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RESEARCH ARTICLE

Exercise training in *ad libitum* and food-restricted old rats: effects on metabolic and physiological parameters

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Abstract Aging is accompanied by a decline in the healthy function of multiple organs, leading to increased incidence and mortality from diseases such as cancer and inflammatory, cardiovascular and neurodegenerative diseases. Dietary restriction is the most effective experimental intervention known to consistently slow the aging process and with positive effects on health span in different organisms, from invertebrates to mammals. Age is also associated with progressive decline in physical activity levels in a wide range of animal species: therefore, regular physical exercise could represent a safe intervention to antagonize aging. In this research we explore the effects of exercise training initiated in late middle aged rats fed with different lifelong dietary regimens: one group was fed ad libitum and the second group was subjected to every-other-day fasting. These two groups might represent examples of "normal" aging and "successful" aging. The study shows the effects of exercise and food restriction and their interaction on plasma levels of total antioxidant capacity, lactate, amino acids, and on products of protein oxidation in soleus and tibialis anterior muscles. In addition, we evaluated body composition measurement by bioelectrical impedance analysis and muscle strength by grasping test. Results show that late-onset exercise training has the potential to improve some metabolic and physiological parameters in rats with the same "chronological age" but different "biological age", without negative effects, and highlight the relevance of a personalised and selected exercise protocol, since the responsiveness to exercise may depend on the individual's "biological age".

Keywords Aging · Dietary restriction · Exercise · Rat

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Introduction

Aging is characterized by a complex interaction of stochastic, environmental, genetic and epigenetic variables. This interaction generates the loss of molecular accuracy and therefore a random accumulation of damage in the organism's cells, tissues, or whole organism during life increases: the probability of disease and death also augments in proportion

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(Rattan 2015). Indeed, aging is accompanied by a decline in the healthy function of multiple organs, leading to increased incidence and mortality from diseases such as cancer and inflammatory, cardiovascular and neurodegenerative diseases (Kennedy et al. 2014). In recent years, there has been increasing interest in interventions to develop realistic and practical methods for maintaining health throughout the life span. DR is the most effective experimental intervention known to consistently slow the aging process and extend median and maximum life span with positive effects on health span in different organisms, from invertebrates to mammals (Kennedy et al. 2007). Observational studies suggest that DR may have beneficial effects also on human longevity (Heilbronn et al. 2006; Lefevre et al. 2009). Recent studies indicate that the benefits of DR on aging are conserved in non-human primates (Mattison et al. 2017). Age is also associated with progressive decline in physical activity levels in a wide range of animal species, ranging from the Caenorhabditis elegans worm (Herndon et al. 2002) to humans (Westerterp 2015), with major metabolic consequences (Chow et al. 2007; Westerterp 2013): therefore, regular physical exercise could represent a safe intervention to delay aging (Cobley et al. 2015). The available data strongly indicate that regular exercise plays a preventive role against lifestyle-dependent diseases (Radak et al. 2004; Goto and Radák 2009) and increase mean life span in rodents (Holloszy et al. 1985). On the other hand, the well-documented beneficial effects of exercise occur in a paradoxical background of biochemical framework: it is well known that exercise increases the production of potentially harmful substances such as reactive oxygen and nitrogen species, other free radicals, acids and aldehydes (Alessio et al. 2000; Sahlin et al. 2010; Powers et al. 2016). The paradox arises as to whether exercise would be recommended to aged population since senescent organisms may be more susceptible to increase of potentially harmful substances during exercise.

In order to obtain further evidence that can gain knowledge about the relationship between exercise and aging, in this research we explore the effects of exercise training initiated in late middle aged rats fed with different lifelong dietary regimens: one group was fed ad libitum (AL) and the second group was subjected to every-other-day fasting (EOD). These two groups might represent examples of "normal" aging and "successful" aging: AL rats are well fed laboratory animals as humans living in affluent western societies, and therefore they may be a model of "normal" aging; EOD rats are laboratory animals submitted to DR, the most effective experimental intervention known to consistently slow aging, and for this reason they may represent subjects who have taken measures to achieve a healthy and "successful" aging. The study shows the effects of exercise and food restriction and their interaction on plasma levels of total antioxidant capacity (TAC), lactate, AAs, and on products of protein oxidation in soleus and tibialis anterior muscles. In addition, we evaluated the effects of treatments on physiological parameters: body composition measurement by BIA and muscle strength by grasping test. The metabolic and physiological dataset obtained from rats with the same "chronological age" but different "biological age" might be useful to understand whether exercise training, initiated in late middle age, may improve physical functions, and consequently quality of life of the elderly population.

Materials and methods

Materials

All reagents were of analytical and HPLC grade. Solvents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Standard molecules and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q (Millipore-Lab, Bedford, MA, USA) purified water was used for all analyses.

Animals

Male Sprague–Dawley rats, raised in the Pisa University Interdepartmental Research Centre on Biology and Pathology of Aging Vivarium, were used. All procedures and animal treatment followed the European Community Directive 2010/63/UE and Italian animal welfare laws, guidelines, and policies. All handing and management procedures were approved by the Independent Ethics Committee of the University of Pisa (Approval number: No. 2A/4155).

Animals were kept in a controlled environment (22 °C, 12/12 h light/dark cycle), had free access to water, and fed AL with standard rodent diet (Teklad,

Harlan, Italy) until 2 months of age. At that time, rats were randomly assigned to dietary treatments: one group was fed AL, the second group was subjected to EOD. At 19 months of age, AL and EOD rats were further divided in two sub-groups as: AL sedentary (ALs), AL exercised (ALe), EOD sedentary (EODs), and EOD exercised (EODe).

