HIGH VOLUME EXERCISE TRAINING IN OLDER ATHLETES INFLUENCES INFLAMMATORY AND REDOX RESPONSES TO ACUTE EXERCISE

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Abstract

To examine whether the volume of previous exercise training in older athletes influences inflammatory, redox and hormonal profiles at rest and in response to acute exercise, forty trained marathon runners were divided into higher-volume (HVG, ~480 min/week) and lower-volume groups (LVG, ~240 min/week). Plasma inflammatory proteins, redox biomarkers and salivary testosterone and cortisol, were assessed at rest and following two maximal acute exercise bouts. At rest, the LVG exhibited higher C-Reactive Protein (CRP), higher protein carbonyls and lower super-oxide dismutase (SOD) activity compared to the HVG (p's<0.05). In response to exercise, Tumour Necrosis Factor (TNF)-α declined similarly in both groups whereas CRP increased differentially (+60% LVG; +24% HVG; p's<0.05). Protein carbonyls decreased and thiols increased similarly in both groups, but SOD declined differentially between groups (−14% LVG; −20% HVG; p's<0.05). Salivary testosterone decreased similarly in both groups, whereas cortisol did not change. To summarise, a higher volume of past exercise training is associated with favorable inflammatory and redox profiles at rest, perhaps mediated by smaller inflammatory responses to acute exercise.

Keywords: aging; exercise training; cytokines; cortisol; testosterone; redox.

Introduction

An aim of sports training is to improve performance, but sometimes this goal is not reached if training intensity, duration, and length of recovery is not appropriate, leading to maladaptive changes in physiology (Gleeson, 2002; Kellmann, 2010; Meeusen et al., 2013). This process includes changes in immunological, inflammatory and hormonal parameters that can be measured at rest, over the course of a day, or in response to acute exercise (Gleeson, 2002; Kellmann, 2010; Meeusen et al., 2013). Although the effects of high volume of training have been well described in young adults, there is limited data for elderly athletes, and it is conceivable that these processes might occur differentially across the life course, perhaps even modulating aging processes.

Aging is associated with a progressive change in most aspects of physiology, including impairments to the cardiovascular, musculoskeletal, immune and endocrine systems (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013). Although many processes implicated in aging and disease are improved, or at least limited, by moderate to high volumes of exercise, evidence is beginning to show that some aging processes might be accelerated by very high volumes of exercise (Simpson et al., 2016; Turner, 2016; Turner, Bennett, Bosch, Griffiths, & Aldred, 2014). For example, it is possible that the prolonged inflammatory profile seen during periods of very high volume training or following extreme exercise (Turner et al., 2014), which in middle-aged or elderly individuals, would be superimposed on the age-associated increase in inflammation, could exacerbate the decline in immune function known as "immunosenescence" (Simpson et al., 2016; Turner, 2016). Even with acute bouts of exercise, there appears to be a threshold by which some damage can occur. For example, it has been shown that exercise-induced lymphocyte apoptosis only occurs at an intensity of around 60% of VO_2max (Navalta, Sedlock, & Park, 2007). Thus, optimizing training loads, and establishing methods to assess the effects of inappropriate volumes and intensities of exercise, might be even more important for middle-aged or elderly athletes compared to their younger counterparts. Perturbations in inflammatory and endocrine parameters, measured during periods of heavy training, and in particular, in response to acute bouts of exercise, have been linked to inappropriate training loads in athletes. These measurements include the magnitude of the exercise-induced change in cortisol and testosterone

levels, as well as plasma inflammatory markers (e.g. IL-6) (Meeusen et al., 2013; Urhausen, Gabriel, & Kindermann, 1998). It is thus hypothesized that high-volume training would exacerbate both inflammatory and endocrine responses to acute bouts of exercise during aging.

One factor implicated in aging, which is also influenced by high volumes of exercise, is altered redox balance: an increase in the levels of reactive oxygen species (ROS) that can sometimes overwhelm our antioxidant defenses leading to oxidative stress (Gutteridge & Halliwell, 2000, 2010; Halliwell, 1996). The antioxidant system is divided into non-enzymatic defenses (e.g., ascorbic acid) and enzymatic defenses, including superoxide dismutase (SOD) and glutathione peroxidase (GPx) that are present within cells but also in extracellular fluids, such as plasma (Gutteridge & Halliwell, 2000; Halliwell, 1996). SOD has an important role of catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide, which is further converted to water by the enzyme catalase (Gutteridge & Halliwell, 2000; Halliwell, 1996). GPx reduces peroxides and hydroxyl radicals into non-toxic forms by concomitant oxidation of reduced glutathione (GSH) into an oxidized form, glutathione disulfide (GSSG) (Gutteridge & Halliwell, 2000; Halliwell, 1996). Measuring the functional capacity of these antioxidant molecules might indicate the ability to cope with large productions of ROS, which in turn can be estimated by assessing the characteristics of plasma proteins. These measurements include plasma protein carbonyls, formed by the oxidation of protein carboxyl groups or by oxidative cleavage of proteins (Berlett & Stadtman, 1997), or the levels of protein thiol groups, which have antioxidant properties (Griffiths et al., 2002). Aging has been associated with the cumulative effects of ROS in most tissues, potentially caused by, or leading to, an impaired antioxidant defense system, resulting in an accumulation of oxidized proteins, lipids and DNA (Finkel & Holbrook, 2000; Jacob, Noren Hooten, Trzeciak, & Evans, 2013). Like aging, prolonged and intense exercise can result in oxidative stress, possibly from the overproduction of ROS by contraction of skeletal muscles, excessive inflammatory activity, and periods of ischemia and reperfusion (Alessio, Goldfarb, & Cutler, 1988; Powers & Jackson, 2008; Radak, Zhao, Koltai, Ohno, & Atalay, 2013; Reid, Shoji, Moody, & Entman, 1992; Sjodin, Hellsten Westing, & Apple, 1990; Vina et al., 2000). It is thought that because endurance exercise can increase ROS production by skeletal muscles, habitual exercise training may upregulate

antioxidant defense system, especially in muscle (Powers, Radak, & Ji, 2016). For example, it has been shown that six months of resistance training attenuated exercise-induced lipid peroxidation in the elderly, likely due to an increase in antioxidant capacity (Vincent, Vincent, Braith, Lennon, & Lowenthal, 2002). Furthermore, a sixteen week progressive endurance training program in inactive older men lowered lipid peroxidation (MDA) and 3-nintrotyrosine (3-NT), and increased GPx and total antioxidant capacity (Fatouros et al., 2004).

