Long-term resveratrol treatment improves the capillarization in the skeletal muscles of ageing C57BL/6J mice

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ABSTRACT

We recently showed that the treatment with Resveratrol (RES) contrasts the effects of ageing on the skeletal muscle (SKM), reduces the appearance of tubular aggregates (TAs), and improves the fatigue resistance. Since fatigue resistance depends on the SKM capillary network, and RES has been described to improve vascularisation, we analysed the SKM capillarization in naturally ageing C57BL/6J male mice, fed with 0.04% RES in the diet for 6 months, which showed a better fatigue resistance in a previous work. Our data show an inverse correlation between the number of capillaries per fibre (CAF) and TAs in both control and treated type IIB fibres, and an increase of CAF in ageing SKM upon RES-treatment. The present work suggests that capillarization is one of the determinants of the development of TAs and fatigue resistance, and that RES can be considered a good candidate to counteract capillary rarefaction in the SKM tissue.

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Capillaries; fibre type; muscle fatigue; resveratrol; sarcopenia; tubular aggregates

Introduction

The natural polyphenol Resveratrol (RES) modulates gene expression according to the energetic state of the cell, resulting in the improvement of insulin sensitivity, mitochondrial number, oxidative defence, and motor function, reducing the effects of ageing on the skeletal muscle (SKM) tissue (Baur et al. 2006; Lagouge et al. 2006). In our previous work we showed that supplementation of RES in the diet reduced the number of Tubular Aggregates (TAs) in the fibres of ageing SKMs (Toniolo et al. 2018). In C57BL/6J male mice, TAs develop in type IIB fibres depending on the age (Agbulut et al. 2000; Chevessier et al. 2004), and on muscle pathologies (Giacomello et al. 2015). Although TAs have been proposed to originate from the sarcoplasmic reticulum due to defective mitochondrial activity (Vielhaber et al. 2001; Schiaffino 2012), the exact mechanism of their biogenesis is still unknown.

In the SKM, fibre type, fibre size, oxidative capacity, and capillarization are strictly correlated morpho-functional properties, which reflect its function. In this complex scenario, the microcirculation plays a crucial role in the delivery of oxygen, nutrients and hormones, and removal of heat, metabolites and waste products, to and from muscle fibres, respectively. Sufficient oxygen supply to the working SKM is vital to muscle contraction, and the capillary network needs to adapt according to the physical performance demand. Interestingly, a poor capillarization has been suggested as one of the determinants of a lower exercise capacity in older adults (Prior et al. 2016; Barnouin et al. 2017).

RES capabilities to modulate capillarization have not been deeply investigated, and studies in humans and animal models have provided controversial results (Ringholm et al. 2013; Diaz et al. 2016; Pollack et al. 2017; Breuss et al. 2019). Based on the evidences that resistance to fatigue is correlated to SKM tissue capillarization, together with data showing that RES is able to improve fatigue resistance (Murase et al. 2009; Wu et al. 2013; Rodriguez-Bies et al. 2016; Toniolo et al. 2018), we hypothesised that an amelioration of oxygen supply could be one of the factors underlying RES effect on fatigue resistance. To test this hypothesis, we further analysed a group of naturally ageing C57BL/6J male mice fed with a chow diet containing 0.04% RES that showed a better fatigue resistance in a previous

investigation (Toniolo et al. 2018), and we explored capillarization, the MyHCIIB expression and the presence of TAs. The analysis of the data suggest for the first time an inverse correlation between the number of capillaries per fibre (CAF) and TAs formation in both control and treated SKMs. Interestingly, RES-treated muscles display a better capillarization, suggesting that RES reduces TAs formation by improving capillarization, and further confirming the capability of RES to protect SKM from age-related dysfunctions.

Materials and methods

Animals

SKM samples used in this study derive from the experiments previously reported (Toniolo et al. 2018), where two groups of 15 C57BL/6J male mice aged 12 months were fed for 6 months either with a standard diet and a standard diet supplemented with RES. For the analyses performed in the present paper, at the end of the experiment 8 control and 9 treated mice were sacrificed by cervical dislocation euthanasia, the tibialis anterior and gastrocnemius muscles were harvested for histological analyses and protein expression respectively. Three C57BL/6J male mice aged 8 months were analysed as young adults controls. The experimental procedures were in accordance with the European legislation on the use and care of laboratory animals (EU Directive 2010/63), approved by the Ethics Committee of the University of Siena, and from Ministero della Salute, Italy (Project n° J-21/10/10).

