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Effects of training regimes at different intensities on performance and oxidative stress in masters athletes

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Lorenzo Pugliese
Matricola R09708

Tutor: Prof. Antonio La Torre
Dipartimento di Scienze Biomediche per la Salute

Coordinatore: Prof. Livio Luzi

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TABLE OF CONTENTS

Contents	1
Abstract	2
Introduction	4
<i>Master athletes</i>	4
<i>Training modalities</i>	8
<i>Reactive oxygen species, ageing and physical exercise</i>	11
First study:	15
<i>Effects of training manipulation on physiological parameters and running performance in masters runners</i>	
Second study:	27
<i>Time-course changes of oxidative stress response to high-intensity discontinuous training versus moderate-intensity continuous training in masters runners</i>	
Third study:	44
<i>Effects of manipulating volume and intensity training in master swimmers</i>	
Fourth study	56
<i>Training effects on ros production determined by electron paramagnetic resonance (EPR) in master swimmers</i>	
References	65
Appendix: Scientific Production	79
Acknowledgements	83

ABSTRACT

Over the last decade, the participation of middle-aged and older ('masters') involved in sport activities at different levels has significantly increased, particularly in long-distance events. Master athletes are typically characterized as people who continue physical training throughout life and in this population, the incidence and risk of chronic and age-related diseases are reportedly lower, and self-rated health is better than in apparently healthy controls. However, regardless of training a decline in peak athletic performance in both endurance and sprint events and for all competitions/disciplines usually occurs with aging. In particular, declines in endurance exercise performance and its physiological determinants with ageing appear to be mediated in large part by a reduction in the exercise training 'stimulus', mainly as a result of increased work and family commitments, few masters still follow structured training programs, and the increased prevalence of exercise training-associated injuries that probably contributes to their reduced training intensity and volume. Furthermore, aging is accompanied by a progressive increase in free radical production (i.e., synthesis of reactive oxygen species) with a concomitant decrease in the enzymatic defence mechanisms, promoting the development of oxidative stress. The chronic repetition of exercise, i.e. exercise training, may have the capability to develop a compensation to oxidative stress in skeletal muscle fibres by means of an adaptation of the antioxidant and repair systems. This might result in a decreased resting level of oxidative damage and an increased resistance to oxidative stress.

In general, the main types of training used to improve endurance exercise performance are: i) continuous training at moderate intensity (CON); and ii) discontinuous training at high intensity (DHIT). Different studies showed that even in sedentary or moderately trained individuals, DHIT might be an efficient strategy to induce adaptations in skeletal muscle and exercise performance that are comparable with conventional endurance training. The first part of the thesis is focus on master runners. First study aims to evaluate if an individualized training schedules characterized by an overall reduction of training volume is able to improve running performance. Moreover, the impact of CON and DHIT training programs on running performance and its main physiological factors in master runners has been evaluated. The second study aims to evaluate the effects of 8-week of DHIT and CON on resting level and time-course changes of several indexes of oxidative stress. The main findings of these studies show that despite a significant reduction of training volume, CON and DHIT, characterized by the same total volume, improve running economy and running performance. Furthermore, both CON and DHIT induced similar beneficial effects, reducing the resting levels of oxidative stress biomarkers in plasma and urine. The second part of the

thesis is focused on master swimmers. The third study aims to compare the effects of two opposite training protocols (low-volume high-intensity vs high-volume low-intensity) in a group of trained master swimmers. The fourth study aims to examine the effects low-volume high-intensity training on ROS production and on antioxidant capacity in master swimmers by applying electron paramagnetic resonance measurement. The results indicate that in master swimmers an increase of training volume may lead to an improvement of indexes of aerobic capacity and middle-long distance performance. A subsequent period of high-intensity low-volume training, besides maintaining previous improvements, may positively affect also short distance performance. Moreover, high intensity training improves antioxidant capacity and significantly decreases baseline ROS production.

INTRODUCTION

Master athletes

Ageing can be considered as an inherent, progressive and decremental process common to all animal biology ¹. Ageing process is potentially due to three theoretically independent, but actually connected factors ^{2, 3}: (i) aging per se acting as a biologic, irreversible process (ii) deconditioning due to the more sedentary lifestyle found in most elderly people, and (iii) effects of co-morbidity, i.e., of primary diseases or injuries that are in principle independent of age, but which accumulate during the lifespan.

Amongst these factors, disuse or physical inactivity seems to be the most overlooked and possibly the most significant ^{1, 4}. Many of the changes in health status and physical performance that have been thought to be the normal result of aging have been found to be actually the result of a long-standing sedentary lifestyle ^{4, 5}. Master athletes are typically characterized as people who continue physical training throughout life. Each sport's national or international governing body determines the age to define a masters athlete. While masters athletes are typically older than 35 years of age, masters' competition in swimming begins at age 25 years, track and field at 35 years, and golf at 50 years ⁶. In this population, the incidence and risk of chronic diseases, e.g. diabetes, metabolic syndrome, or coronary heart disease, are reportedly lower, and self-rated health is better ⁷ than in apparently healthy controls. Moreover, master athletes may be able to maintain or even increase athletic performance they achieved at younger ages and are considered an adequate model to determine the "successful aging" where decrements in physiological function capacity can be genuinely attributed to human ageing process and they are not the result of pathologies that may arise from confounding factors (i.e. sedentary lifestyle) ^{1, 8}. Masters athletes participate in organized competitive sport for a number of reasons. Previous research reviewed in Reaburn et al. ⁶ from masters swimming, athletes competing at multisport events, or older people involved in regular exercise and sport has shown that masters athletes participate for enjoyment, competition, physical fitness, health benefits, social, travel, stress relief, personal challenge, and skill development reasons. However, the factors of enjoyment, health and fitness benefits, social, and competition appear the primary drivers for involvement. An increasing proportion of these active older individuals are becoming recreational or competitive athletes focused on sports performance. For example, the inaugural World Masters Games held in Toronto, Canada had 8,305 participants across 22 sports, whereas in the 2013 World Masters Games held in Torino participated about 25,000 athletes across 28 events.

Indeed, master athletes typically train for 10 hours or more per week, and they often do so over decades. However, regardless of training a decline in peak athletic performance in both endurance and sprint events and for all competitions/disciplines usually occurs with aging⁹⁻¹². As for endurance performance, age-related declines in endurance events have been observed in running¹⁰, orienteering¹³, indoor rowing¹⁴, and swimming¹⁵. The age-related decrease in endurance performance of elite level masters endurance athletes appears curvilinear from age 35 years until approximately age 60-70 years and exponential thereafter^{9, 16}. In general, the magnitude of decline in endurance running performance with age is greater in women than in men^{15, 17, 18}. However, interpretation of this apparent widening of sex differences with advancing age is confounded by the relatively smaller number of female versus male runners in the older groups. Indeed, such increasing sex differences with age are absent in the endurance swimming events, where approximately equal number of men and women compete throughout the age range¹⁵. Performance in endurance events is dependent upon three main physiological factors: i. maximal oxygen consumption ($\dot{V}O_{2max}$), ii. exercise intensity at which a high fraction of the maximal oxygen consumption can be sustained and iii. exercise economy^{17, 19}.