The present study investigated whether the volume of habitual exercise training, in athletes over 60 years of age, influences inflammatory, redox and hormonal profiles at rest and in response to the accumulated effect of two maximal exercise bouts separated by four hours of rest. We hypothesize that high-volume trained athletes (480 min/week) would report larger inflammatory, redox, and neuroendocrine responses to acute exercise as compared to low-volume trained athletes (240 min/week).

Methods

Subjects

The sample size required for the present study was calculated utilizing G*Power software (version 3.1.9), based on previous studies that analyzed the effects of training volume in older subjects (Cannon & Marino, 2010). Results indicated that 11 subjects in each group would provide a statistical power greater than 0.85 for all variables. Forty-two physically active male and female marathon runners were recruited for this study (Table 1). Recruitment criteria consisted of being at least 60 years of age, and if female, being in the post-menopausal period. All participants self-reported to eat a normal diet. Exclusion criteria were the presence of osteoarthritis, joint diseases, heart disease, gastrointestinal disease, liver disease, autoimmune diseases, infections during the last two weeks, presence or history of neoplasias, neurodegenerative diseases, mood disorders, severe orthopedic or lung disorders, medication use (beta blockers, glucocorticoids, antidepressants, etc.), and supplementation with proteins, vitamins, minerals or antioxidants.

Participants were divided into two groups. A higher-volume training group (n=21) consisted of individuals who had both taken part in marathons in previous years (2011, 2012, 2013 or 2014) and who undertook more than 24 h of training per month. A lower-volume training group (n = 21) consisted of individuals who self-reported to undertake less than 24 h per month of training using a questionnaire (Prompt Questions for Physical Activity; developed at Anhembi Morumbi University, School of Medicine).

From the forty-two individuals enrolled in the study, two had signs of myocardial ischemia in response to the first bout of exercise, and were excluded from the study and all analyses. Thus the higher-volume group consisted of nineteen individuals and the lower-volume group consisted of twenty-one individuals. All participants were fully informed about the procedures and possible risks involved before providing written and informed consent. The study was approved by an Institutional Review Board (reference: 293-035). All experimental procedures were in accordance with the declaration of Helsinki regarding human experimentation.

Pre-experimental procedures

Prior to arriving at the laboratory, participants were instructed to refrain from consuming alcohol and caffeine for 48 hours, refrain from undertaking exercise for 24 hours, and to eat their habitual diet. All participants were regular caffeine drinkers and consumption was not restricted prior to this study. Participants arrived at the laboratory at midday, in a fed state, having eating their habitual breakfast at least two hours before. Participants rested for 10 min before anthropometric data were collected, including weight, height and body composition using skinfold calipers. Percentage body fat was calculated with the Petroski (1996) formula for men (Benedetti, Borges, Petroski, & Goncalves, 2008; Pereira, da Silva, Santos, Petroski, & Geraldes, 2013; Vasconcelos Fde, Cordeiro, Rech, & Petroski, 2010) and the Tran & Weltman (1989) formula for women (Tran & Weltman, 1989). The short version of POMS (Profile of Mood States, the Brunel Mood Scale) was used to assess psychological state (Table 1) (Kellmann, 2010; Purvis, Gonsalves, & Deuster, 2010; Shacham, 1983).

Experimental protocol

Participants were observed in a controlled laboratory setting over approximately 4.5 hours whereby inflammatory, redox and hormonal profiles were characterized at rest, and after undertaking two maximal bouts of exercise, separated by four hours. The rationale for this design was to cause a disturbance to allostasis by the accumulated effect of two maximal physiological stressors, interspersed with a short and controlled recovery period, that may be insufficient for individuals already exhibiting disruptions to allostasis (e.g., perhaps due to regular very high-volume exercise training).

After undertaking anthropometric measurements, a resting blood sample was collected and participants provided a saliva sample (see: blood and saliva collection). Participants then undertook the first of two standardized maximal exercise tests on a treadmill. Each exercise test consisted of walking at 3.4 km h^{-1} 0% incline as a warm-up for five minutes, followed by an intensive ramp protocol, whereby the treadmill speed increased by 0.1 km h^{-1} every 4 sec (i.e., a 1.5 km h^{-1} increase each minute) until exhaustion. Throughout exercise, heart rate was monitored by electrocardiography and ratings of perceived exertion were recorded using the Borg scale.

After the first exercise test, participants rested for 4 h in the laboratory whereby only light stretching or seated rest was allowed. Participants did not consume any food during this period, but were asked to drink 500 mL of water. Following this recovery period, a second exercise test, identical to the first, was undertaken. Upon exhaustion, participants returned to a seated position, and after a 5 min period of rest, a venous blood sample was collected and participants provided an un-stimulated saliva sample (see blood and saliva collection). Test-rest reliability was not determined.

Blood and saliva collection

Blood samples were collected by venepuncture of an antecubital vein and five milliliters of blood was drawn into an EDTA tube. Immediately after collection, blood samples were centrifuged at 400g for 5 minutes to aliquot plasma. Un-stimulated saliva samples were collected using sublingual

cotton (3 minute collection), and transferred into a tube without preservatives. Saliva was centrifuged at 400g for 5 minutes for separation from the cotton, and the supernatant was aliquoted. All samples were stored at −80°C until analysis.

Total plasma protein determination

Plasma protein concentration was determined with the Bradford assay (Bio-Rad, Hercules, CA) and used to normalize enzymatic and oxidative damage measurements. Data were expressed as μg/μL.