Experimental protocol

Animal were administered either a standard diet or a standard diet (control) supplemented with 0.04% RES (treated), based on studies demonstrating the effectiveness of similar concentrations in reducing age-related decline on skeletal muscle and vascular function (Baur et al. 2006; Pearson et al. 2008). The food intake monitored in control and treated mice resulted to be 3.5 ± 1.2 and 4 ± 1.4 gr per mouse per day respectively, corresponding to approximately 1.6 mg of RES per mouse per day. Being the mice mean weight 30 gr, the RES dose corresponded to 50–55 mg/Kg RES per day (Toniolo et al. 2018).

The present study was aimed at simulating the treatment of middle-aged subjects, which undergo physiological muscle ageing. Therefore, the experimentation entailed the treatment of naturally ageing C57BL/6J mice, aged 12 months, which according to previous studies correspond approximately to 55 years old humans (middle age), and at the end of the treatment, 18 months, their age corresponds approximately to 65 years old humans (old age) (Flurkey et al. 2007; Dutta and Sengupta 2016). C57BL/6J male mice aged 8 months were analysed as young adults controls based on evidences that at this age mice have reached the complete maturity (Flurkey et al. 2007; Dutta and Sengupta 2016), and type IIB fibres have not, or few, TAs (Agbulut et al. 2000; Chevessier et al. 2004; Giacomello et al. 2015).

Antibodies

In immunohistochemistry experiments the primary antibody against the MyHC IIB, clone BF-F3 (Developmental Studies Hybridoma Bank, Iowa, USA), was utilised at 4 µg/ml, and the anti-mouse biotinylated secondary antibody raised in horse (Vector Laboratories, Burlingame, CA USA) was used at the dilution of 1:200. In western blot analyses, the primary antibody against the Vascular-Endothelial Growth Factor (VEGF, Thermofisher) was used at the dilution of 1:100, and the anti-mouse Horse Radish Peroxidase conjugated secondary antibody raised in goat (Sigma Aldrich, Italy) was used at the dilution of 1:5000.

Cryostat sectioning

Tibialis anterior muscles were 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). Histological experiments entailed the analysis of the entire tibialis anterior muscles to avoid sampling errors.

Alkaline phosphatase stain

Capillaries were detected by Alkaline Phosphatase staining, as previously described (Kamei et al. 2010). The incubation solution consisted in 50 mM Tris-HCl pH 9.5 containing 0.14 mg/ml Nitro Blue Tetrazolium (Sigma Aldrich, Italy), 0.07 mg/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP, Sigma Aldrich, Italy), and 2.3 mM Magnesium Chloride. Air-dried sections were incubated in freshly prepared incubation solution at 37 °C for 30 minutes, and extensively washed in water (Kamei et al. 2010). Sections were counterstained in 0.5% eosin, washed in water, dehydrated in ethanol,