Amongst these factors, a progressive reduction in $\dot{V}O_{2max}$ appears to be a key physiological mechanism associated with declines in endurance performance with advancing age⁹. $\dot{V}O_{2max}$ is estimated to decline approximately 10% per decade after the age of 25 years in healthy sedentary aging individuals of both sexes regardless of activity level^{18, 20-25}. Early investigations suggested the rate of decline in $\dot{V}O_{2max}$ of masters endurance athletes to be only half that observed in sedentary aging individuals^{21, 26}. However, more recent studies have suggested accelerated decline in $\dot{V}O_{2max}$ in older endurance athletes when expressed as per cent decrease from early adulthood^{18, 22-25, 27, 28}. In fact, endurance trained men and women possess higher initial $\dot{V}O_{2max}$ values at baseline^{23, 24} and demonstrate greater absolute ($\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) rates of decline in $\dot{V}O_{2max}$ with age than healthy sedentary adults^{18, 23-25}, probably as a result of greater reductions in habitual exercise with ageing compared with sedentary adults.

Both central (maximal heart rate and maximal stroke volume) and peripheral (maximal arteriovenous oxygen difference) factors may play a role in age-related declines in $\dot{V}O_{2max}$. Centrally, an age-related decrease in maximum heart rate (HR_{max}) is commonly observed in male and female endurance athletes^{6, 9, 20}. HR_{max} declines with age at a rate uninfluenced by exercise training or sex of approximately 3-5% per decade^{20, 29}. As for maximal stroke volume a significant age-related decline in maximal stroke volume in

both the endurance-trained men and women has been observed³⁰. However, compared to age matched sedentary controls, the available research suggests that maximal stroke volume of both male and female masters endurance athletes is elevated³¹ suggesting that long-term physical training may maintain a high level of cardiac function and stroke volume in this population. Consequently, the decreased maximal cardiac output and $\dot{V}O_2\text{max}$ observed in masters athletes appears related to age-related decreases in HRmax rather than significant changes in stroke volume or cardiac morphology. Peripheral adaptations includes arterio-venous oxygen difference that is influenced by a variety of factors including muscle mass, the capacity of the blood to transport and relinquish oxygen (blood volume, hemoglobin), and the capacity of the working tissues to take up and utilize oxygen (capillarization, muscle fiber type, aerobic enzyme activity). In sedentary adults, maximal arterio-venous O_2 difference clearly declines with advancing age, consistent with the marked reductions in capillary density and mitochondrial enzyme activities observed with ageing in this group³². Reductions in peripheral oxygen extraction during maximal exercise also appears to contribute to the decline in $\dot{V}O_2\text{max}$ with age in endurance exercise-trained adults, as maximal arterio-venous O_2 difference declines modestly (5-10%) over a span of ~30 years in this group³³⁻³⁵. It remains to be determined if the reduction in maximal arterio-venous O_2 difference with ageing in endurance athletes reflects reductions in maximal oxygen delivery to or extraction by the active muscles. However, older endurance-trained athletes can oxygenate blood in the lungs to a similar extent as young athletes, and their contracting muscles are capable of extracting oxygen as much as their younger counterparts³⁴. Furthermore, muscle oxidative enzyme activities and capillarization (expressed per area or per fibre) are similar between young and older endurance-trained adults³⁶. Thus, it is likely that maximal oxygen delivery, rather than oxygen extraction, is a major contributor to the age-related reduction in maximal arterio-venous O_2 difference in endurance-trained adults. As skeletal muscle mass is closely related to maximal aerobic capacity among healthy humans across the adult age range³⁷, a decline in maximal arterio-venous O_2 difference may be secondary to an age-related loss of muscle mass. Cross-sectional data demonstrated loss rates in $\dot{V}O_2\text{max}$ of approximately 9% per decade for both men and women that were reduced to 4% per decade when controlling for changes in lean body mass and fat mass³⁸. Similarly, a recent longitudinal investigation demonstrated that maintenance of lean body mass was associated with maintenance of $\dot{V}O_2\text{max}$ in men master runners³⁹. Rosen et al.⁴⁰ utilised statistical

modelling to suggest that 35% of the decline in $\dot{V}O_{2\max}$ with age was due to age-associated declines in lean body mass.

A reduction in the ability to sustain a high fraction of one's maximal oxygen consumption during submaximal exercise, typically evaluated using the blood lactate threshold, also contributes to the reduction in endurance performance with ageing. In older runners, it appears that endurance running performance is correlated with both $\dot{V}O_{2\max}$ and velocity at lactate threshold in male^{41, 42} and highly trained older female runners^{43, 44}. Wiswell et al.⁴³ determined that 60% of the variability in performance for runners aged 23-47 year was explained by the running velocity at which lactate threshold occurred, whereas $\dot{V}O_{2\max}$ explains 74% of the variability for the runners aged 37-56 years. Absolute work rate or running speed at lactate threshold declines with advancing age in endurance athletes^{41, 44-46}. However, lactate threshold has been shown to not change or even increase with increasing age when expressed relative to the percentage of $\dot{V}O_{2\max}$ ^{41, 44, 46, 47}.

Exercise economy is measured as the steady-state oxygen consumption while exercising at a specific submaximal exercise intensity below the anaerobic threshold⁴⁸ and has been shown to be a stronger predictor of endurance performance than $\dot{V}O_{2\max}$ in a homogenous group of endurance athletes^{49, 50}. The few studies focused on masters endurance athletes^{44, 50} concluded that exercise economy does not change with age in masters endurance athletes suggesting that this factor does not contribute significantly to age related decreases in endurance performance.

In conclusion, amongst the main physiological determinants of endurance exercise performance, a progressive reduction in $\dot{V}O_{2\max}$ appears to be the primary mechanism associated with declines in endurance performance with age. A reduction in lactate threshold velocity and muscle mass, also contributes to the reduction in endurance performance with ageing, although this may be secondary to decreases in $\dot{V}O_{2\max}$. In contrast, exercise economy does not change with age in endurance-trained adults. However, apart from these physiological factors, the age-related declines in endurance performance have been suggested to be due to⁶: decreased training volumes and intensities as a result of increased work and family commitments, behavioural factors such as reduced motivation to train, few masters athletes having coaches, and masters athletes spending less overall time in training than international caliber younger athletes. In addition, the increased prevalence of exercise training-

associated injuries among masters athletes also probably contributes to their reduced training intensity and volume ⁵¹.

