T. wrote the paper.

Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

Agbulut, O., et al., 2000. Age-related appearance of tubular aggregates in the skeletal muscle of almost all male inbred mice. Histochem. Cell Biol. 114 (6), 477–481.

Allen, D.G., Lamb, G.D., Westerblad, H., 2008. Impaired calcium release during fatigue. J. Appl. Physiol. 104 (1), 296–305.

Bastin, J., Djouadi, F., 2016. Resveratrol and myopathy. Nutrients 8 (5).

Baur, J.A., et al., 2006. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444 (7117), 337–342.

Boncompagni, S., et al., 2006. Progressive disorganization of the excitation-contraction coupling apparatus in aging human skeletal muscle as revealed by electron microscopy: a possible role in the decline of muscle performance. J. Gerontol. A Biol. Sci. Med. Sci. 61 (10), 995–1008.

Carter, H.N., Chen, C.C., Hood, D.A., 2015. Mitochondria, muscle health, and exercise with advancing age. Physiology (Bethesda) 30 (3), 208–223.

Chabi, B., et al., 2008. Mitochondrial function and apoptotic susceptibility in aging skeletal muscle. Aging Cell 7 (1), 2–12.

Chevessier, F., et al., 2004. Tubular aggregates are from whole sarcoplasmic reticulum origin: alterations in calcium binding protein expression in mouse skeletal muscle during aging. Neuromuscul. Disord. 14 (3), 208–216.

Chung, J.H., Manganiello, V., Dyck, J.R., 2012. Resveratrol as a calorie restriction mimetic: therapeutic implications. Trends Cell Biol. 22 (10), 546–554.

Ciciliot, S., et al., 2013. Muscle type and fiber type specificity in muscle wasting. Int. J. Biochem. Cell Biol. 45 (10), 2191–2199.

Conley, K.E., Jubrias, S.A., Esselman, P.C., 2000. Oxidative capacity and ageing in human muscle. J. Physiol. 526 (Pt 1), 203–210.

Cristea, A., et al., 2010. Effects of aging and gender on the spatial organization of nuclei in single human skeletal muscle cells. Aging Cell 9 (5), 685–697.

Dutta, S., Sengupta, P., 2016. Men and mice: relating their ages. Life Sci. 152, 244–248.

Elashry, M.I., et al., 2017. The effect of caloric restriction on the forelimb skeletal muscle fibers of the hypertrophic myostatin null mice. Acta Histochem. 119 (5), 582–591.

Engel, W.K., 1964. Mitochondrial aggregates in muscle disease. J. Histochem. Cytochem. 12, 46–48.

Engel, W.K., Bishop, D.W., Cunningham, G.G., 1970. Tubular aggregates in type II muscle fibers: ultrastructural and histochemical correlation. J. Ultrastruct. Res. 31 (5–6), 507–525.

Feige, J.N., Auwerx, J., 2008. Transcriptional targets of sirtuins in the coordination of mammalian physiology. Curr. Opin. Cell Biol. 20 (3), 303–309.

Feige, J.N., et al., 2008. Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. Cell Metab. 8 (5), 347–358.

Fortes, M.A., et al., 2016. Housekeeping proteins: how useful are they in skeletal muscle diabetes studies and muscle hypertrophy models? Anal. Biochem. 504, 38–40.

Giacomello, E., et al., 2015. Deletion of small ankyrin 1 (sAnk1) isoforms results in structural and functional alterations in aging skeletal muscle fibers. Am. J. Phys. Cell Phys. 308 (2), C123–C138.

Hamalainen, N., Pette, D., 1993. The histochemical profiles of fast fiber types IIB, IID, and IIA in skeletal muscles of mouse, rat, and rabbit. J. Histochem. Cytochem. 41 (5), 733–743.

Hoeks, J., Schrauwen, P., 2012. Muscle mitochondria and insulin resistance: a human perspective. Trends Endocrinol. Metab. 23 (9), 444–450.

Hollmann, W., et al., 2007. Physical activity and the elderly. Eur. J. Cardiovasc. Prev. Rehabil. 14 (6), 730–739.

Hori, Y.S., et al., 2011. Resveratrol ameliorates muscular pathology in the dystrophic mdx mouse, a model for Duchenne muscular dystrophy. J. Pharmacol. Exp. Ther. 338 (3), 784–794.