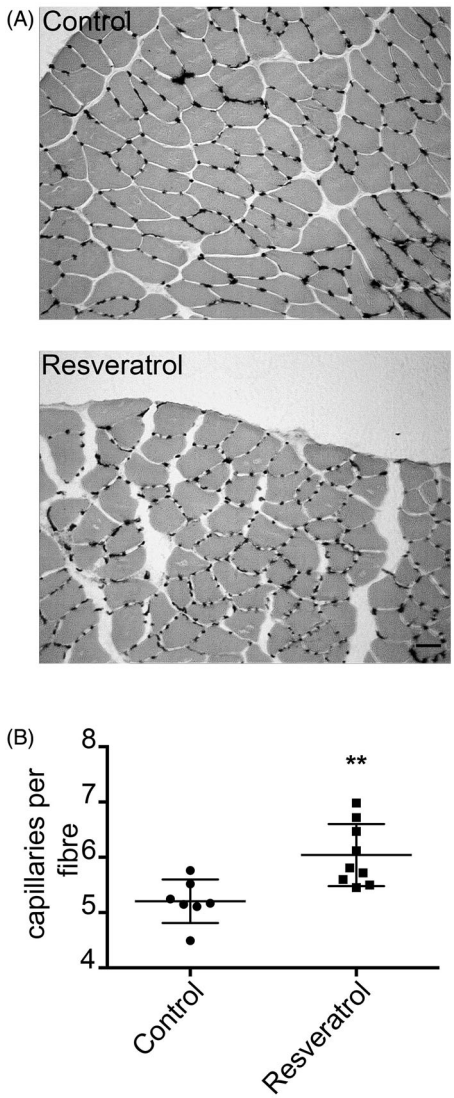

Figure 1. Histological analysis of tibialis anterior cross sections in RES-treated and control mice to detect capillaries. (A) Alkaline phosphatase stained cross sections from tibialis anterior muscles suggest a better capillarization in RES supplemented mice. Bar 50 μm. (B) The mean of CAF is significantly increased in RES-treated compared to control mice (**$p < .01$; data were obtained counting 200 fibres per tibialis anterior muscle from 9 treated and 7 untreated samples).

clarified in BioClear clearing reagent (BioOptica, Milano, Italy), and mounted with a resinous medium. Images were acquired with a Leica Orthoplan microscope (Leica, Germany) equipped with a Leica DC-100 digital camera (Leica, Germany).

Toluidine blue stain

Tissue sections were incubated for 2 minutes in a 0.1% Toluidine Blue solution, washed in water, dehydrated in ethanol, clarified in BioClear clearing reagent (BioOptica, Milano, Italy) and mounted with a resinous medium. Images were acquired with a Leica Orthoplan microscope (Leica, Germany) equipped with a Leica DC-100 digital camera (Leica, Germany).

Immunohistochemistry reactions

Sections were fixed with 3% para-formaldehyde, blocked with 5% normal horse serum in PBS to avoid non-specific binding of the antibodies, and incubated with primary antibodies in a humidified chamber at room temperature for 2 hours. Specimens were washed three times with PBS and then incubated 1 hour with an anti-mouse biotinylated secondary antibody raised in horse, washed and incubated with VECTASTAIN ABC Reagent (Vector Laboratories, Burlingame, CA USA) for 30 minutes. After washing, the sections were incubated in Diamino Benzidine solution (SigmaFastDAB; Sigma Aldrich, Italy) until development of the desired staining. Sections were then washed in water, dehydrated in ethanol, clarified in BioClear clearing reagent (BioOptica, Milano, Italy), and mounted with a resinous medium. Images were acquired with a Leica Orthoplan microscope (Leica, Germany) equipped with a Leica DC-100 digital camera (Leica, Germany).

Image analyses

Images were processed with Fiji free software (Schindelin et al. 2012). The number of capillaries per fibre was determined by counting the alkaline phosphatase positive spots surrounding each fibre. The percentage of fibres with TAs was determined on Toluidine Blue stained sections.

Western Blot

Western blot analysis were performed on gastrocnemius muscles that were snap-frozen in liquid nitrogen and subsequently homogenised and lysed for 60 minutes at 4 °C in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-Deoxycholate, 1% NP40) supplemented with Protease inhibitor cocktail (Sigma Aldrich, Italy). Samples were centrifuged at 10,000 g for 10 minutes at 4 °C to remove insoluble material, and soluble protein concentration was measured by the Bradford assay (BioRad, Italy).