At 24 months of age, rats were sacrificed under pentobarbital anesthesia (50 mg/kg body weight, i.p.). Food was withdrawn 16 h before experimentation. The age of 24 months for sacrifice was chosen, because it represents approximatively the mean life span for Sprague–Dawley rats fed ad libitum (Masoro 1980).

Blood was collected from the posterior vena cava into test tube containing 0.25 M EDTA, centrifuged, and plasma samples were stored at − 80 °C until analysis. Soleus (slow-twitch muscle, composed predominantly of red fibers) and tibialis anterior (fast-twitch muscle, composed predominantly of white fibers) muscles were dissected out, snap frozen in liquid nitrogen, and stored at − 80 °C until analysis.

Exercise training programme

The exercise protocol was designed in accordance with the basic principles of training in humans: specificity, progressive overload, and variable intensity (Spiering et al. 2008; Goutianos et al. 2015). A strict control of health and animal welfare before, during, and after each training session was performed. Rats were not daily trained, but only three times a week to minimize the potentially evoked stress effects of exercise and to allow the recovery of liver and muscle glycogen.

Exercise was performed using a converted human treadmill with 5 separate running lanes with an incline of 0°; an acrylic block was placed on the ledge at the end of the belt so as to make it difficult for rats to remain there. All rats assigned to trained groups were adapted to walking on a treadmill (2 weeks: three times a week for 20 min at 4 m/min), before the beginning of the exercise protocol. Exercise training programme was composed of two phases with sessions of 30 min three times a week. Training phase 1 (8 weeks) was set with a gradually increasing running speed and time, as follows: weeks 1–2 (4 min at 7 m/min, 6 min at 9 m/min, 10 min at 12 m/min, 6 min at 9 m/min, 4 min at 7 m/min); weeks 3–4 (5 min at 7 m/min, 20 min at 12 m/min, 5 min at 7 m/min); weeks 5–6 (5 min at 9 m/min, 20 min at 15 m/min, 5 min at 9 m/min); weeks 7–8 (5 min at 12 m/min, 20 min at 15 m/min, 5 min at 12 m/min) (Ben et al. 2009, 2010). Training phase 2 (2 weeks) was set with the running speed returned to the level at the beginning of phase 1 to reach adaptation at a stable training load. Training phases 1 and 2 were repeated to complete 5 months of exercise. When necessary for rats to run, their tails were stimulated using a soft bristle brush. To control for the stress of handling and exposure to the treadmill, sedentary animals (ALs and EODs groups) were placed on the stationary treadmill three times a week, 5 min per session, during the length of the study.

Body composition measurement by bioelectrical impedance analysis

The effects of treatments on body composition in fat free mass and fat were measured by BIA. BIA procedure was conducted as described by Skalicky et al. (2001) at the beginning and at the end of exercise training programme. A tetrapolar impedance (model 101 RJL, Clinton T., MI) was used. Three consecutive measurements were performed. The equation validated against chemical carcass analysis by Skalicky et al. (2001) was used for the calculation of fat free mass and fat (in grams).

Grasping test

The grasping test is a simple non-invasive method designed to evaluate rodent forelimb muscle strength in vivo by taking advantage of the animal's tendency to grasp a horizontal metal bar (Smith et al. 1995). The rat was placed over a base plate in front of a grasping bar, and to perform the evaluation the animal was pulled by the tail with increasing force. The rat could seize a grid attached to a force transducer until the animal lost its grip. The bar was attached to a force transducer (Ugo Basile Grip-Strength Meter), and the force produced during the pull on the bar was measured three times during each test. The grasping test was performed at the beginning and at the end of exercise training programme by the same investigator. Results are given as quotient between grip strength and animal body weight.

Plasma analysis

Measurement of total antioxidant capacity

TAC assay considers the cumulative action of all the antioxidants present in plasma and may help in the measurement of physiological, environmental, and nutritional factors of the redox status. TAC was measured by the method based on the absorbance of the stable, colored 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation) ($ABTS^{\cdot+}$), as described by Erel (2004). The reduced ABTS molecule is oxidized to $ABTS^{\cdot+}$ using hydrogen peroxide alone in acetate buffer (30 mmol/L, pH 3.6) and in this solution, the concentrate (deep green) $ABTS^{\cdot+}$ molecules stay stable for a long time. When it is diluted with a more concentrated acetate buffer at high pH values (400 mmol/L, pH 5.8), the color is spontaneously and slowly bleached. Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations. The bleaching rate is inversely related with the TAC of the sample and this reaction can be monitored spectrophotometrically. The reaction rate was calibrated with Trolox, which is widely used as a traditional standard for TAC measurements assays. Results are given as mmol Trolox equivalent/L.

Determination of lactate threshold

Before sacrifice, all rats performed an incremental test (IT) for evaluation of blood lactate threshold (LT) to verify the effect of treatments on physical capacity as described by Pilis et al. (1993) and Carvalho et al. (2005) with modifications. This is considered an important marker of exercise intensity at which the transition from aerobic to anaerobic metabolism occurs. The LT value was determined through identification of the upward inflection point on the blood lactate concentration versus running speed curve. The animals were allowed to rest for at least 30 min in individual cages, with free access to water. After this period, the rats were submitted to an initial warm-up period of 10 min at low speed (3 m/min) to remove the excess blood lactate accumulated during the manipulation preceding the test (Langfort et al. 1996). After a passive recovery during 3 min they were submitted to the IT, starting at the speed of 6 m/min, with an increase of 3 m/min at the end of every 3-min stage (Takahashi et al. 2012). The IT finished when the animal reached exhaustion. Blood samples were collected from the tail vein between each stage. Lactate concentration was determined by an enzymatic–amperometric method, using a Lactate Scout analyzer (SensLab GmbH, Germany). Blood lactate concentration at different steps was plotted as a function of the corresponding running speed. Results are given as mmol/L.