Taken together, these results suggest that high-intensity training and maintenance of training volume may mediate the age-related declines in $\dot{V}O_2\text{max}$ and endurance performance. However, the greatest challenge to masters athletes is to balance an adequate stimulus to the body to promote high performance while preventing excessive fatigue that may lead to injury ⁵. Thus, master athletes should focus on quality of training rather than quantity ⁵² and this implicates the selection of the most appropriate exercise modalities and intensities.

Training modalities

Endurance athletes often seek the most effective training methods to enhance the most important physiological determinants of endurance performance ⁵³. In general, the main types of training used to improve endurance exercise performance are: i) continuous training (CON) at low- to moderate-intensity characterized by high volumes of training (> 30 min) with intensities between 60% and 80% of $\dot{V}O_2\text{peak}$ or below the “anaerobic threshold” (AT) with a nearly constant O_2 consumption and without a “slow component” in O_2 kinetics ⁵³⁻⁵⁷ and ii) discontinuous high intensity training (DHIT) characterized by repeated exercises performed at intensity corresponding to $\dot{V}O_2\text{peak}$ (or slightly lower) or above AT or “all-out”, exercise bouts are separated by brief periods of low-intensity work or inactivity that allow a partial but often not a full recovery ^{53-55, 57-59}.

When individuals are untrained and commence a period of training characterized by continuous low- to moderate-intensity exercise ‘aerobic’ fitness typically improves ^{54, 57}. Improvements in exercise capacity are associated with changes in cardiovascular, muscular and metabolic responses to exercise ⁶⁰⁻⁶². Cardiovascular changes include increases in working muscle capillary density, rises in blood volume and resultant decreases in heart rate at similar absolute exercise intensities ^{54, 57, 62-64}. Muscular changes with endurance training include greater muscle glycogen storage, increases in $Na^+ - K^+$ ATPase pump activity, and rises in most mitochondrial enzymes, with little change in glycolytic enzymes. ^{54, 57, 60, 65, 66}. Submaximal endurance training increases the size and number of mitochondria in skeletal muscle resulting in increase in the maximal capacity of muscle to generate ATP *via* oxidative phosphorylation ⁶⁷. Lower plasma lactate concentrations at similar relative work rates typically observed after endurance training are also due to a greater mitochondrial capacity to oxidise fat. ^{66, 67}. Thus, with previously untrained and recreationally

individuals, blood flow, oxygen delivery, oxygen extraction and fat metabolism in working muscles increase after a long period (weeks to months) submaximal exercise-training. As a result, muscle contraction becomes more efficient and physical work capacity increases. However, when submaximal endurance training becomes habitual, such as for the endurance athlete, further improvements in exercise performance with an increase in training volume do not normally occur^{54, 55, 68, 69}. Indeed, the muscle of trained athletes has three to four times higher oxidative enzyme activity, up to three times more capillaries per muscle fibre, and a greater percentage of slow twitch fibres when compared with untrained muscle⁷⁰. In these individuals, additional improvements in endurance performance and associated physiological markers appear to require a different training stimulus than simply an increase in volume^{53, 54, 57, 69}.

In contrast to submaximal exercise training, DHIT is normally achieved through the use of intervals. Buchheit and Laursen⁵⁸ recently defined DHIT as either repeated short (<45 s) to long (2-4 min) bouts of rather high- but not maximal-intensity exercise, or short (<10 s, repeated-sprint sequences) or long (>20-30 s, sprint interval session) all-out sprints, interspersed with recovery periods. As such, maximal, all-out sprint training is classified as a form of high-intensity training at the highest end of the intensity spectrum⁷¹. When compared on a matched-work basis or when estimated energy expenditure is equivalent, DHIT can serve as an effective alternate to traditional endurance training, inducing similar or even superior changes in a range of physiological, performance and health-related markers in both healthy individuals and diseased populations^{54, 55, 71-75}. However, one of the most interesting aspects of high intensity training is that performance can be improved, or at least maintained, under conditions of reduced weekly volume. In fact, growing evidence suggests this type of training stimulates physiological remodeling comparable with moderate-intensity continuous training despite a substantially lower time commitment and reduced total exercise volume^{71, 76}.

As few as 6 sessions of HIT over 2 weeks, totaling ~ 15 min of “all-out” cycle exercise, has been shown to increase the maximal activity of mitochondrial enzymes and improve performance during tasks that rely heavily on aerobic energy provision^{59, 77}. Other adaptations documented after several weeks of HIT include an increased resting glycogen content, a reduced rate of glycogen utilization and lactate production during matched-work exercise, an increased capacity for whole-body and skeletal muscle lipid oxidation, enhanced peripheral vascular structure and function, improved exercise performance as measured by time-to-exhaustion tests or time trials⁷⁶. The impact of low-volume DHIT programs on maximal oxygen uptake in healthy adults has been investigated. A recent meta-analysis by Weston et al.⁷⁸

show that when compared with control, moderate improvements in $\dot{V}O_{2\max}$ were likely for active non-athletic males and possible for sedentary males and active nonathletic females. A small improvement in $\dot{V}O_{2\max}$ was likely for sedentary females. The effect on athletic males was unclear. With the exception of a possible moderate additional increase in $\dot{V}O_{2\max}$ for subjects with a lower baseline value. The comparison of DHIT with endurance training was considered unclear.

Given the oxidative phenotype that is rapidly upregulated by DHIT, it is plausible that metabolic adaptations to this type of exercise could be mediated, in part, through signalling pathways normally associated with endurance training. A key regulator of oxidative enzyme expression in a number of cell types, including skeletal muscle, is peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α), a transcriptional coactivator that serves to coordinate mitochondrial biogenesis^{67, 79}.

In this respect, acute low-volume DHIT increases PGC-1 α mRNA by several-fold when measured 3 h post-exercise^{80, 81}. This is comparable with the acute increase in PGC-1 α mRNA expression observed after a bout of continuous endurance-type exercise^{82, 83}. Similar to endurance exercise⁸⁴, acute DHIT may activate PGC-1 α by increasing its nuclear translocation⁸¹. The increase in nuclear PGC-1 α following low-volume DHIT coincides with increased mRNA expression of several mitochondrial genes, suggesting that a program of mitochondrial adaptation is engaged with these short bursts of intensity exercise.

Several signalling pathways have been linked to exercise induced activation of PGC-1 α and mitochondrial biogenesis, including calcium/calmodulin-dependent protein kinase (CaMK), 5'-adenosine monophosphate-activated protein kinase (AMPK), the p38 mitogen-activated protein kinase (MAPK)⁸⁵.

The first of these mentioned molecular signals is mediated by the prolonged rise in intramuscular calcium, such as that which occurs during prolonged endurance exercise or high exercise training volumes. These high calcium concentrations activate the CaMK, a mitochondrial biogenesis messenger. The upstream signals that activate PGC-1 α and mitochondrial biogenesis in response to low-volume HIT is probably related to robust changes in intramuscular ATP:ADP/AMP ratio following exercise⁸⁶ and the concomitant activation of AMPK^{80, 81}. Activation of p38 mitogen-activated protein kinase (MAPK), possibly via increased generation of reactive oxygen species (ROS)⁸⁷, may also be involved.