Protein samples (15 μg of total protein/lane) were boiled in Laemmli Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.004% Bromophenol

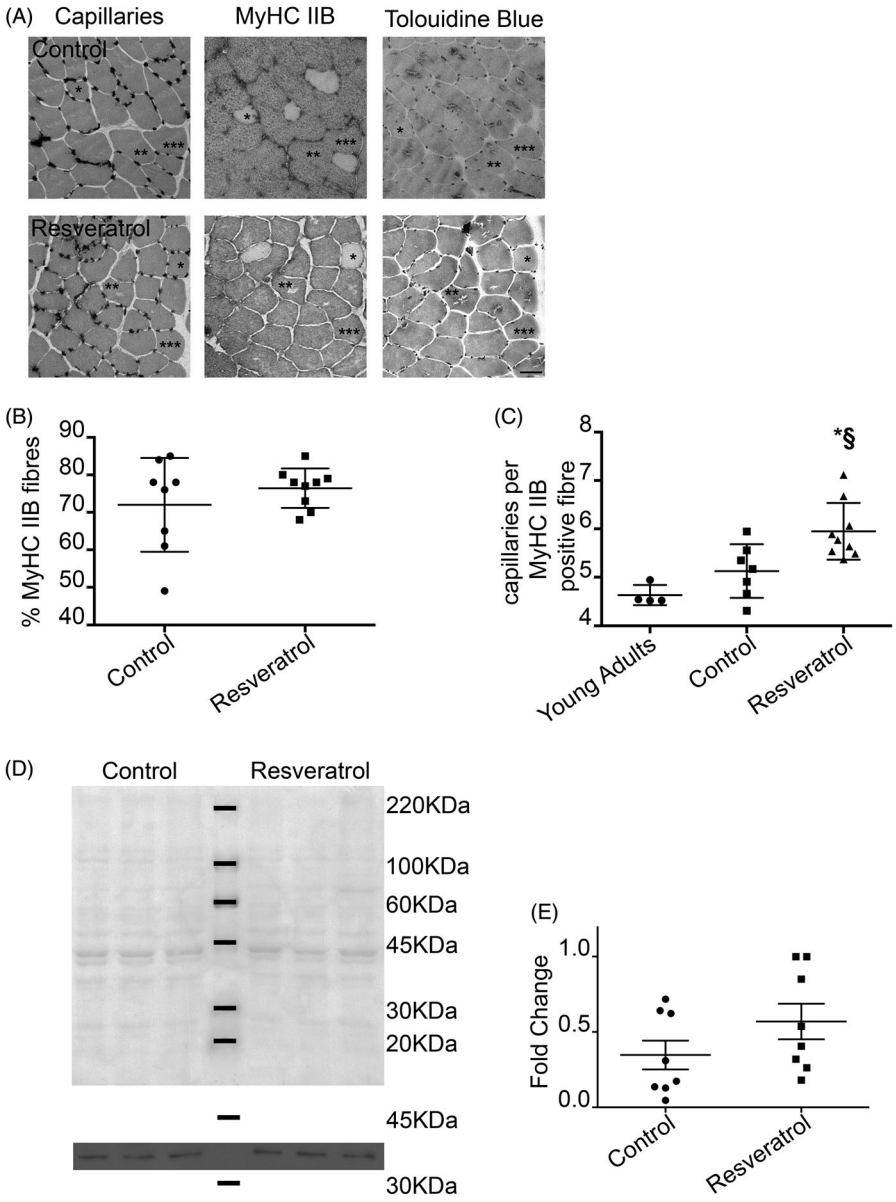

Figure 2. Capillaries, MyHC IIB, tubular aggregates and VEGF expression in RES-treated and control mice. (A) Tibialis anterior cross sections from RES-treated and untreated mice were stained with alkaline phosphatase, BF-F3 antibody and Toluidine Blue. A representative picture with treated and untreated samples is reported. One asterisk (*) highlights a MyHC IIB negative fibre, two and three asterisks (**, ***) highlight type IIB fibres with or without tubular aggregates, respectively. Bar 50 μm. (B) The percentage of type IIB fibres is not significantly different in control and treated animals (n = 8 control and $n = 9$ RES-treated mice). (C) The CAF in type IIB fibres was counted in 4 young adults, 7 control and 9 RES-treated mice. The CAF was not significantly different in young adults and control mice, but was significantly increased in RES-treated mice compared young adults ($^{\S}p < .01$) and control mice ($^{*}p < .05$). (D) Western blot analysis on gastrocnemius lysates with an anti VEGF antibody. (E) VEGF expression in RES-treated mice did not display statistically significant differences ($n = 8$ control and $n = 9$ RES-treated mice).