Measurement of amino acid levels

The plasma free AA levels change with exercise, in particular branched chain-AA (BCAA) and aromatic AA (AAAr). BCAAs, which include leucine (Leu), isoleucine (Ile) and valine (Val), are readily metabolized in the muscle. AAAr, including phenylalanine (Phe), tryptophan (Trp) and tyrosine, are degraded into the liver. AA concentrations were assayed using a high-performance liquid chromatography (HPLC) procedure as described by Donati et al. (2009). AA separation was carried out on a 4.6 × 250 mm Bio-Sil ODS-5S column (particle size, 5 mm) in a Beckman HPLC system (equipped with 32 Karat software). AAs were determined by measuring the fluorescence of dansylated derivative with a Jasco spectrofluorometer (340 nm excitation, 525 emission). Norvaline was added as an internal standard to all samples. Levels of BCAA and AAAr are given as mmol/L.

Soleus and tibialis anterior protein carbonyl content

The measurement of protein carbonyl (PC) content has been used as a sensitive assay for oxidative damage to proteins in animal tissue. Muscles were weighed and diluted 20% w/v in potassium phosphate buffer (pH 6.7) with EDTA, and homogenized with an AEG SB2E-680, Germany. The introduction of PC groups into proteins by oxidative mechanisms was assayed by reaction of PC groups with primary amines to form semi-stable Schiff bases through reaction with 2,4-dinitrophenylhydrazine (DNPH), as described by Levine et al. (1990). PC content was calculated using the DNPH molar extinction coefficient (22,000/M/cm). Results are given as nmol/mg protein.

Statistical analysis

The analysis of variance (ANOVA) test was used to evaluate differences among multiple conditions. If positive, the Tukey test was used to test for their statistical significance. Student's t test was used to evaluate differences between two conditions. Values of $P < 0.05$ were considered to be statistically significant.

Results

Rats' characteristics

Data related to body weight and food intake of the rats during the experiment are presented in Table 1. EOD treatment significantly lowered body weight and food intake. In addition to a significant overall age and EOD effect, there was also an age and EOD interaction both in body weight and in food intake. These data were not modified by exercise training programme. Survival percentage of EOD animals at the end of study was 90% and was not changed by training, whereas that of ALs rats was 50% and that of ALe rats was 70%.

Body composition

Body composition changes are shown in Fig. 1. EOD rats kept the body fat content lower compared to AL rats up the end of protocol. No significant differences in body composition between sedentary and treadmill-trained groups were observed, although EODe rats showed an increase in fat free mass respect to EODs rats.

Grasping test

Data obtained from the grasping test are presented in Fig. 2. The grip strength normalized to the animal body weight was similar for the AL rats during the experiment period. EOD rats had values higher respect to AL rats and the difference increased significantly at 24 months of age ($P < 0.01$).

Plasma analysis

TAC

At 19 months of age, before exercise period, plasma TAC concentration in the food-restricted rats was

Table 1 Body weight and food consumption in Sprague–Dawley rats submitted to different diet regimens and exercise

	ALs	ALe	EODs	EODe
Body weight (g)[a,b,c]				
6 months	529 ± 7^{dw}	–	451 ± 5^{dy}	–
12 months	627 ± 10^{ew}	–	497 ± 6^{ez}	–
19 months	673 ± 10^{fw}	673 ± 10^{dw}	507 ± 8^{ey}	507 ± 8^{dy}
24 months	633 ± 16^{efw}	630 ± 14^{dw}	508 ± 7^{ey}	485 ± 8^{dy}
Food intake (g/day)[a,b,c]				
6 months	21.6 ± 0.3^{dw}	–	16.8 ± 0.1^{dy}	–
12 months	23.6 ± 0.2^{ew}	–	17.6 ± 0.2^{dy}	–
19 months	24.2 ± 0.7^{ew}	24.2 ± 0.7^{dw}	16.5 ± 0.2^{dy}	16.5 ± 0.2^{dy}
24 months	22.3 ± 0.5^{dew}	22.0 ± 0.5^{dw}	16.5 ± 0.4^{dy}	16.1 ± 0.4^{dy}

Values represent the mean ± SEM

ALs ad libitum sedentary group, *ALe* ad libitum exercised group, *EODs* diet restricted sedentary group, *EODe* diet restricted exercised group

[a]Significant age effect ($P < 0.01$)

[b]Significant diet effect ($P < 0.01$)

[c]Significant age by diet effect ($P < 0.01$)

[def]Means in the same column across age groups with different superscripts are significantly different ($P < 0.05$)

[wy]Means in the same row across diet groups with different superscripts are significantly different ($P < 0.05$)