Consequently, the high mitochondrial oxidative capacity, improved fat oxidation potential, and increased glucose transport capacity in the skeletal muscle of endurance athletes may be achieved through either high volumes of endurance training, high intensities of endurance training or various combinations of both⁵⁵. At the molecular level, it may be the blend of signals induced from combined high-volume training and

high-intensity training that elicits either a stronger or more frequent promotion of the aerobic muscle phenotype through PGC-1 α mRNA transcription⁵⁵.

Reactive oxygen species, ageing and physical exercise.

A “ free radical ” is a term defining an atom (or molecule) that contains one or more unpaired electrons that is capable of independent existence. Reactive oxygen species (ROS) are compounds (or atoms) which may be free radicals containing oxygen or non-radical but have reactive derivatives of oxygen, like hydrogen peroxide and others. Reactive nitrogen species (RNS) are nitrogen radicals and non-radical nitrogen reactive molecules with a nitrogen reactive center⁸⁸⁻⁹⁰. The occurrence of one unpaired electron results in high reactivity by their tendency to give or subtract electrons to attain stability. As a consequence, ROS can in turn be stabilized by subtracting electrons to neighbouring molecules (e.g. lipids, proteins, DNA).

Cells are exposed to a large variety of ROS from both exogenous and endogenous sources. Exogenous sources of ROS can be identified in UV and gamma radiations, microbes, allergens, car exhausts, certain food, tobacco smoke, air pollutants, drugs and alcohol when assumed in a great amount. Nevertheless, despite the extremely strong exposure of whole our organism to ROS coming from exogenous sources, endogenous ROS play the most important and extensive role, since, in the time course of life, each body cell is continuously exposed to them. The major responsible of ROS production are in mitochondria, enzymes are another endogenous source of ROS^{91, 92}. While most enzymes produce ROS as by-product of their activity (e.g. xanthine oxidase) some of them are designed to produce ROS (e.g. nitric oxide synthase yields nitric oxide radicals, NADPH oxidase complex utilizes electrons to produce superoxide radicals from the oxygen molecule. It is reported that ~2 % of the O₂ used by the mitochondrial electron transport chain creates ROS and in particular the superoxide anion (O₂^{•-}), due to its incomplete reduction^{91, 92}. The O₂^{•-} is very unstable and is rapidly converted either spontaneously or after its export into the cytoplasm by mitochondrial and cytoplasmic superoxide dismutases (SOD), to the more stable hydrogen peroxide (H₂O₂), and then further to water by catalase (CAT) or to the very reactive hydroxyl radical in the presence of transition metals (e.g. Fe²⁺). Five enzymes complexes are localized on the inner mitochondrial membrane. Complexes I-IV (the electron transport chain) are involved in transporting electrons through a series of proteins REDOX reactions, with the final destination being a an oxygen molecule. Under normal situation, this oxygen is the converted to water in complex IV, and the energy stored in the proton gradient is used to drive ATP production in complex V. However, during this process, it would occur an inadequate

coupling of the electron transfer between the complexes I and III: complex I (namely, the iron-sulfur clusters) releases the ROS ($O_2^{\cdot-}$) only towards the mitochondrial matrix, whereas complex III (the ubiquinol oxidation site) releases superoxide into both matrix and outside the inner membrane. So a small percentage of the oxygen consumed by mitochondria at complex IV is converted to ROS rather than water⁹³.

At appropriate concentration, ROS are known to act as important signalling molecules. ROS have been recognized as activating multiple pathways that influence gene expression^{88, 94}. ROS are also produced by immune cells during the process of respiratory burst in order to eliminate antigens⁹⁵. Consequently, ROS are essential to wellbeing, having various regulatory roles in cells. However, oxygen radicals are also known to damage DNA and lipids, and oxidize proteins⁹⁶⁻⁹⁸. For this reason, a network of antioxidant defence mechanism is present in the body^{99, 100}. In general, antioxidants are often reducing agents (intracellular and extracellular) able to react with free radicals and reactive species minimizing their harmful actions. Antioxidants can be both synthesized in vivo and absorbed through diet. They can be divided into two groups: enzymatic and non-enzymatic. The main enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). Each of these enzyme is responsible for the reduction of a different ROS, and they are located in different cellular compartments. The non-enzymatic group includes glutathione, vitamin C, vitamin E, carotenoids, uric acid, and similar. Further, there is an evidence that bilirubin can act as antioxidant to help neutralize certain free radicals¹⁰¹.

Oxidative stress is generally defined as an imbalance that favours the production of ROS and/or RNS over their inactivation by antioxidant defence systems¹⁰². Whilst small fluctuations in the steady-state concentration of these oxidants may actually play a role in intracellular signalling¹⁰³, uncontrolled increases in the steady-state concentrations of these oxidants lead to free radical-mediated chain reactions which indiscriminately target proteins, lipids, and DNA⁹⁶⁻⁹⁸, together with functional impairment of metabolic process as the mitochondrial respiratory chain^{104, 105}.

As a consequence, the delicate balance between advantageous and detrimental ROS effects plays a great physio-pathological importance. In fact, conditions of oxidative stress (i.e. when oxidants overwhelm antioxidants) are associated with a number of neurological diseases (e.g. Parkinson's disease, Huntington's disease, Alzheimer's disease, Amyotrophic Lateral Sclerosis and various peripheral neuropathies), pathogenesis of diseases states such as atherosclerosis, diabetes, ischemia/reperfusion

injury, inflammatory diseases, cancer, cardiovascular and/or respiratory insufficiency^{103, 106, 107} as well as the normal aging process¹⁰⁸.

There is scientific evidence that aging is accompanied by a progressive increase in free radical production [i.e., synthesis of reactive oxygen species, (ROS)] with a concomitant decrease in the enzymatic defense mechanisms, promoting the development of oxidative stress¹⁰⁹⁻¹¹².

The aging muscle would be also more sensitive to exercise induced muscle damage, would be less able to regenerate¹¹³, and would produce more ROS because of the greater proportion of type 1 fibers, which have the greatest oxygen consumption¹¹⁴. It has been demonstrated that aging induces an imbalance in the intracellular levels of prooxidants and antioxidants, which in turn elevates oxidative stress and increases oxidative damage¹⁰⁹. Nevertheless, the elderly who are physically active benefit from exercise-induced adaptation in cellular antioxidant defense systems¹¹¹. Improved muscle mechanics, strength, and endurance make them less vulnerable to acute injury and chronic inflammation. Indeed, moderate levels of oxidative stress are essential for the organisms to adapt and reach a new level of hormesis even if the balance of oxidants and antioxidants becomes more fragile in advance age¹¹⁵.