Blue, 5% β-mercaptoethanol) for 5 minutes at 95 °C before loading. Samples were separated on 10% SDS-PAGE gels, and, were electrophoretically transferred onto nitrocellulose membranes (BioRad, Italy). Membranes were then blocked for 1 h in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% non-fat dry milk and incubated for 1 hour with the specific primary antibodies at room temperature. After extensive washings, membranes were incubated for 1 hour at room temperature with the secondary antibodies conjugated with horseradish peroxidase (GE-Healthcare, Little Chalfont, UK). Immunodetection was performed with a chemiluminescence kit (ECL kit, GE-Healthcare, Little Chalfont, UK). Immunoreactivity on photographic films was analysed by means of Fiji free software (Schindelin et al. 2012). Sample loading was normalised by staining membranes with Ponceau S

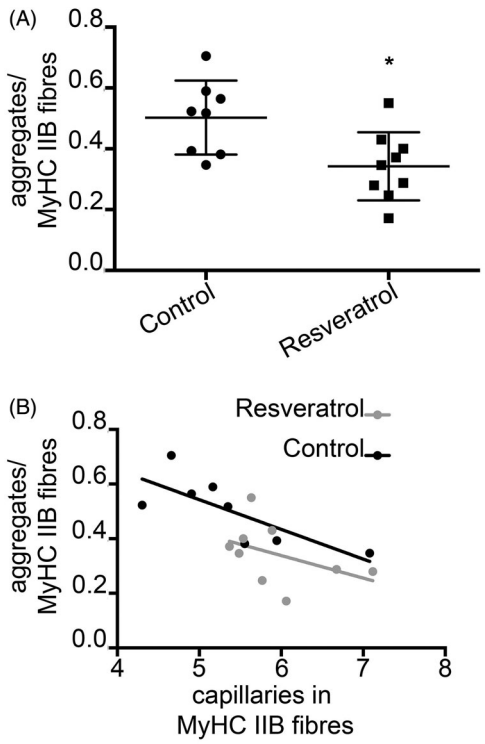

Figure 3. Potential correlation between tubular aggregates and capillaries. (A) The number of TAs per muscle was normalised by the percentage of type IIB fibres for each mouse. The normalised number of TAs significantly decreased from 0.50 ± 0.04 in the control ($n = 8$) to 0.34 ± 0.04 in RES-treated mice ($n = 9$; $^{*}p < .05$). (B) Analysis of the correlation between the mean CAF (X axis) and aggregates (Y axis). The regression line extrapolated from the data (black dots and black line for control mice; grey dots and grey line for RES-treated mice) suggests an inverse correlation between capillaries number and TAs development in tibialis anterior muscles.

(Toniolo et al. 2018). Differences in treated and control samples are reported as fold change.

Statistical analyses

Student's *t* test and correlation analyses 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.

Results

Capillaries in control and RES-treated mice

The number of capillaries for each fibre was assessed in control and RES-treated mice by subjecting cross-sections from tibialis anterior muscles to alkaline phosphatase histochemistry (Figure 1(A)), and counting the number of capillaries surrounding each fibre. The mean number of capillaries per fibre (CAF) was significantly increased from 5.206 ± 0.149 in the control group, to 6.041 ± 0.187 in the RES-treated mice (Figure 1(B)).

After the staining of serial cross-sections with the anti-MyHC IIB antibody (Figure 2(A,B)), capillaries were counted in fibre dependent fashion. The CAF in type IIB fibres was significantly higher in RES-treated mice compared to control (Figure 2(A,C)), in a measure comparable to what observed when capillaries were analysed independently of the fibre type (Figure 1(B)), suggesting that the increase of CAF was not dependent on a fibre type switch.

Moreover, in control mice aged 8 months and 18 months, the CAF in type IIB fibres did not display a significant difference (Figure 2(C)), suggesting that in this time window the capillary network is not yet affected, and that RES is capable to induce the formation of new capillaries.

Vascular Endothelial Growth Factor (VEGF) expression in skeletal muscles from control and RES-treated mice

Aimed at understanding the mechanisms underlying the increase of the capillaries number, we measured the levels of VEGF, a regulator of blood vessel formation, in the gastrocnemius lysates from RES-treated and untreated animals by means of western blot analysis. We observed that the levels of VEGF did not display any statistical difference (Figure 2(D,E)).