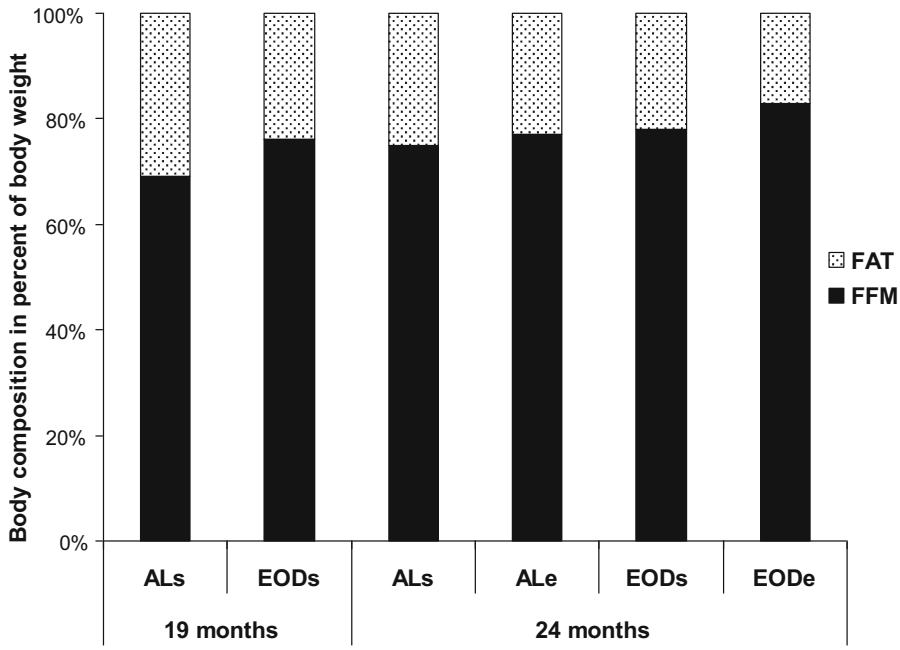

Fig. 1 Changes in body composition in Sprague–Dawley rats submitted to different diet regimens and exercise. Results are given as percent changes in body weight. Results represent the mean of at least five cases. *FAT* fat mass, *FFM* fat free mass, *ALs* ad libitum sedentary group, *ALe* ad libitum exercised group, *EODs* diet restricted sedentary group, *EODe* diet restricted exercised group

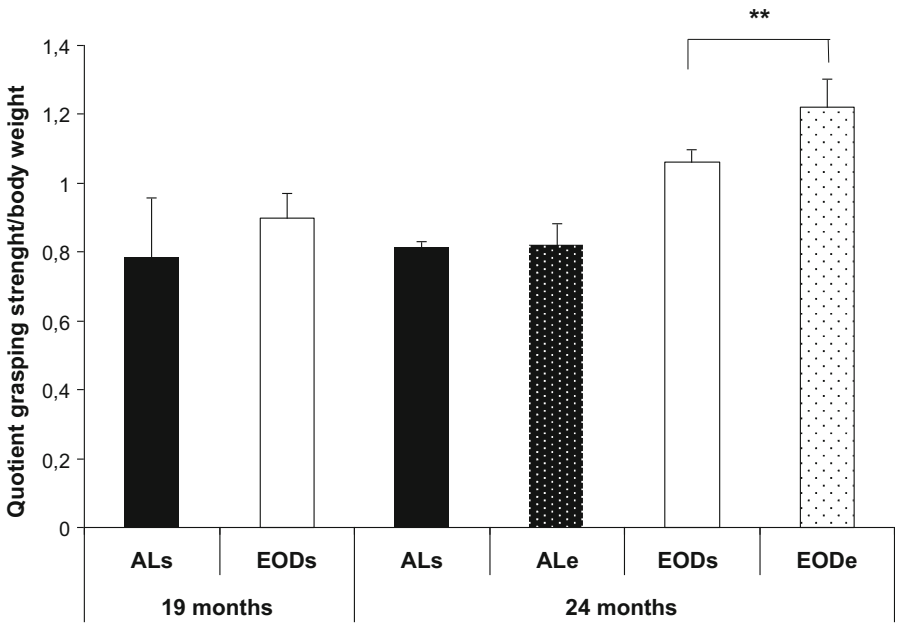

Fig. 2 Grasping force in Sprague–Dawley rats submitted to different diet regimens and exercise. Results represent the mean ± SEM of at least five cases. Two-way ANOVA statistical analysis (age × diet) in sedentary rats—age main effect: N.S; diet main effect: N.S.; age by diet interaction: N.S. Two-way ANOVA statistical analysis (diet × exercise)—**diet main effect: ($P < 0.01$); exercise main effect: N.S. diet by exercise interaction: N.S. *ALs* ad libitum sedentary group, *ALe* ad libitum exercised group, *EODs* diet restricted sedentary group, *EODe* diet restricted exercised group

significantly less than in the fed ad libitum (AL: 0.46 ± 0.04 mmol Trolox equivalent/L; EOD: 0.33 ± 0.02 mmol Trolox equivalent/L; $P < 0.05$). Figure 3 shows that the effect of diet treatment was significant until 24 months of age; no significant age-related change was observed, although there was a