Physical exercise is perhaps one of the most characteristic examples demonstrating that ROS are not necessarily harmful, considering that the well-known benefits of regular exercise on human organism accompanied by repeated episodes of oxidative stress^{116, 117}. We are aware that physical exercise is associated with a dramatic increase in oxygen uptake by the whole body and in particular by skeletal muscle. It has been reported an increase of 10-15-fold in the rate of whole body oxygen consumption and an increase of more than 100-fold in the oxygen flux in active muscles during whole-body aerobic exercise, so resulting in increased ROS formation shifting the cellular environment from a reduced to an oxidized state independently of physical activity (aerobic, anaerobic or resistance types of activities)¹¹⁸⁻¹²⁰.

Most of the oxygen consumed by the body is utilized in the mitochondria for substrate metabolism and ATP production. An increased ATP demand accompanying exercise increases both aerobic and/or anaerobic metabolism. Many factors might contribute to the oxidative stress induced by exercise and a variety of factors can influence the oxidative rate, such as muscle groups recruited, modes of contraction, exercise intensity, exercise duration, and the exercising population. During aerobic exercise, the generation of ROS increases according to a higher O₂ consumption and, consequently, a higher electron leakage from the electron transport chain¹¹⁹. Recent reports have indicated the potential role that blood may play at rest or during exercise on ROS production. Some important factors, that contribute to the oxidative stress during

exercise, are easily visible in the blood: increase in blood temperature, decrease in blood pH, decrease in blood oxygen partial pressure and increase in the concentration of blood lactate ¹²¹⁻¹²³. The whole blood, or parts of it (plasma ¹²⁴, erythrocytes ¹²⁵, neutrophils ^{124, 126}, lymphocytes ¹¹⁷, platelets ¹²⁷) have reported an increased production of various reactive species after exercise. However, the majority of the relevant human studies have measured the redox status of the plasma (plasma is about 55% of blood volume; its composed mostly water, about 90% and contains dissolved proteins, glucose, lipids, mineral ion), this is probably for the reasons: the assumption that plasma better reflects tissue redox status and the easiness of plasma collection.

Blood is able to produce ROS during exercise, yet it is equally evident that skeletal muscle is able to produce reactive species during increased contractile activity. Referring to the appropriate level of reactive species, ROS are important molecules for muscle contraction. At the molecular level, the exercise activates p38 γ MAPK (mitogen-activated protein kinase) which promotes PGC-1 α activity and expression in control of mitochondrial biogenesis and angiogenesis in skeletal muscle . The promoting effects of regular exercise on different cellular functions include the up-regulation of antioxidant, oxidative damage repairing systems, neurogenesis, and induction of trophic factors ¹²⁸.

FIRST STUDY

Effects of training manipulation on physiological parameters and running performance in masters runners.

Introduction

The world population has been experiencing significant ageing. The number of older persons increased from 202 million in 1950 to 841 million in 2013, and the older population will almost triple by 2050 ¹²⁹. In parallel with these changes in demographical age, the number of middle-aged and older ('masters') involved in sport activities, especially at different level is continuously increasing, particularly in long-distance events ⁹. For example, U.S. marathon finishers aged over 40 years were 37,180 (26% of estimated U.S. marathon finishers) in 1980 reaching the number of 254,270 (47% of estimated U.S. marathon finishers) in 2013 ¹³⁰. Master athletes are typically characterized as people who continue physical training throughout life and in this population, the incidence and risk of chronic and age-related diseases are reportedly lower, and self-rated health is better than in apparently healthy controls ⁷. In addition, master athletes are still capable of accomplishing exceptional performances for their age categories ⁹. Notwithstanding, despite training and regular participation in sporting competitions, exercise performance inevitably declines with ageing ^{6, 9, 12, 15}. The age-related decrease in performance of masters endurance athletes has been shown to be mainly linked to a decline in $\dot{V}O_2\text{max}$, a decrease in neuromuscular function, and a reduction in the lactate threshold, whereas running economy (RE) is preserved with ageing ^{6, 9, 12}. Decline in endurance performance and its physiological determinants appear to be mediated in large part by a reduction in the exercise training 'stimulus' (i.e. exercise-training intensity, session duration and weekly frequency) with advancing age ^{6, 9}. In fact, masters athletes are often "busier" than young athletes and the increases in job- and family-related responsibilities may impinge on the availability of time and energy for the intensive training required to remain competitive ⁹. In addition, few master athletes still have coaches or follow structured training programs and the increased prevalence of exercise training-associated injuries in this population also probably contributes to their reduced training intensity and volume ^{6, 51}.

In general, the main types of training used to improve endurance exercise performance are: i) continuous training (CON) at low- to moderate-intensity characterized by high volumes of training (> 30 min) with intensities between 60% and 80% of $\dot{V}O_2\text{peak}$ or below the "anaerobic threshold" (AT) with a nearly constant O_2 consumption and without a "slow component" in O_2 kinetics ⁵³⁻⁵⁷ and ii) discontinuous high

intensity training (DHIT) characterized by repeated exercises performed at intensity corresponding to $\dot{V}O_{2peak}$ (or slightly lower) or above AT or "all-out", exercise bouts are separated by brief periods of low-intensity work or inactivity that allow a partial but often not a full recovery^{53-55, 57-59}. Until a few years ago, it was widely believed that DHIT was prerogative of elite athletes accustomed to sustain training periods of CON alternating with periods of DHIT, especially during competitive season⁵⁵. Instead, in sedentary or moderately trained subjects were prescribed primarily exercises of low-moderate intensity and high-volume as it was considered safer and, with reason, effective to improve aerobic metabolism^{60, 131, 132}. However, different studies showed that even in sedentary or moderately trained individuals, DHIT might be an efficient strategy to induce adaptations in skeletal muscle and exercise performance that are comparable with conventional endurance training^{55, 73, 74, 77, 133-136}.

To the best of our knowledge, there are no data on the effects of training program with different intensity and volume characteristics in master runners with several years of training experience. Thus, the aims of this study are: i) to evaluate if an individualized training schedules characterized by an overall reduction of training volume is able to improve performance and ii) to compare the impact of CON and DHIT training programs on running performance and the main physiological factors related to endurance performance in master runners^{6, 9}.

Materials and methods

Participants

Thirty-four male masters runners (age: 47.2 ± 7.4 years, height: 1.75 ± 0.06 m, body mass: 70.0 ± 8.8 kg, BMI: 23.0 ± 1.9) participated in the study. Athletes had a training experience of 15 ± 4 years and in the last 6 months their average training volume was ~ 50 km \cdot wk⁻¹ with a training frequency of 3-4 sessions per week. Participants were not involved in a structured training programs and they independently managed their own training for at least 5 years. All of them competed at regional level on distances between 10-km to Marathon. Before the start of the study, participants were screened medically (history, physical examination, and resting ECG) to ensure that there were no contraindications to study participation. One participant left the study for a cardiovascular problem incompatible with exercise discovered during preliminary test. No subject participated in any study prior to this experiment. Each athlete was fully informed about the aims, methods and risks associated with participation and gave his written informed

consent before the start of the study. All procedures were in accordance with the Declaration of Helsinki and the study was approved by the local Ethics Committee.