Plasma inflammatory proteins

Plasma levels of C-reactive protein (CRP, Cat.# KHA0031), Interleukin (IL)-6 (Cat.# KHC0061) and Tumour Necrosis Factor (TNF)-α (Cat.# KHC3011) were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions (Invitrogen, CA, USA). All samples were assayed in duplicate. Detection limits were 10 pg/mL (CRP), 2 pg/mL (IL-6) and 1.7 pg/mL (TNF-α). The intra- and inter-assay coefficients of variation were less than 10%.

Activity of antioxidant enzymes

Superoxide dismutase (SOD) and Glutathione Peroxidase (GPx) enzyme activity was measured in plasma. The activity of SOD was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation with the absorbance at 480 nm measured using a spectrophotometer. All samples were assayed in duplicate and values were expressed as units of SOD activity per milligram of protein. The activity of GPx was assessed using t-butyl hydroperoxide and GSH as substrates with the absorbance measured at 340 nm uisng a spectrophotometer. All samples were assayed in duplicate and values were expressed as units of GPx activity per milligram of protein (Wendel, 1981). The intra- and inter-assay coefficients of variation were less than 10%.

Oxidative stress biomarkers

Plasma protein carbonylation was measured based on the reaction with dinitrophenylhydrazine (DNPH) (Levine et al., 1990). Briefly, proteins were precipitated by the addition of 20 % trichloroacetic acid (Wajswelner, Metcalf, & Bennell) and solubilized in DNPH. All samples were assayed in duplicate, with absorbance measured at 370 nm using a spectrophotometer and the values expressed as nmol of carbonyls per milligram of protein. Total plasma reduced thiol (-SH) concentration was measured by diluting plasma 1:1 in PBS followed by incubation for 60 minutes at 25 °C with 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB or Ellman's reagent) in ethanol. All samples were assayed in duplicate, absorbance was measured at 412 nm using a spectrophotometer, and values expressed as mmols of SH per milligram of protein (Ellman, 1959). The intra- and inter-assay coefficients of variation were less than 10%.

Salivary cortisol and testosterone

Samples were analyzed in duplicate by radioimmunoassays (Coat-A-Count® Cortisol Kit - Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) according to manufacturer instructions. The sensitivity of these assays was 0.1 nM. All samples were assayed in duplicate and values were expressed as nmol/L. The intra- and inter-assay coefficients of variation were less than 10%.

Statistical analysis

All variables tested for normal distribution using the Kolmogorov-Smirnov test. For continuous variables, the differences between groups were analyzed by two-way ANOVA for determining both Group (higher-volume vs. lower-volume) and Time (baseline vs. post-exercise) effects. At baseline, differences between groups were assessed by independent t tests. Effect sizes are reported as eta-squared (η^2). Conventionally, η^2 values of 0.01, 0.06 and 0.14 are considered small, medium and large effect sizes, respectively. Non-normally distributed variables were log transformed.

Correlations were determined using Spearman's rank correlation coefficient (r_s). Statistical significance was accepted as $p<0.05$. Data were analyzed using the Statistical Package for Social Sciences 20 (IBM SPSS Inc., Chicago, IL, USA).

Results

Participant characteristics at baseline

The characteristics of the participants at baseline are presented in Table 1. The higher-volume group self-reported significantly more exercise training per month ($p<0.0001$), longer distance of running per week (<0.0001), and higher speed ($p<0.001$) than the lower-volume training group. There were no significant differences in terms of age, number of males/females, body mass index, and % body fat. Both groups exhibited favorable psychological profiles, as shown by high values of the vigor construct and the low values of the confusion and tension constructs.

At baseline, plasma IL-6 and TNF-α were similar between groups, however, the lower-volume training group exhibited higher CRP levels (+17.85%, $p<0.0001$) compared to the higher-volume group (Table 1 and Figure 1). At baseline, both groups exhibited similar plasma total thiol levels and glutathione peroxidase activity, but plasma protein carbonyl concentration was greater (+23%, $p=0.07$), and SOD activity was less (−4%, $p=0.06$) in the lower-volume group compared to the higher-volume training group. Both groups exhibited similar salivary levels of stress-related hormones (cortisol and testosterone) at baseline (Table 1). Total plasma protein concentration did not differ between groups at baseline: lower-volume (8.31±1.23 μg/μL) vs. high–volume (8.13±1.33 μg/μL; $F=0.17$, $p=0.68$).

Exercise test characteristics

The higher-volume training group maintained a higher top speed compared to the lower-volume group during the first and second exercise tests (Group effect; $p<0.0001$) (Table 2). There were no Time or Group × Time interaction effects for running speed. No differences in exercise

duration, heart rate or ratings of perceived exertion were found in the response to each test in either group (Table 2).

Inflammatory response to exercise

Plasma TNF-α levels decreased over time from baseline to the sample collected after the second exercise test in both groups (Time effect: $F=6.15$, $p=0.01$, $\eta^2 = 0.08$; Figure 1A). Plasma IL-6 did not change over time in either of the groups (Figure 1B). There were neither Group effects nor Group × Time effects for these cytokines. CRP levels increased significantly over time in both groups (Time effect: $F=83.82$, $p<0.0001$, $\eta^2 = 0.53$). The magnitude of this change in CRP was greater in the lower-volume group (+60% increase) compared to higher-volume group (+24% increase) (Group × Time interaction: $F=20.62$, $p<0.0001$, $\eta^2 = 0.22$; Figure 1C).