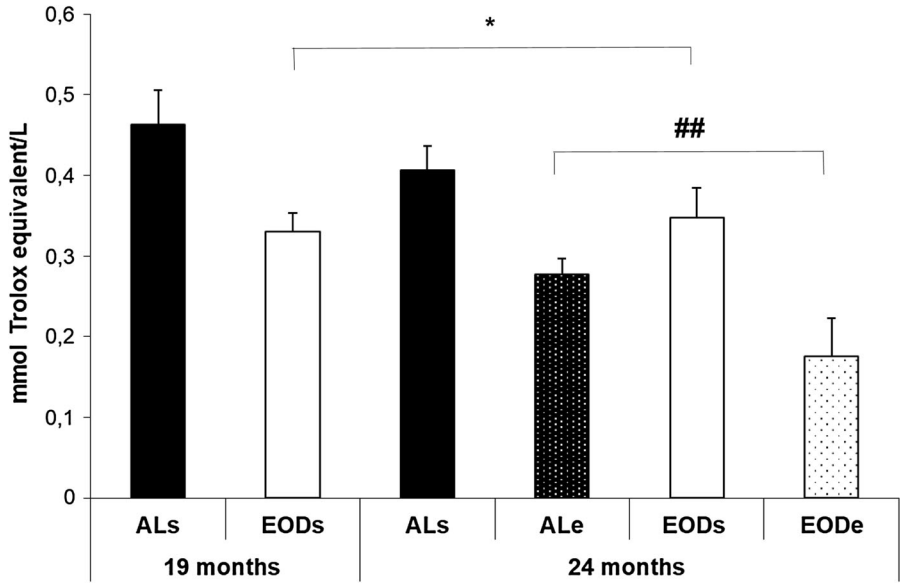

Fig. 3 Effects of diet regimens and exercise on total antioxidant capacity (TAC) plasma concentration in Sprague–Dawley rats. Results represent the mean ± SEM of at least five cases. Two-way ANOVA statistical analysis (age × diet) in sedentary rats—age main effect: N.S; *diet main effect: ($P < 0.05$); age by diet interaction: N.S. Two-way ANOVA statistical analysis (diet × exercise)—diet main effect: N.S.; ##exercise main effect: ($P < 0.01$); diet by exercise interaction: N.S. *ALs* ad libitum sedentary group, *ALe* ad libitum exercised group, *EODs* diet restricted sedentary group, *EODe* diet restricted exercised group

trend toward decreasing in AL rats. Exercise training programme lowered significantly plasma TAC levels in AL and EOD rats ($P < 0.01$), and no significant effect of diet treatment was detected.

LT

Data obtained on evaluation of blood LT are presented in Fig. 4. The lactate concentration at rest was similar in all groups. The velocity at LT was 9 m/min for EODs and ALs groups, 12 m/min for ALe group, and 18 m/min for EODe group. ALs group had reached exhaustion at 15 m/min, whereas ALe and EODs groups reached exhaustion at 18 m/min and only rats in EODe group run until 21 m/min. At the end of the IT test the blood lactate concentrations were lower in the trained groups than in the sedentary ones ($P < 0.05$).

AAs

AA levels are showed in Fig. 5. BCAA levels at 19 months of age were significantly higher in EOD than AL rats ($P < 0.01$, only Ile was not statistically

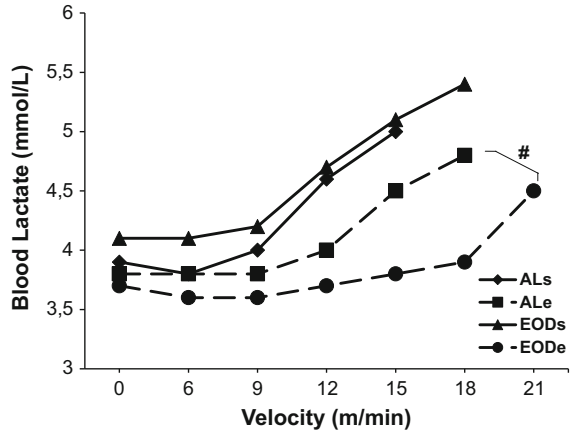

Fig. 4 Determination of plasma lactate threshold in Sprague–Dawley rats submitted to different diet regimens and exercise. Results represent the mean ± SEM of at least five cases (error bars are the same size or smaller than the symbols). Two-way ANOVA statistical analysis (diet × exercise)—diet main effect: N.S.; #exercise main effect: ($P < 0.05$); diet by exercise interaction: N.S. *ALs* ad libitum sedentary group, *ALe* ad libitum exercised group, *EODs* diet restricted sedentary group, *EODe* diet restricted exercised group

significant). At 24 months of age, a significant decrease in BCAA values was observed in AL and