Study design

Participants were randomly assigned to three different groups using a spreadsheet¹³⁷ in order to reduce difference between group means for $\dot{V}O_{2\text{peak}}$, gas exchange threshold (GET) and 5-km time trial. The three groups were: continuous moderate intensity training (CON) (n = 11); discontinuous high-intensity training (DHIT) (n = 11) and control group (CTRL) (n=12). All participants were tested before (PRE) and after 8-weeks of training (POST).

Thirty of 35 participants successfully completed the protocol. Four subjects left the study: one subject for knee pain (CON group), and three for muscle injury occurred during training (one in DHIT and two in CTRL).

Training characteristics

CON and DHIT groups trained 3 times per week during a 8-weeks periods in a 400-m outdoor track. Three different training sessions were prepared based on GET. Total distance achieved during each session was controlled in order to obtain an identical training volume. For CON sessions were: 1) 64.5 min at 70% GET, 2) 58.5 min at 80% GET, 3) 54 min at 90% GET. For DHIT: 1) 18 x (1 min at 120% GET, 2 min at 65% GET), 2) 18 x (1 min at 130% GET, 2 min at 65% GET), 3) 18 x (1 min at 140% GET, 2 min at 65%GET). All athletes in CON and DHIT groups received a detailed training plan before the start of the study and they trained under the supervision of an expert. As for CTRL, participants were asked to maintain training habits during 8-weeks. Competitions were not allowed during training period for all groups.

Tests and procedures

Participants were instructed to arrive at the laboratory in a rested and fully hydrated state, about 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. In addition, they were told to avoid alcohol and caffeine products intake 48 h before the exercise test. All laboratory exercise testing sessions were carried out in a well-ventilated climatized laboratory at 19-21°C on a motorized treadmill (Jaeger, Germany) set at a 1% gradient. Subjects initially performed a ramp incremental exercise test (IE) for the determination of $\dot{V}O_{2\text{peak}}$ and GET. The protocol began with

subjects running at $8 \text{ km}\cdot\text{h}^{-1}$ for 6 min; then the belt speed was increased by $1 \text{ km}\cdot\text{h}^{-1}$ every minute until volitional exhaustion. The peak values of the main cardiovascular, respiratory and metabolic parameters were taken as the highest 30-s mean value attained prior to the subject's volitional exhaustion. The GET was determined as described previously¹³⁸. Maximal speed (V_{max}) reached at the end of IE was also recorded.

At least 48 hours after the incremental test, subjects performed two 6-min constant load exercises (CLE) at moderate intensity (60% of $\dot{V}O_{2\text{peak}}$) for determination of metabolic energy cost of running (Cr). Each CLE was separated at least with 20 minutes of rest. As for running performance, participants completed a 5-km endurance running time trial on a 400-m outdoor track. Running test was performed two times separated from at least 5 days and the best performance recorded. During trials, subjects received information regarding the number of laps to go. Performance time was measured using a manual stopwatch.

Measurements

Pulmonary ventilation ($\dot{V}E$, in BTPS), O_2 consumption ($\dot{V}O_2$), and CO_2 output ($\dot{V}CO_2$), both in STPD, were determined breath-by-breath by a metabolic cart (Vmax29c; SensorMedics, Bilthoven, The Netherlands). Expiratory flow was determined by a mass flow sensor (hot wire anemometer). $\dot{V}O_2$ and $\dot{V}CO_2$ were determined by continuously monitoring PO_2 and PCO_2 at the mouth throughout the respiratory cycle and from established mass balance equations. Respiratory exchange ratio (RER) was calculated as $\dot{V}CO_2/\dot{V}O_2$. HR was determined from the ECG signal. At rest and at various times (1, 3, 5 and 7 min) during recovery, 20 μL of capillary blood was obtained from a preheated earlobe for the determination of blood lactate concentration ($[La^-]_b$) by an enzymatic method (Biosen 5030; EKF, Cosmed, Italy). The rate of metabolic energy expenditure was calculated by multiplying the subject's net $\dot{V}O_2$ values (in $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) (measured minus resting) during CLE per the caloric equivalent (in $\text{kJ/l } O_2$) determined from the respiratory exchange ratio. Metabolic energy cost of running (Cr) were finally calculated (in $\text{J}\cdot\text{kg}^{-1}\cdot\text{m}^{-1}$) as the ratio between energy expenditure and the speed ($\text{m}\cdot\text{min}^{-1}$) during CLE^{139, 140}.

Statistical analysis

Results are expressed as means \pm SD. The data were analyzed using a two-way ANOVA for repeated measures (groups \times time). Post-hoc analysis was completed using Bonferroni multiple comparisons. When significant effects of time were found, a student t-test for paired data was used to determine differences between PRE and POST. A stepwise multiple regression analysis of the PRE data has been performed in order to extract a set of physiological variables which provided the optimal prediction of 5-km time trial. Significance level was set at $P < 0.05$.

Results

Training volume. During the 8 weeks, training volume was significantly reduced in CON ($34.1 \pm 3.1 \text{ km}\cdot\text{wk}^{-1}$) and DHIT ($33.3 \pm 2.8 \text{ km}\cdot\text{wk}^{-1}$) compared to CTRL ($51.8 \pm 13.4 \text{ km}\cdot\text{wk}^{-1}$). (Figure 1).

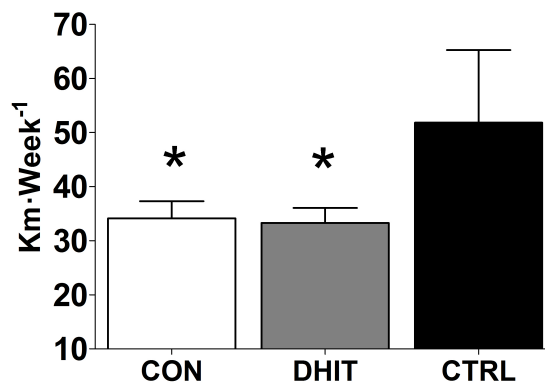


Figure 1. Training volume differences between groups. CON, continuous moderate intensity training; DHIT, discontinuous high-intensity training; CTRL, control group. * significantly different from CTRL ($P < 0.05$)

Incremental exercise. Mean (\pm SD) of the main cardiovascular, respiratory and metabolic parameters obtained in CON, DHIT and CTRL are shown in Table 1. All groups attained peak HR values around 95% of the age predicted maximum. Thus, taking into account also RER and $[\text{La}^-]$ peak values, it can be assumed that maximum exercise capacity had in fact been reached in each condition.