Oxidative stress biomarkers and antioxidant enzyme activity

In both groups, plasma protein carbonyl concentration decreased over time from baseline to the sample collected after the second exercise test (Time effect; $F=8.10$, $p=0.006$, $\eta^2 = 0.11$; Figure 2A), whereas plasma total thiol levels increased (Time effect; $F=6.34$, $p=0.01$, $\eta^2 = 0.09$; Figure 2B). There were no group effects for protein carbonyl or total thiol levels. A Group × Time interaction was identified for carbonyl levels, although this only approached statistical significance ($F=3.37$, $p=0.06$, $\eta^2 = 0.05$). Plasma SOD activity decreased over time in both groups (Time effect; $F=91.24$, $p<0.0001$, $\eta^2 = 0.66$; Figure 2C) and there was a Group × Time interaction ($F=4.34$, $p<0.05$, $\eta^2 = 0.09$), indicating a larger magnitude change for SOD activity in the higher-volume training group (−20%) as compared lower-volume training group (−14%). No Group effects were observed for SOD levels. No differences were observed in GPx activity (Figure 2D). Total plasma protein concentration increased significantly following exercise (Time effect: $F=117.19$, $p<0.0001$, $\eta^2 = 0.62$), however with similar magnitude in both groups (Group × Time interaction: $F=0.002$, $p=0.97$): low-volume (13.32±2.70 μg/mL) vs. high-volume (13.11±2.54 μg/mL). However, analysis of protein carbonyls, thiols, SOD and GPx are

expressed relative to protein concentration, thus the changes in total protein concentration are not driving the changes reported above.

Salivary hormonal responses to exercise

Salivary testosterone decreased (−55%) from baseline to the sample collected after the second exercise test in both groups (Time effect: F=25.95, p<0.0001), and consequently, as cortisol levels did not change, there was an increased cortisol/testosterone ratio (+90%) after the second exercise test for both groups (Time effect: F=8.16, p=0.005; Table 3). There were no Group or Group × Time effects for salivary measurements.

Relationships between variables

We next sought to investigate whether there were correlations between previous training volume inflammatory, redox and endocrine variables. As expected, training volume was positively correlated with total distance ran per week (r_s = 0.61, p<0.0001), top speed (r_s = 0.42, p<0.006), and negatively correlated with average time spent per km (r_s = −0.42, p<0.006) (data not shown). Although training volume was negatively correlated with CRP levels (r_s = −0.42, p<0.006), no other associations were observed for the remaining inflammatory, endocrine and redox variables (data not shown).

Discussion

The present study shows that the volume of exercise training undertaken by older athletes appears to influence the inflammatory and redox response to the accumulated effect of two maximal bouts of acute exercise. Adults engaged in lower-volume training (approximately four hours per week) exhibited a larger exercise-induced inflammatory response, whereby CRP increased by 60% following exercise, compared to a 24% increase exhibited by adults engaged in higher-volume training (approximately eight hours per week). In addition, the activity of the antioxidant enzyme SOD declined post-exercise in both training groups, but to a different magnitude based on training history: a −14% decline in the lower-volume group, but a −20% decline in the higher-volume group. All other

responses to exercise (e.g., plasma TNF-α, IL-6, protein carbonyl and protein thiol levels, GPx activity, and salivary testosterone and cortisol) were similar between groups.

This investigation also confirmed that older athletes engaged in regular higher-volume exercise, exhibited lower levels of systemic inflammation (e.g., as shown by plasma CRP) at baseline compared to the lower-volume training group. This finding is consistent with previous studies that reported an inverse relationship between cardiorespiratory fitness and CRP in adults (Church et al., 2002; Stewart et al., 2007). In addition, CRP has been associated with endothelial injury and increased risk for developing coronary heart disease (Joshi et al., 2012; Pai et al., 2004). Resting plasma CRP values of <1.0 mg/L, 1.0-3.0 mg/L and >3.0 mg/L are typically considered to represent low, average and increased risk for cardiovascular disease respectively (Ridker, 2003). The CRP levels of approximately 0.24 – 0.64 mg/L in the present study indicate that the individuals examined are very healthy, which might be expected considering the study population undertook 2-4 times the recommended volume of exercise per week (World-Health-Organisation, 2010). Thus, it might be concluded by measuring CRP in plasma at rest, that even very high volumes of exercise (i.e., approximately eight hours per week in the higher-volume group) does not exacerbate the age-associated increase in inflammatory activity, but instead might prevent "inflammaging" which has been associated with frailty, cardiovascular disease, and overall mortality in older adults (Franceschi et al., 2000; Koenig et al., 1999). The plasma TNF-α and IL-6 baseline levels in this study are in within a similar range to those previously reported in healthy older adults at rest (Bruunsgaard, Bjerregaard, Schroll, & Pedersen, 2004; Forsey et al., 2003; Lima et al., 2015; Toft et al., 2002): TNF (1.5 – 50 pg/mL) and IL-6 (2 – 50 pg/mL). Considering that biomarkers of inflammation have been negatively associated with cardiorespiratory fitness, muscle mass, and muscle function (Beyer, Mets, & Bautmans, 2012; Della Gatta, Garnham, Peake, & Cameron-Smith, 2014), then high-volume exercise interventions would reduce morbidity and mortality from diseases with an inflammatory etiology (Beyer et al., 2012; Koenig & Wanner, 1999).

Other investigations have shown that a single session of acute vigorous exercise increases the systemic levels of TNF-α, IL-6 and acute phase reactants such as CRP in untrained adults (Brown,

Davison, McClean, & Murphy, 2015). For instance, it has been shown that plasma IL-6 levels increase up to more than 100-fold following concentric or eccentric exercise in young adults (Pedersen & Bruunsgaard, 2003). Muscle contractions induce production and release of IL-6, but not TNF-α, into the circulation, in both young and older adults. In the present study, exercise did not change the levels of IL-6 and there was a small decrease in TNF-α in the higher-volume training group. This could also be interpreted as reflecting an impaired inflammatory response to a stressor. In support, it has previously been shown that the IL-6 response to eccentric exercise is less pronounced in older adults compared with young subjects (Toft et al., 2002). In addition, plasma IL-6 was not correlated with muscle damage in the elderly, supporting the hypothesis that aging is associated with impaired repair mechanisms including cell migration (Toft et al., 2002). In addition, a previous study has also suggested that the exercise-induced increase in plasma IL-6 in response to habitual exercise is attenuated by previous exercise training (Croft et al., 2009).