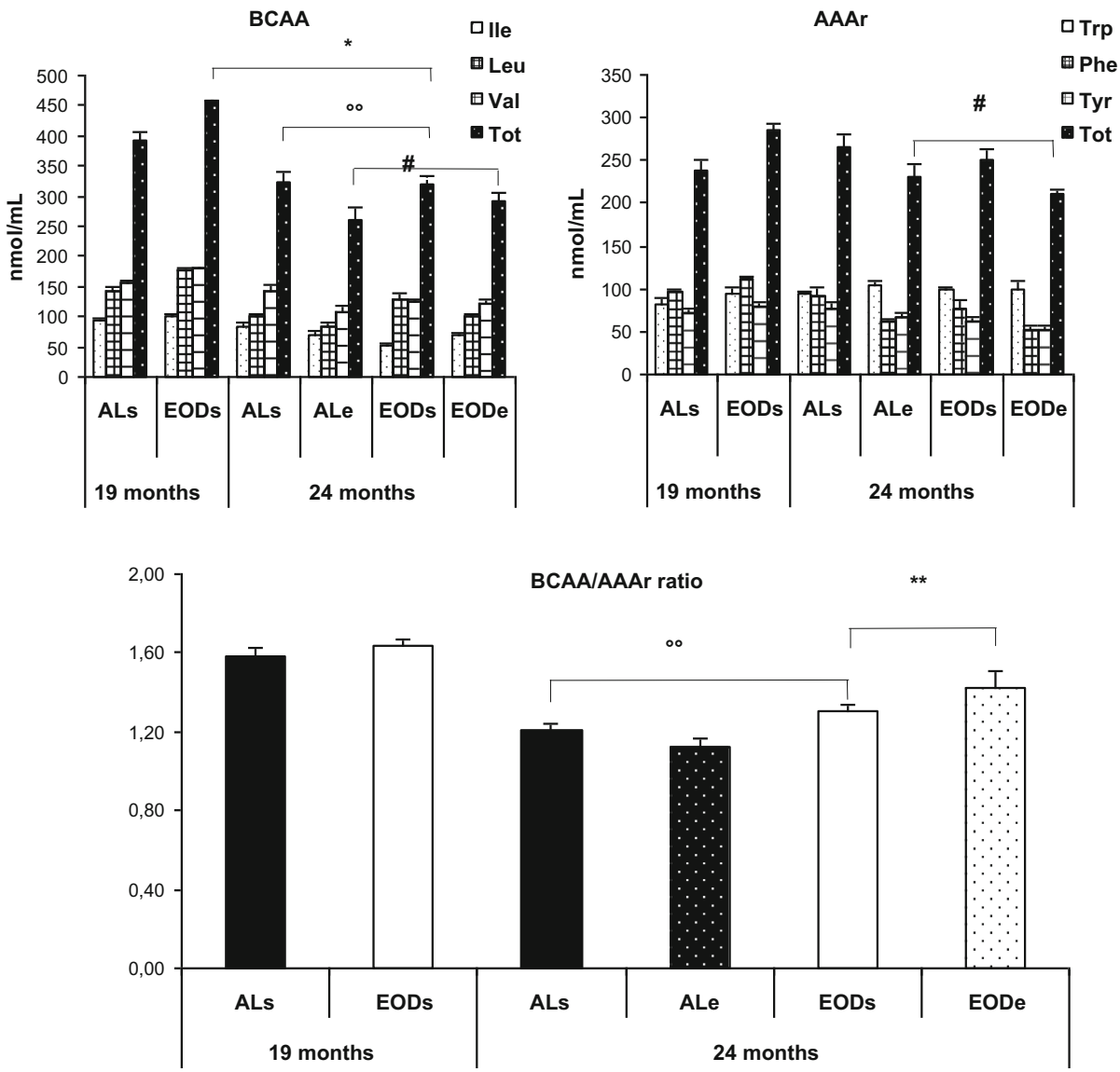

Fig. 5 Effects of diet regimens and exercise on branched chain amino acids (BCAA), aromatic amino acids (AAAr) and BCAA/AAAr ratio in plasma of Sprague–Dawley rats. Results represent the mean ± SEM of at least five cases. Two-way ANOVA statistical analysis (age × diet) in sedentary rats: *BCAA Tot*—°°age main effect: ($P < 0.01$); *diet main effect: ($P < 0.05$); age by diet interaction: ($P < 0.05$); Post-ANOVA Tukey test ($P < 0.05$): 19 months versus 24 months; AL versus EOD. Ile: age main effect: ($P < 0.01$); diet main effect: N.S.; age by diet interaction: ($P < 0.05$); Post-ANOVA Tukey test ($P < 0.05$): 19 months versus 24 months. Leu: age main effect: ($P < 0.01$); diet main effect: ($P < 0.01$); age by diet interaction: N.S. Val: age main effect: ($P < 0.01$); diet main effect: N.S; age by diet interaction: ($P < 0.01$); Post-ANOVA Tukey test ($P < 0.05$): 19 months versus 24 months *AAAr Tot*—age main effect: N.S.; diet main effect: N.S.; age by diet interaction: ($P < 0.05$). Phe: age main effect: ($P < 0.05$); diet main effect: N.S.; age by diet interaction: ($P < 0.05$). Post-ANOVA Tukey test ($P < 0.05$): 19 months versus 24 months *BCAA/AAAr ratio*—°°age main effect: ($P < 0.01$); diet main effect: N.S.; age by diet interaction: N.S Two-way ANOVA statistical analysis (diet × exercise): *BCAA Tot*—diet main effect: N.S.; exercise main effect: ($P < 0.05$); diet by exercise interaction: N.S. Leu: diet main effect: ($P < 0.05$); exercise main effect: ($P < 0.05$); age by diet interaction: N.S *AAAr Tot*—diet main effect: N.S.; #exercise main effect: ($P < 0.05$); diet by exercise interaction: N.S. Phe: diet main effect: N.S.; exercise main effect: ($P < 0.01$); diet by exercise interaction: N.S. *BCAA/AAAr ratio*—**diet main effect: ($P < 0.01$); exercise main effect: N.S.; diet by exercise interaction: N.S. *Ile* isoleucine, *Leu* leucine, *Val* valine, *Phe* phenylalanine, *Trp* tryptophan, *Tyr* tyrosine, *ALs* ad libitum sedentary group, *ALe* ad libitum exercised group, *EODs* diet restricted sedentary group, *EODe* diet restricted exercised group

EOD groups ($P < 0.01$), and the effect of diet treatment was significant only in Leu levels ($P < 0.01$). Exercise training programme lowered significantly BCAA levels in AL and EOD rats ($P < 0.05$), and the effect of diet treatment was significant only in Leu levels ($P < 0.05$).

As far as AAAr are concerned, there were no significant differences in the Trp and Tyr plasma levels in all experimental groups, while Phe values showed a significant decrease with aging ($P < 0.05$) and after exercise ($P < 0.01$). A significant decrease in BCAA/AAAr ratio was observed between 19 and 24 months of age in ALs and EODs rats ($P < 0.01$). After exercise training programme the ratio was significantly higher in EOD than AL rats ($P < 0.01$).

PC groups

The effects of exercise training programme on biomarker of muscle protein oxidation are presented in Fig. 6. PC groups were affected differentially by exercise and diet treatment, depending on muscle type. Exercise did not affect soleus PC levels either in AL or EOD rats, while difference was observed between diet groups: PC content in the AL groups was significantly lower than that in the EOD groups ($P < 0.01$). Exercise induced a significant increase of PC groups in tibialis anterior muscle ($P < 0.05$); the effect of diet treatment was not significant, although a more pronounced increase was observed in the AL group.