Table 1. Mean±SD of the main cardiovascular, respiratory and metabolic parameters obtained during the incremental test

	CON		DHIT		CTRL	
	PRE	POST	PRE	POST	PRE	POST
$\dot{V}O_{2peak}$ (L·min ⁻¹)	3.24±0.33	3.30±0.34	3.50±0.39	3.51±0.38	3.37±0.44	3.40±0.42
HR max (bpm)	173±10	170±10	175±14	172±13	174±9	173±11
$\dot{V}E$ (L·min ⁻¹)	116.5±12.8	112.9±16.2	121.1±17.7	129.5±24.6	119.8±14.2	118.1±15.4
R Q	1.19±0.04	1.20±0.07	1.16±0.07	1.18±0.08	1.18±0.02	1.19±0.06
[La ⁻]b (Mm)	7.81±1.57	8.01±1.64	7.9±0.8	8.44±0.5	8.4±1.1	8.2±0.9

$\dot{V}O_{2peak}$, peak oxygen uptake; HRmax, maximal heart rate; $\dot{V}E$, pulmonary ventilation; [La⁻]b, blood lactate concentration; R, respiratory exchange ratio

$\dot{V}O_{2peak}$ did not change after training in all groups (CON, 47.6±4.2 mL·kg⁻¹·min⁻¹ vs. 48.0±6.5 mL·kg⁻¹·min⁻¹; DHIT, 48.8±5.5 mL·kg⁻¹·min⁻¹ vs. 49.0±4.4 mL·kg⁻¹·min⁻¹; CTRL 48.4±4.4 mL·kg⁻¹·min⁻¹ vs. 48.3±4.0 mL·kg⁻¹·min⁻¹, in PRE and POST respectively).

Vmax was significantly higher after training only in DHIT (16.4±1.8 km·h⁻¹ vs 17.4±1.3 km·h⁻¹ in PRE and POST respectively; P) no differences were found for CON (16.4±1.5 km·h⁻¹ vs 16.9±1.4 km·h⁻¹ in PRE and POST respectively) and CTRL (16.3±1.9 km·h⁻¹ vs 16.5±1.6 km·h⁻¹ in PRE and POST respectively) (Figure 2).

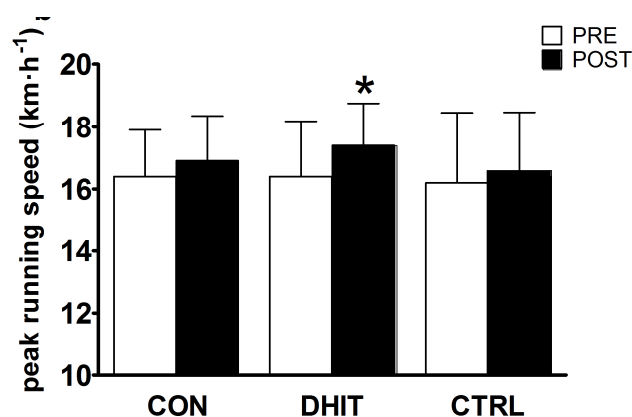


Figure 2. Peak treadmill running speed (Vmax) PRE (white bars) and POST (black bars) 8 weeks of training. CON, continuous moderate intensity training; DHIT, discontinuous high-intensity training; CTRL, control group. * significantly different from PRE (P<0.05)

As for GET, no significant differences were found in POST (88.6±3.2%, 88.8±4.6%, 88.8±3.8% of $\dot{V}O_{2peak}$ in CON, DHIT and CTRL respectively) respect to PRE (87.6±6.1%, 86.8±4.9%, 87.6±6.3% of $\dot{V}O_{2peak}$ in CON, DHIT and CTRL respectively) in all groups. However, the speed at GET significantly improved POST respect to PRE in CON (15.1±1.1 km·h⁻¹ vs 14.6±1.4 km·h⁻¹, respectively) and DHIT (14.9±1.1 km·h⁻¹ vs 14.1±1.2 km·h⁻¹, respectively) whereas no differences were found in CTRL (14.2±1.3 km·h⁻¹ vs 14.0±1.6 km·h⁻¹, respectively) (Figure 3).

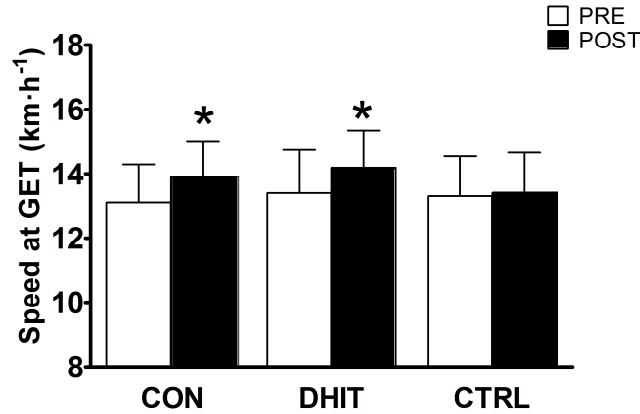


Figure 3. Speed at GET ($\text{km}\cdot\text{h}^{-1}$) PRE (white bars) and POST (black bars) 8 weeks of training. CON, continuous moderate intensity training; DHIT, discontinuous high-intensity training; CTRL, control group. * significantly different from PRE ($P<0.05$)

Constant load exercise. Mean (\pm SD) overall $\dot{V}\text{O}_2$ values, $\dot{V}\text{E}$, HR, RER, and $\Delta[\text{La}]_b$ at the end of moderate exercise are presented in Table 2. All variables are significantly different POST training respect to PRE in both CON and DHIT group, whereas no change was found for CTRL. The metabolic energy cost of running are shown in Figure 4. CON and DHIT significantly decreased Cr by 4.6% and 3.9% after training. No change was found for CTRL.

Table 2. Mean \pm SD of the main cardiovascular, respiratory and metabolic parameters obtained during the constant load exercise.

	CON		DHIT		CTRL	
	PRE	POST	PRE	POST	PRE	POST
$\dot{V}\text{O}_2$ ss ($\text{mL}\cdot\text{Kg}^{-1}\cdot\text{min}^{-1}$)	33.9 \pm 2.8	32.6 \pm 2.2*	33.1 \pm 3.3	31.7 \pm 3.1*	33.3 \pm 2.3	33.0 \pm 2.1
HR max (bpm)	137 \pm 12	132 \pm 13*	129 \pm 11	125 \pm 10*	136 \pm 13	134 \pm 14
$\dot{V}\text{E}$ ($\text{L}\cdot\text{min}^{-1}$)	61.2 \pm 9.4	57.1 \pm 10.2*	60.1 \pm 8.2	57.7 \pm 6.3*	59.7 \pm 8.3	58.1 \pm 9.0
R Q	0.92 \pm 0.05	0.89 \pm 0.04*	0.94 \pm 0.02	0.91 \pm 0.03*	0.92 \pm 0.02	0.91 \pm 0.04
$\Delta[\text{La}]_b$ (Mm)	0.7 \pm 0.4	0.4 \pm 0.3*	0.7 \pm 0.4	0.3 \pm 0.3*	0.7 \pm 0.5	0.5 \pm 0.5

$\dot{V}\text{O}_{2\text{ss}}$, oxygen consumption at steady state; HR, heart rate; $\dot{V}\text{E}$, pulmonary ventilation; $\Delta[\text{La}]_b$, delta blood lactate concentration; RER, respiratory exchange ratio. * significantly different from PRE ($P<0.05$).