In the present study, the lower-volume training group exhibited at baseline higher levels of protein carbonyls (+23%) and lower SOD activity (−4%) compared to the higher-volume group. This finding might indicate that the individuals undertaking approximately four hours of exercise per week (i.e., the lower-volume group) were less adapted to their training, or at least exhibited more exercise-induced muscle damage than the individuals undertaking approximately eight hours of exercise per week. Exercise-induced muscle damage is associated with prolonged deterioration of muscle strength, edema, oxidative stress, recruitment of inflammatory cells with increased secretion of pro-inflammatory cytokines, and leakage of muscle proteins into circulation (Fatouros & Jamurtas, 2016). In addition, such exercise-induced damage to muscles is accompanied by increased circulatory levels of pro-inflammatory cytokines (e.g. TNF-α and IL-1β), indicating that muscle damage may cause cytokine secretion by cells other than muscle (Suzuki et al., 2002; Toft et al., 2002). In the present study, the older athletes engaged in lower-volume training exhibited a higher CRP at rest and a *larger* increase in plasma CRP following acute bouts of exercise than those engaged in higher-volume training. Data presented here highlight two key points: (i) regular high-volume exercise appears to decrease systemic inflammation measured at rest, and (ii) lower volumes of exercise in older adults

may result in higher stressor-induced increases in certain inflammatory markers, as shown by measuring acute phase reactants such as CRP.

In response to acute exercise, however, SOD activity post-exercise declined to a significantly different level based on training history: a −14% decline in the lower-volume group, but a −20% decline in the higher-volume group. Individuals that follow a regular program of exercise have positive changes in antioxidant systems (Radak, Taylor, Ohno, & Goto, 2001), as shown here by the increased SOD and lower carbonyl levels in the higher-volume training group at baseline. In a similar study, a high fitness level group of older adults (66 years) had increased SOD and GPx both at rest and following an acute bout of acute exercise as compared to a low fitness level group (Bouzid, Hammouda, Matran, Robin, & Fabre, 2015). In addition, SOD activity in response to acute eccentric exercise was found to be significantly higher in young (20 years) compared to older adults (58 years) (Nordin, Done, & Traustadottir, 2014), suggesting that signal transduction in response to acute exercise may be impaired with aging. In the present study, due to the characteristics of the exercise bouts, it is more likely that the activity of SOD declined because ROS production was low (Di Meo & Venditti, 2001). It has been hypothesized that repeated periods of oxidative stress, as seen with some forms of exercise, may be needed to increase resistance to oxidative stress in older individuals (Nordin et al., 2014). Furthermore, it has been shown that older adults who exercise regularly had a lower oxidative profile and better ability to resist to an oxidative challenge (Traustadottir et al., 2012). The implications of these findings are unclear. Despite a decline in SOD activity post-exercise, other evidence from this study suggests that there was, by other mechanisms, an adaptive exercise-induced "antioxidant response" (Turner et al., 2013; Wadley et al., 2015). For example, in response to exercise, plasma total thiol concentration increased in both groups to a similar magnitude. The assay employed in this study is a non-specific measure of all thiol groups in plasma, to which both free and bound thiols, likely originating from cysteine, homocysteine, reduced glutathione, cysteinylglycine or albumin, will be detected (Biswas, Chida, & Rahman, 2006; Giustarini, Dalle-Donne, Lorenzini, Milzani, & Rossi, 2006; Rossi, Giustarini, Milzani, & Dalle-Donne, 2009). However, considering only reduced thiols are detected by this assay (Ellman, 1959; Griffiths et al., 2002), and that molecules such

as cysteine have a strong reducing capacity, this increase in plasma thiol concentration could be interpreted as an increased capacity to buffer ROS post-exercise, as has been shown previously in rodents and humans (Alessio et al., 1988; Powers & Jackson, 2008; Radak et al., 2013; Reid et al., 1992; Sjodin et al., 1990; Vina et al., 2000). A thiol-specific "antioxidant response" might negate the requirement to increase the concentration or activity of other antioxidant molecules (e.g., GPx, as shown in the present study) and consequently, this milieu might prevent oxidative damage to proteins (and other molecules, such as lipids and DNA). In line with this interpretation, the present results show that protein carbonyl concentration declined from baseline to the sample collected after the second maximal exercise test, further supporting the idea that these bouts of exercise did not elicit a substantial increase in ROS. Decreases in plasma protein carbonyl concentration are common in the literature (but often not discussed) and are likely a result of an imbalance between production and clearance of oxidized proteins, mediated in part by the plasma proteasome system, or possibly plasma protein redistribution, tissue uptake or excretion (Wadley, Chen, Lip, Fisher, & Aldred, 2016).

Previous studies have explored associations between cardiovascular training, redox and inflammatory status. It has been shown that a sixteen week progressive endurance training program in inactive older men lowered lipid peroxidation (MDA) and 3-nintrotyrosine (3-NT), while increasing antioxidant defenses (GPx and total antioxidant capacity) (Fatouros et al., 2004). In addition, a single bout of aerobic exercise also attenuated oxidative markers in skeletal muscle of both inactive and active older adults (Bori et al., 2012; Radak et al., 2009). The marker of DNA damage, 8-Oxo-7,8 dihydroguanine (8-oxoG), accumulates in the genome over time and it may lead to the development of aging-related diseases. In response to a single bout of aerobic exercise, the 8-oxoG level was lastingly elevated in sedentary young and old subjects, but returned rapidly to pre-exercise levels in the DNA of physically active individuals independent of age (Radak et al., 2011). In support of this human data, exercise training in rats decreased DNA damage, increased DNA repair mechanisms, and increased resistance to oxidative stress in skeletal muscle (Radak et al., 2002).