Hyatt, J.P., et al., 2016. Muscle-specific myosin heavy chain shifts in response to a long-term high fat/high sugar diet and resveratrol treatment in nonhuman primates. Front. Physiol. 7, 77.
Ikeda, S., et al., 2006. Muscle type-specific response of PGC-1 alpha and oxidative enzymes during voluntary wheel running in mouse skeletal muscle. Acta Physiol (Oxford) 188 (3–4), 217–223.
Lagouge, M., et al., 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell 127 (6), 1109–1122.
Lopez-Lluch, G., Navas, P., 2016. Calorie restriction as an intervention in ageing. J. Physiol. 594 (8), 2043–2060.
Madreiter-Sokolowski, C.T., Sokolowski, A.A., Graier, W.F., 2017. Dosis facit sanitatem-concentration-dependent effects of resveratrol on mitochondria. Nutrients 9 (10).
Marzetti, E., Leeuwenburgh, C., 2006. Skeletal muscle apoptosis, sarcopenia and frailty at old age. Exp. Gerontol. 41 (12), 1234–1238.
Marzetti, E., et al., 2018. Age-related changes of skeletal muscle mass and strength among Italian and Taiwanese older people: results from the Milan EXPO 2015 survey and the I-Lan Longitudinal Aging Study. Exp. Gerontol. 102, 76–80.
Murase, T., et al., 2009. Suppression of the aging-associated decline in physical performance by a combination of resveratrol intake and habitual exercise in senescence-accelerated mice. Biogerontology 10 (4), 423–434.
Murgia, M., et al., 2017. Single muscle fiber proteomics reveals fiber-type-specific features of human muscle aging. Cell Rep. 19 (11), 2396–2409.
Nakata, R., Takahashi, S., Inoue, H., 2012. Recent advances in the study on resveratrol. Biol. Pharm. Bull. 35 (3), 273–279.
Narkar, V.A., et al., 2008. AMPK and PPARdelta agonists are exercise mimetics. Cell 134 (3), 405–415.
Pearson, K.J., et al., 2008. Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. Cell Metab. 8 (2), 157–168.
Ringholm, S., et al., 2013. Effect of lifelong resveratrol supplementation and exercise training on skeletal muscle oxidative capacity in aging mice; impact of PGC-1alpha. Exp. Gerontol. 48 (11), 1311–1318.
Rodriguez-Bies, E., et al., 2016. Resveratrol primes the effects of physical activity in old mice. Br. J. Nutr. 116 (6), 979–988.
Rosenberg, I.H., 1997. Sarcopenia: origins and clinical relevance. J. Nutr. 127 (5 Suppl), 990S–991S.
Ryall, J.G., Schertzer, J.D., Lynch, G.S., 2008. Cellular and molecular mechanisms underlying age-related skeletal muscle wasting and weakness. Biogerontology 9 (4), 213–228.
Schiaffino, S., 2012. Tubular aggregates in skeletal muscle: just a special type of protein aggregates? Neuromuscul. Disord. 22 (3), 199–207.
Schiaffino, S., Reggiani, C., 2011. Fiber types in mammalian skeletal muscles. Physiol. Rev. 91 (4), 1447–1531.
Subbaramaiah, K., et al., 1998. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J. Biol. Chem. 273 (34), 21875–21882.
Sung, M.M., Dyck, J.R., 2015. Therapeutic potential of resveratrol in heart failure. Ann. N. Y. Acad. Sci. 1348 (1), 32–45.
Talmadge, R.J., Roy, R.R., 1993. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. J. Appl. Physiol. 75 (5), 2337–2340.
Vielhaber, S., et al., 2001. Defective mitochondrial oxidative phosphorylation in myopathies with tubular aggregates originating from sarcoplasmic reticulum. J. Neuropathol. Exp. Neurol. 60 (11), 1032–1040.
Wu, R.E., et al., 2013. Resveratrol protects against physical fatigue and improves exercise performance in mice. Molecules 18 (4), 4689–4702.
Yoshimura, Y., et al., 2017. Interventions for treating sarcopenia: a systematic review and meta-analysis of randomized controlled studies. J. Am. Med. Dir. Assoc. 18 (6), 553e1–553e16.