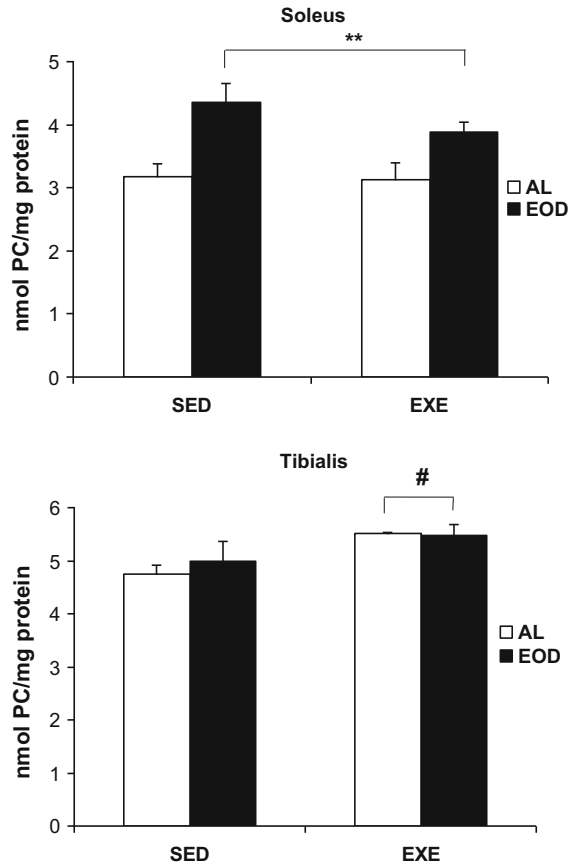

Fig. 6 Effects of diet regimens and exercise on soleus and tibialis anterior protein carbonyl content in Sprague–Dawley rats. Results represent the mean ± SEM of at least five cases. Two-way ANOVA statistical analysis (diet × exercise): *Soleus*—**diet main effect: ($P < 0.01$); exercise main effect: N.S.; diet by exercise interaction: N.S. *tibialis anterior*—diet main effect: N.S.; #exercise main effect: ($P < 0.05$); diet by exercise interaction: N.S. *Sed* sedentary groups, *Exe* exercised groups, *AL* ad libitum rats, *EOD* restricted rats

Discussion

In the present study, the effects on physiological and metabolic parameters of late-onset exercise training were investigated in rats with the same "chronological age" but different "biological age" as a result of different lifelong dietary regimens: the positive effects of DR on longevity and on health span are well known (Liang et al. 2018), but the effects of exercise in late age have not been yet studied in detail.

Physiological parameters

The pattern of changes over time in body weight, food intake and survival percentage are comparable to patters shown in our previous studies on DR effects (Cavallini et al. 2002; Bonelli et al. 2008). Exercise training programme did not cause significant changes, only the survival percentage in AL rats showed an increase: given that our findings is based on a limited number of animals (10 rats for experimental group), the result from such analyses should thus be treated with caution. However, these data support the idea that a regular exercise may beneficial affect and delay biological aging (Radak et al. 2005), even when initiated in old animals (Cobley et al. 2015). The body composition was measured by BIA and was comparable to patterns shown in other studies on the same rat strain (Skalicky et al. 2001): after 19 months of age the fat content tended to stay stable, exercise caused a

slight increase in free fat mass only EOD rats, probably DR preserves lean body mass from age-related modifications as observed by McKiernan et al. (2012) in aged rhesus monkeys. Therefore, muscle fibers from EOD rats might be better poised to endure and adapt to changes like exercise training programme. Interestingly, data obtained with grasping test further support the protective role of DR on skeletal muscle mass: indeed, EOD rats showed a higher grip strength respect to AL rats until 24 months, and the training improved their grip strength. The age-related loss of muscle strength has been defined as dynapenia and was related to deficits in muscle quality and neuromuscular control (Manini and Clark 2012). It is known that the anti-aging effect of DR involved the activation of autophagy, a required function in cell housekeeping during fasting, which can remove damaged macromolecules, organelles and membranes selectively, acting as an alternative source of energy and participating in cell quality control (Bergamini et al. 2007; Hubbard et al. 2012). A recent paper suggests that autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance, and this cell function is very active and more intense when exercise is performed in fasted state (Martin-Rincon et al. 2018).

Plasma metabolic parameters

TAC is a sensitive and reliable marker for detecting plasma changes in vivo oxidative stress that may not be detectable through the measurement of a single, specific antioxidant (Erel 2004), and is used to evaluate a number of physiological conditions in humans and animals (Ghiselli et al. 2000). An interesting finding in this study was that plasma TAC concentrations in EOD rats were less than in AL rats. On the basis of such data, it seems reasonable to suggest that DR-induced decrease in reactive oxygen species (ROS) production may result in a lessening of the requirement for TAC. It has been reported that exercise decreased plasma TAC in rats: our results are in line with these findings (Ficicilar et al. 2003). A growing number of reports indicate that exercise-induced ROS production is required to promote exercise training response in skeletal muscle and contributes to exercise-induced skeletal muscle adaptation (Davies et al. 1982; Gomez-Cabrera et al. 2008).

Blood LT, defined as the point at which blood lactate concentration increases exponentially with increasing exercise intensity, has shown to be a useful tool in the exercise prescription (Billat et al. 2003). Many researches on exercise physiology have been conducted with laboratory animals and the "aerobic/anaerobic" transition has been used to ascertain endurance capability and measure adaptations to training (Gobatto et al. 2001; Billat et al. 2004). Our incremental training was found to be efficient in improving physical fitness of the rats in 8 weeks. The late lactate increase in trained animals suggests improvement of the aerobic metabolism (Sjödin and Jacobs 1981), and the increase in the time of exhaustion indicates improvement in the cardiac performance, through increased cardiac output (Li et al. 2018). Mammalian skeletal muscle fibers are subject to significant changes during postnatal development and aging (Schiaffino and Reggiani 2011) and lactate levels are known to be influenced by the muscle fiber composition (Kitada et al. 2015). During aging, rat skeletal muscles undergo a type 2B to 2X switching in fast muscles and a type 2A to type 1 switching in slow muscles (Larsson et al. 1995). Previous studies have reported a strong relationship between the number of slow muscle fibers and LT value (Ivy et al. 1980). As expected, EOD rats shown better performance than AL rats: probably the fiber type transitions increase the amount of oxidative type I fibers which are more resistant to fasting than type II glycolytic fibers (Wang and Pessin 2013).