Tubular aggregates, fibre type and capillaries in control and RES-treated mice

In naturally ageing C57BL/6J male mice, TAs develop exclusively in fibres expressing MyHC IIB (Agbulut et al. 2000), therefore we counted the number of TAs in tibialis anterior muscles from treated and untreated mice, and we normalised the number of TAs by the percentage of type IIB fibres for each mouse. According to our previous observations (Toniolo et al. 2018), the normalised number of TAs significantly decreased from 0.50 ± 0.04 in control to 0.34 ± 0.04 in RES-treated mice (Figure 3(A)). The analysis of the correlation between CAF and TAs for each mouse (Figure 3(B)) revealed that, the higher was the number of capillaries per type IIB fibre, the lower was the normalised number of TAs. The regression lines extrapolated from the data had a negative coefficient with a narrow 95% confidence interval of the slope (–0.1993 to –0.01824 for controls, −0.2364 to 0.07112 for RES-treated), suggesting an inverse correlation between

capillaries number and TAs in both control and RES-treated muscles.

Discussion

SKM ageing entails the modification of its morphological and functional properties potentially leading to a condition named sarcopenia, which consists in a decrease of muscle mass with loss of strength and altered fatigue resistance (Rosenberg 1997).

The natural polyphenol RES has been demonstrated to protect the SKM from age-related dysfunctions, ameliorate the histological properties of muscle fibres, and improve resistance to fatigue (Wu et al. 2013; Rodriguez-Bies et al. 2016; Toniolo et al. 2018). The emerging evidence that sarcopenic individuals can present an altered capillary network (Prior et al. 2016; Wan et al. 2017) oriented our work towards the investigation of RES capabilities to affect the capillarization of the SKM fibres. Our data demonstrate that, in the tibialis anterior muscles of naturally ageing mice fed with RES, the mean CAF was increased compared to controls. This increase was independent of the fibre type, since type IIB fibres, that usually have a more limited number of capillaries due their glycolytic metabolism, displayed a significantly higher CAF compared to controls.

RES has been suggested to promote angiogenesis via the Trx-1-HO-1-VEGF pathway (Kaga et al. 2005). However, in our experimental conditions, the levels of VEGF were not significantly different in treated and untreated muscles. This can be the result of the achievement of a steady state due to the long-term treatment, or, since SKM angiogenesis is the result of a fine interplay of molecular actors at different levels, other angiostatic molecules or pathways can be involved in this modification (Gliemann et al. 2014). In this context, beside the Trx-1-HO-1-VEGF pathway, RES has been suggested to positively modify vessels function and angiogenesis by interacting with several pathways that lead to a reduction of the nitric oxide and inflammatory citokines levels, or the maintenance of endothelial cells integrity (Chen and Tseng 2007; Gliemann et al. 2014; Diaz et al. 2016). Not last, the better capillarization could be also the results of upstream effects on hemodynamic properties of the treated subjects (Bresciani et al. 2014), that can positively influence the level of capillarization in the periphery of the organisms as the skeletal muscle district (Sullivan et al. 1990).

In our previous work we showed that supplementation of RES in the diet reduced the number of TAs, which in C57BL/6J male mice develop exclusively in type IIB fibres (Agbulut et al. 2000), probably due to the accumulation of oxidative stress by-products (Schiaffino 2012). The presented data show that control and RES-treated mice fibres have similar, inverse correlation between capillaries number and TAs formation. Although this observation does not provide direct evidences, it suggests that the blood flow could have a major role in the formation of TAs, and that RES could reduce the presence of TAs via the better capillarization, rather than directly act on TAs.

Given that in the SKM the blood flow has a key-role by providing the appropriate oxygen supply, and removing the waste products produced by metabolic processes and contraction events, it can be speculated that a compromised capillarization can affect the cell equilibrium and contribute to the TAs onset. In RES-treated muscles, the improved capillarization allows both efficient oxygen supply and removal of by-products, resulting in a better prooxidant-antioxidant equilibrium that prevents or delays the formation of TAs.