Exercise training programs may also influence aspects of endocrine function either acutely or chronically. For example, it has been shown that salivary cortisol is increased transiently during a half

marathon in master endurance athletes (Piacentini et al., 2015). However, it has also been shown that regular exercise is associated with reduced hypothalamic-pituitary-adrenal activity (Heaney, Carroll, & Phillips, 2014; Vaczi et al., 2014). In the present study there was no change (or at least a small but statistically non-significant increase) in salivary cortisol and there was a significant decrease in salivary testosterone in response to the accumulated effect of two bouts of maximal exercise. This response was similar in both the lower- and higher-volume training groups. While it is appealing to speculate that a small output from the hypothalamic-pituitary-adrenal axis (particularly in the case of cortisol) is related to adaptation in response to regular exercise (Struder et al., 1998) (even in the low-training group who undertook approximately four hours of exercise per week), the lack of a cortisol response is likely to be governed by other factors. For example, the magnitude of cortisol release from the adrenal gland during exercise is known to correlate positively with the duration and intensity of exercise (Gabriel, Schwarz, Steffens, & Kindermann, 1992; Hansen, Wilsgard, & Osterud, 1991; Hill et al., 2008). Although the intensity of exercise in the present study was maximal, the duration spent at this intensity was very short. In addition, cortisol is known to exhibit a very pronounced diurnal rhythm, whereby the highest concentration is seen approximately 30 minutes after waking, and the lowest concentration before sleep at night (Heaney, Phillips, & Carroll, 2010; Stalder et al., 2016). Thus, although participants provided saliva samples between approximately 12:30-16:30, the levels of cortisol would be declining over this period, which might limit any small stressor-induced output from the adrenal gland, given the short duration of exercise employed.

When interpreting the results of this study, it should be considered that we did not assess and control for exercise-induced changes in plasma volume. Although it is possible that a decrease in plasma volume following exercise might have amplified some of our results (e.g., on average the +40% increase in plasma CRP post-exercise) other parameters that also increased post-exercise (e.g., plasma total thiol groups) were expressed relative to total plasma protein concentration and would therefore be unaffected. Moreover, such changes in plasma volume would only have very modest effects on our results, as such exercise-induced fluid shifts are typically small (e.g., −10% change) (Zouhal et al., 2001).

There are some limitations to this study. First, no inactive control group was recruited because of potential cardiac damage upon challenge. Indeed, two elderly athletes were excluded because of signs of myocardial ischemia in response acute exercise – as identified by a senior cardiologist. Second, we were unable to measure oxygen uptake and associated variables during exercise due to a fault with our gas analysis system that was not detected until all data had been collected. However, based on our heart rate and ratings of perceived exertion data, exercise was confirmed as being of maximal intensity. Finally, habitual diet was not assessed or controlled, and it is known that some dietary parameters can affect immunological and redox measurements. Thus, providing standardized diets to our participants may have reduced the variation in our data.

CONCLUSION

Comparing elderly adults engaged in lower-volume training (approximately four hours per week) to those engaged in higher-volume training (approximately eight hours per week) reveals different inflammatory and redox and profiles at rest and in response to exercise. Differences at rest included the lower-volume group exhibiting higher CRP (+60%), higher protein carbonyls (+23%) and lower SOD activity (−4%), compared to the higher-volume group. Differences in response to exercise included the lower-volume group exhibiting a larger increase in CRP (+60% higher-volume; +24% lower-volume group) and a smaller decrease in the activity of the antioxidant enzyme SOD (−20% higher-volume; −14% lower-volume group).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Figure 1. Inflammatory proteins at baseline and following two bouts of maximal exercise separated by four hours of rest. Statistically significant differences are indicated: * (TNF-α: Time effect, F=6.15, p=0.01) or *** (CRP: Time effect, F=83.82, p<0.0001). ### (CRP: Group × Time interaction, F=20.62, p<0.0001). Data were analyzed by two-way ANOVAs and are presented as mean ± SE.

Figure 2. Redox profile at baseline and following two bouts of maximal exercise separated by four hours of rest. Statistically significant differences are indicated: * (Thiol: Time effect, F=6.34, p=0.01), ** p<0.01 (Carbonyl: Time effect, F=8.10, p=0.006), *** (SOD: Time effect, F=91.24, p<0.0001). Group × Time interactions are also reported as indicated: # (Carbonyl, F=3.37, p=0.06) and ## (SOD, F=4.34, p<0.05). Data were analyzed by two-way ANOVAs and are presented as mean ± SE.