Among plasma free AAs, BCAA (Ile, Leu and Val) are key regulators of protein synthesis. Unlike other AAs, BCAA are not metabolized in the liver, and therefore after the ingestion they are almost immediately put into circulation and made available to the body (Dato et al. 2019). BCAA catabolism is mainly located in skeletal muscle and the brain, but also adipose tissue can metabolize substantial amounts of BCAA (Herman et al. 2010). This factor may be responsible for different BCAA plasma levels in AL and EOD rats at the beginning of the experiment: AL rats showed more FFA than EOD, after 19 months of age the fat content tended to remain stable. Age and exercise training lowered significantly BCAA in AL and EOD rats. It is interesting to note that a diet significant effect was observed only on Leu levels: probably the different meal distribution between groups might affect the Leu plasma levels, as

suggested by Norton et al. (2017). In addition, this result highlights that the protective role of DR on skeletal muscle mass might be associated with an effect on Leu levels: Leu is the main stimulator for protein synthesis in the skeletal muscle and improves whole body glucose metabolism, with action on glucose muscle uptake, body weight and food intake (Valerio et al. 2011). Furthermore, the decrease in the BCAA plasma concentrations may be relate to fatigue, especially in old animals. Indeed, no significant changes was observed in Trp levels: Trp is a precursor of serotonin, and some studies have shown that excessive serotonin induced fatigue (Cordeiro et al. 2017). Since the transporter for Trp and BCAA is the same (Fernstrom 2005), when BCAA levels decrease, a larger amount of Trp can entry into the brain and cause fatigue. In this perspective, also BCAA/AAAr ratio showed an age-related decrease: the ratio was significantly higher in EOD rats than AL rats. Unlike BCAA, AAAr undergo liver metabolism process and BCAA/AAAr ratio is used as an indicator of liver function (Holecek et al. 1996): the liver of EOD rats might be affected by "anticipatory activity", when animals show increased locomotor activity 2–3 h before food access, which depend on a food-entrainable oscillator (Díaz-Muñoz et al. 2000). The authors suggest that hepatic metabolism in DR rats is modulated with a different pattern from AL rats, and during "anticipatory activity" the liver of food-restricted animals optimizes the processing of nutrients to daily feeding with an anticipatory function in the control of energy balance. Furthermore, DR may enhance liver autophagy during the longer time period of fasting and remove damaged macromolecules, organelles and membranes selectively, acting as an alternative source of energy (Donati et al. 2001).

PC groups

PC groups are well known as a useful and reliable marker for assessing oxidative stress in skeletal muscle (Çakatay et al. 2003). Our findings show that this marker was affected differentially by exercise and diet treatment, depending on muscle type. Unexpectedly, levels of PC were higher EOD rats, especially in soleus muscle. DR animals are more active than AL animals at all ages, as reported by several studies (Duffy et al. 1989; Yamada et al. 2013). Increased protein oxidation might be associated with an elevated metabolic rate in muscle tissue involved in spontaneous activity. DR increases the turnover of cell components and disposal of damaged protein or organelles by autophagic and proteasomal degradation (Bonelli et al. 2008; Hubbard et al. 2012): therefore, the accumulation of damaged protein should be reduced. The age-related muscle fiber type transitions and metabolic shifts in aging muscle can offer an explanation for the increase of PC groups (Larsson et al. 1995; Schiaffino and Reggiani 2011), especially in slow-twitch muscles, rich in myoglobin and oxidative enzymes. Furthermore, PC groups may be considered intermediate products of oxidation, since further oxidation and cross-linking results in the formation of fluorescent age pigments (Sitte et al. 2000). In the AL animals, where autophagic and proteasomal degradation are impaired, oxidized proteins might remain more time within cells and be subjected to more modifications and cross-linking, lowering free PC groups to be detected by the assay. However, exercise does not seem to change a pattern already defined by age and diet treatment, although in EOD soleus muscle was observed a decrease of PC groups, probably training might further improve the activity of proteasomal complex (Radak et al. 2019). The increase of PC levels in AL and EOD tibialis anterior muscle may highlight the major susceptibility of type II fibers to aging, both in natural aging than accelerated-mimetic aging models (Wang and Pessin 2013; Yanar et al. 2019).

Conclusion

A considerable amount of knowledge regarding the relationship between exercise and aging is derived from animal research, particularly rodents. In this regard, our results show that late-onset exercise training has the potential to improve performance and metabolic parameters in rats with the same "chronological age" but different "biological age". Furthermore, our results indicate that physical exercise does not have negative effect on the majority of the testing parameters, does not increase the difference between animals with different "biological age", and might be useful to separate the influence of exercise from those that occur solely due to aging. Interestingly, a recent study shows that rat responses to exercise adequately reflect human ones in blood

parameters linked to various organs, tissues, functions, and diseases (Goutianos et al. 2015). Based on such data, our results might represent a spur for future studies, using rat model to understand how exercise training, initiated in late middle age, may improve physical functions, and consequently quality of life of the elderly population. Many critical questions still remain regarding the relationship of aging and exercise, but our results highlight the relevance of a personalised and selected exercise protocol, since the responsiveness to exercise may depend on the individual's "biological age".

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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