RES has been demonstrated to improve resistance to fatigue (Wu et al. 2013; Rodriguez-Bies et al. 2016; Toniolo et al. 2018). Interestingly, it has been reported that the resistance to fatigue is positively correlated to capillarization (Ballak et al. 2016), and in turn, a poor vascular function is a key factor in the decline of muscle activity in sarcopenic individuals (Prior et al. 2016; Wan et al. 2017). Based on these evidences, it can be speculated that the better resistance to fatigue reported in previous work (Wu et al. 2013; Rodriguez-Bies et al. 2016; Toniolo et al. 2018), is at least partially, ascribable to a better CAF.

Despite the increasing evidences showing that RES improves vascular function (Diaz et al. 2016; Pollack et al. 2017), the effects of RES dietary supplementation in animal models and humans is still poorly investigated and controversial (Gliemann et al. 2014; Ballak et al. 2016; Diaz et al. 2016). Most probably, the dose and the time window of RES administration could be key-factors to observe a beneficial action (Bosutti and Degens 2015; Rodriguez-Bies et al. 2016; Madreiter-Sokolowski et al. 2017). The present study entailed the administration of 50–55 mg/Kg RES per day in the diet for a six-month period (Toniolo et al. 2018). Although the data on RES and RES metabolites distribution in the body for such an experimental protocol have not been deeply investigated, it has been reported that the administration for 15–16 weeks of 400 mg/Kg/day RES results in a range of 10–120 ng/L of RES in the plasma (Lagouge et al. 2006), and the administration of 2.5 mg/Kg/day RES resulted in

0.11 ± 0.09 ng/ml RES (Sun et al. 2007). It is conceivable that plasma levels in the present experimental conditions fall in an intermediate concentration. Interestingly, it has been demonstrated that RES is rapidly absorbed via the gastrointestinal tract, concentrating mainly in liver and kidneys (Vitrac et al. 2003). A 12 weeks treatment with 0.04% RES in the diet, resulted in the accumulation of RES and its derivatives in the liver concentration of 2.17 µmol/Kg tissue (Desquiret-Dumas et al. 2013). Although very few works analysed RES metabolites in the SKM tissue, studies on rat models showed that RES derivatives are found in decreasing concentrations in liver, adipose and skeletal muscle tissue, with the last one presenting about 1/100 of the liver concentrations (Andres-Lacueva et al. 2012). A study entailing a low dose treatment, shows that RES concentration in the liver is 5.59 ± 3.45 nmol/kg, and in the skeletal muscle 0.72 ± 0.50 nmol/kg (Sun et al. 2007). The discrepancies in the data can be ascribable to several factors, such as the mesurement of RES or RES metabolites, the dose provided, and the duration of treatment. In this context, since RES metabolites have been shown to undergo bioaccumulation in the cardiac muscle tissue, directly influencing the functional effects on cardiac hemodynamics (Bresciani et al. 2014), it appears evident that the treatment length is fundamental in the evaluation of RES effects. Further studies are needed to fully understand the most suitable time window for RES administration in the mouse model.

Altogether, the present observations provide the basis for a future investigation on RES ability to positively modulate capillarization, not only in skeletal muscle ageing, but also in other pathologies that involve SKM capillary rarefaction.

Conclusions

The presented data suggest that in the tibialis anterior of ageing C57BL/6J mice, the development of tubular aggregates in type IIB fibres is inversely correlated with the capillarization. The long-term treatment with Resveratrol induces a better capillarization in ageing mice, and a reduction of tubular aggregates, further confirming the capability of Resveratrol to protect the skeletal muscle tissue from age-related alterations.

Disclosure statement

No potential conflict of interest was reported by the author(s). The funding 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.

Author contributions

L. Toniolo and E. Giacomello conceived and designed the experiments. L. Toniolo, L. Formoso, E. Crea and E. Giacomello performed the experiments; L. Toniolo, L. Torelli and E. Giacomello analysed the data; A. Bergamo and G. Sava contributed with helpful advice, instrumentation, reagents and materials; E. Giacomello and L. Toniolo wrote the paper.

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