Figure 1

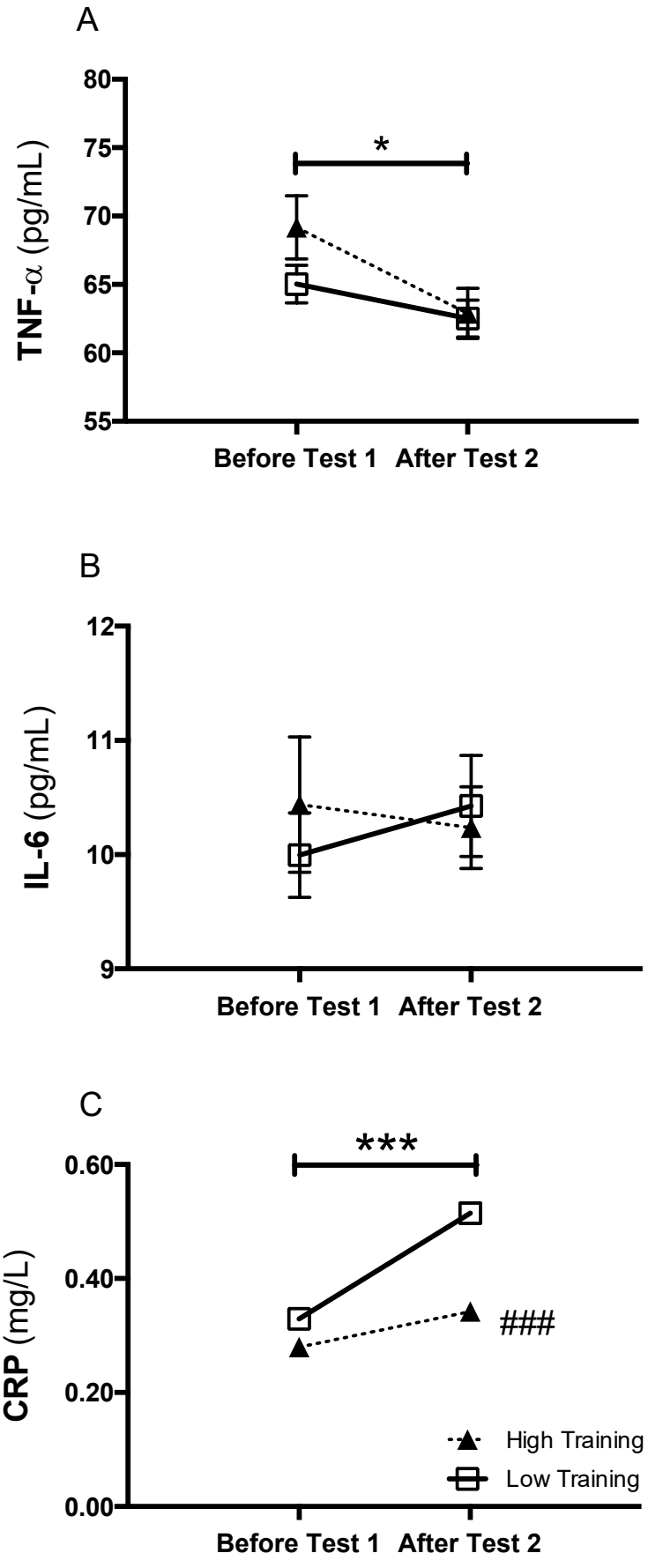
A
80
75
70
65
60
55
TNF-α (pg/mL)
*
Before Test 1
After Test 2
B
12
11
10
9
IL-6 (pg/mL)
Before Test 1
After Test 2
C
***
0.60
0.40
0.20
0.00
CRP (mg/L)
###
High Training
Low Training
Before Test 1
After Test 2

Figure 2

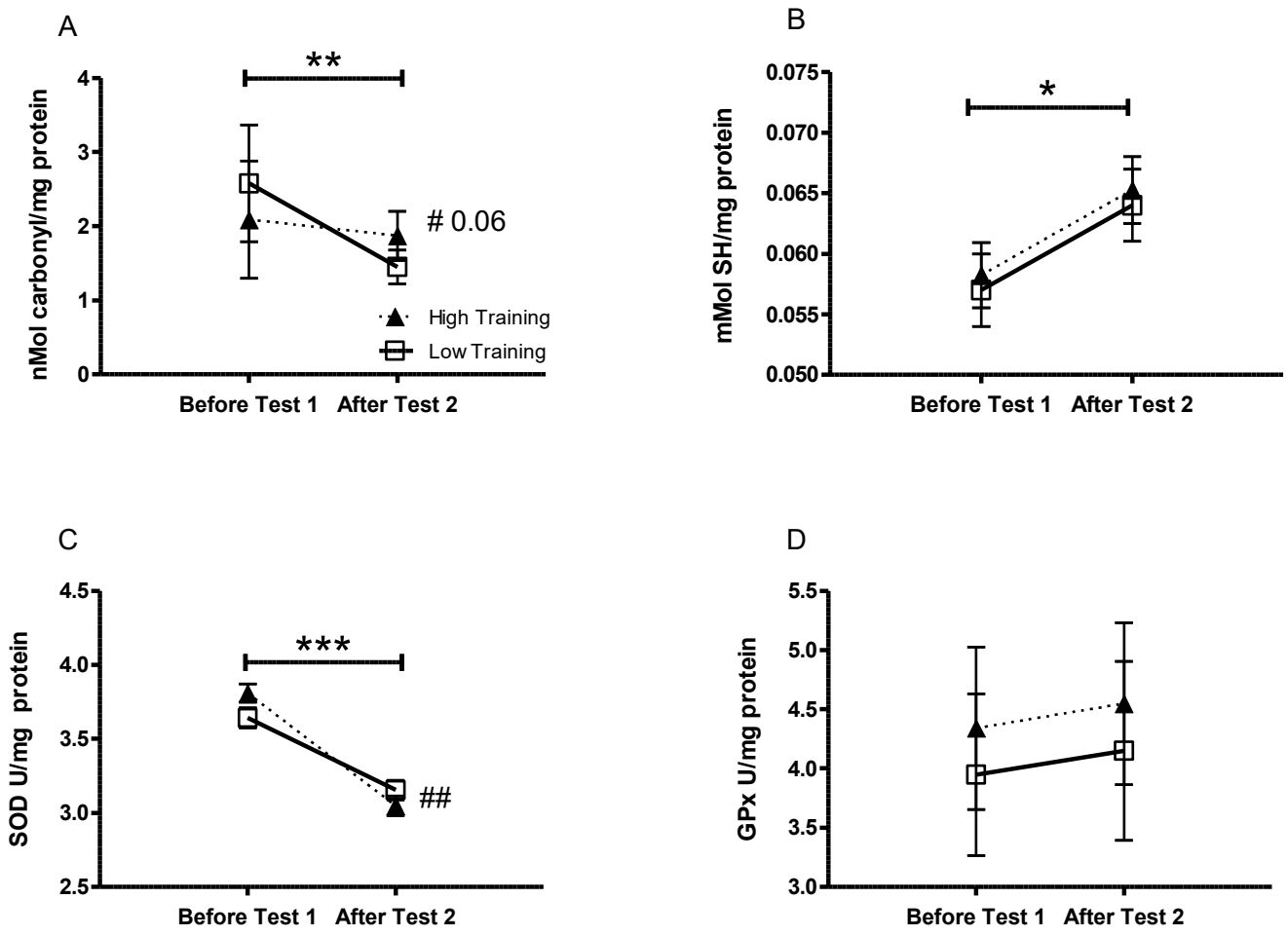
A
**
nMol carbonyl/mg protein
4
3
2
1
0
0.06
High Training
Low Training
Before Test 1
After Test 2
B
*
mMol SH/mg protein
0.075
0.070
0.065
0.060
0.055
0.050
Before Test 1
After Test 2
C
***
SOD U/mg protein
4.5
4.0
3.5
3.0
2.5
##
Before Test 1
After Test 2
D
GPx U/mg protein
5.5
5.0
4.5
4.0
3.5
3.0
Before Test 1
After Test 2

Table 1. Anthropometric, psychological, inflammatory, redox and hormonal profiles of participants at rest (baseline) for each group.

	Lower volume Training	**Higher volume Training**	***P-value***	***Effect size (η^2)***
Age (yrs)	65 ± 5	66 ± 4	0.47	0.013
Gender (F/M)	11 / 10	10 / 11	0.76	0.14
Weight (Kg)	73.9 ± 15.5	70.5 ± 14.3	0.47	0.013
Height (cm)	165.9 ± 10.9	163.4 ± 7.1	0.38	0.019
BMI	26.7 ± 3.5	26.4 ± 4.6	0.81	0.001
% Fat (Kg)	33.4 ± 7.9	29.2 ± 8.6	0.11	0.06
Training Volume (h/month)	16.9 ± 4.8	32.4 ± 8.6	**0.0001**	0.34
Distance/week (km)	43.67 ± 9.44	81.71 ± 9.88	<**0.0001**	0.80
Time/km (min)	5.71 ± 0.93	4.88 ± 0.50	**0.001**	0.24
Tension	1.4 ± 2.6	2.0 ± 2.2	0.45	0.014
Depression	0.4 ± 1.2	0.1 ± 0.4	0.31	0.025
Anger	0.6 ± 1.5	0.7 ± 2.1	0.86	0.001
Vigor	11.8 ± 2.1	10.5 ± 3.9	0.18	0.044
Fatigue	1.2 ± 1.9	1.2 ± 1.8	1.00	0.001
Confusion	1.1 ± 1.7	0.7 ± 1.7	0.38	0.019
CRP (mg/mL)	0.33 ± 0.05	0.28 ± 0.03	**<0.0001**	0.48
IL-6 (pg/mL)	9.99 ± 1.70	10.44 ± 2.65	0.53	0.001
TNF-α (pg/mL)	65.02 ± 6.36	69.17 ± 10.33	0.13	0.02
Thiols (mMol/mg protein)	0.057 ± 0.0036	0.058 ± 0.0079	0.78	0.004
Carbonyl (nMol/mg protein)	2.58 ± 0.79	2.09 ± 0.79	0.07	0.001
SOD (U/mg)	3.64 ± 0.28	3.80 ± 0.13	0.06	0.004
GPx (U/mg)	3.95 ± 2.83	4.33 ± 2.85	0.69	0.005
Cortisol (nmol/L)	9.48 ± 2.89	8.70 ± 2.30	0.34	0.03
Testosterone (nmol/L)	0.27 ± 0.17	0.25 ± 0.16	0.74	0.001
Ratio	52.49 ± 40.20	52.36 ± 39.48	0.99	0.01

Legend: BMI, body mass index. CRP, C-reactive protein. GPx, Glutathione Peroxidase. IL-6, interleukin-6. SOD, superoxide dismutase. Psychological profiles were assessed by the Profile of Mood States Questionnaire. Data are shown as mean ± SD. Data were analyzed by independent samples T tests.

Table 2. Exercise test characteristics.

				GROUP			TIME		
		During Test one	**During Test two**	**F**	***p***	***Effect Size (η^2)***	**F**	***p***	***Effect Size (η^2)***
Top speed (km/h)	LVG HVG	9.61 ± 2.20 11.69 ± 3.00	9.10 ± 1.78 11.42 ± 3.33	13.20	**<0.0001**	0.140	0.21	0.86	0.001
Time (min)	LVG HVG	5.34 ± 2.09 6.29 ± 1.30	6.06 ± 3.20 6.19 ± 1.34	0.11	0.74	0.001	0.55	0.46	0.007
HR1 (bpm)	LVG HVG	152 ± 20 162 ± 23	150 ± 24 155 ± 21	2.42	0.12	0.03	0.77	0.38	0.010
HR2 (%max)	LVG HVG	98 ± 12 106 ± 15	97 ± 14 101 ± 12	5.32	0.27	0.020	0.92	0.34	0.024
RPE	LVG HVG	16.6 ± 2.83 18.58 ± 0.94	17.10 ± 1.99 17.87 ± 0.91	8.99	**0.004**	0.163	0.06	0.81	0.054

Legend: Data are presented in mean ± SD (two-way ANOVA, df=1,78). HR: Heart Rate. RPE: Ratings of perceived exertion. LVG, Low-Volume Group. HVG, High-Volume Group. Group × Time interaction effects are discussed in the text.

Table 3. Salivary hormone responses to exercise

				GROUP			TIME		
		Before Test one	After Test two	F	*p*	***Effect Size* (η^2)**	F	*p*	***Effect Size* (η^2)**
Cortisol (nmol/L)	Low-volume	9.48 ± 2.89	11.44 ± 6.23	2.79	0.09	0.035	2.36	0.13	0.029
	High-volume	8.70 ± 2.30	9.36 ± 2.51						
Testosterone (nmol/L)	Low-volume	0.27 ± 0.16	0.12 ± 0.05	0.11	0.73	0.001	25.95	**<0.0001**	0.250
	High-volume	0.25 ± 0.12	0.12 ± 0.06						
Ratio	Low-volume	52.49 ± 40.20	152.27 ± 215.02	1.11	0.30	0.002	8.16	**0.005**	0.180
	High-volume	52.36 ± 39.48	98.58 ± 54.81						

Legend: Data are presented as mean ± SD (two-way ANOVA, df=1,78). Data were collected at baseline and following two-bouts of maximum exercise.