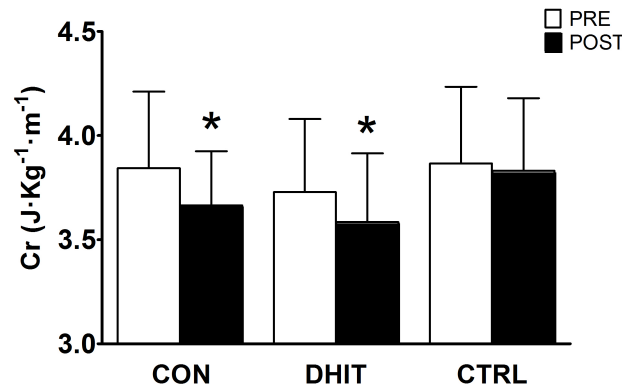


Figure 4. Metabolic energy cost of running during submaximal exercise PRE (white bars) and POST (black bars) 8 weeks of training CON, continuous moderate intensity training; DHIT, discontinuous high-intensity training; CTRL, control group. * significantly different from PRE ($P < 0.05$)

Running performance. 5-km time trial results are shown in Figure 5. Respect to PRE, the time to cover 5 km was statistically lower POST both in CON (1304 ± 109 s vs. 1264 ± 85 s, $P = 0.004$) and DHIT (1282 ± 155 s vs. 1254 ± 140 s, $P = 0.015$). Performance did not change in CTRL (1320 ± 149 s vs. 1309 ± 142 s, in PRE and POST respectively). Results of the Pearson product moment-correlation analysis showed that all variables measured were significantly correlated with 5-km time trial (Table 3).

Table 3. Relationships between $\dot{V}O_{2peak}$, V_{max} , GET, Speed at GET, Cr and 5-km time trial (n=30)

Variables	$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	V_{max} ($km \cdot h^{-1}$)	GET ($mL \cdot kg^{-1} \cdot min^{-1}$)	Speed at GET ($km \cdot h^{-1}$)	Cr ($J \cdot kg^{-1} \cdot min^{-1}$)
5-km time trial (s)	-0.733**	-0.856**	-0.695**	-0.831**	0.428*

$\dot{V}O_{2peak}$, maximal oxygen uptake; V_{max} , maximal speed at the end of incremental test; GET, gas exchange threshold; Cr, energetic cost. ** $P < 0.001$, * $P < 0.05$

The stepwise multiple regression analysis revealed that V_{max} ($km \cdot h^{-1}$), GET ($mL \cdot kg^{-1} \cdot min^{-1}$) and Cr ($J \cdot kg^{-1} \cdot min^{-1}$) are the variables selected for prediction of the 5-km time trial. The analysis showed that 72.4% of the variance in 5-km time trial could be explained by V_{max} alone ($P < 0.0001$), and the addition of GET and Cr to the prediction equation increased this significantly to 79.0% (V_{max} and GET) ($P < 0.0001$) and 85.5% (V_{max} , GET and Cr) ($P < 0.0001$). Table 4 summarizes the linear multiple regression equations obtained.

Table 4. Linear multiple regression equations (n=30)

5-km time trial (s) = 2353.979 - 64.350 Vmax

5-km time trial (s) = 2690.008 - 51.091 Vmax - 13.166 GET

5-km time trial (s) = 2181.979 - 26.394 Vmax - 23.977 GET + 146.629 Cr

Vmax, maximal speed at the end of incremental test; GET, gas exchange threshold; Cr, energetic cost.

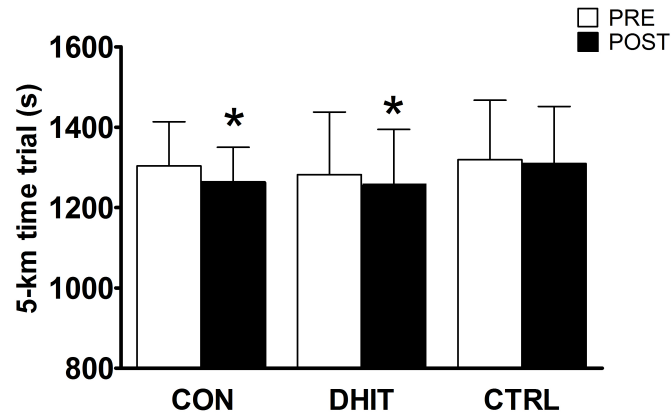


Figure 5. 5-km time trial performance PRE (white bars) and POST (black bars) 8 weeks of training. CON, continuous moderate intensity training; DHIT, discontinuous high-intensity training; CTRL, control group. * significantly different from PRE (P<0.05)

Discussion

The first aim of this study was to evaluate the consequences of a reduction of training volume on running performance in master runners. Results show that a significant reduction of self-managed training volume (about 35%) replaced by a controlled training program significantly improved 5-km time trial. Before the intervention the whole group affirmed to undertake a combination of both high-intensity (~ 4-10% of total) and high-volume (~ 90% of total) training sessions for a training amount of about 50 km·wk⁻¹. However, subjects did not follow any individualized training program and training intensity, as well as training volume, were based only on the participants' experience. Consequently, after some years of similar training contents and intensities, a plateau of performance was reached as suggested by the results of CTRL group. The variables of training should be manipulated from day to day with the goals to maximize physiological capacity over time and to continue to obtain performance improvements⁵⁷. In fact, the intracellular signaling impact of a given exercise stress (intensity x duration) almost certainly decays with training¹⁴¹. It has been suggested that the reduction¹⁴¹ in the exercise training stimulus with advancing age (especially the minimum training intensity to elicit adaptive responses) may have a role in the decline of peak performance^{18, 24, 142, 143}. This issue emphasizes the importance of quality of training rather than

quantity⁵² in master runners in order to obtain improvements on running performance and this implicates the selection of the most appropriate exercise modalities and intensities.

Thus, the second aim of this study was to investigate the effects of continuous training at moderate intensity vs. discontinuous training at high intensity on the main physiological determinants of endurance performance. Some studies comparing the physiological impact of CON and DHIT on sedentary, apparently healthy, young adults and moderately trained individuals showed that both training modalities may similarly improve exercise performance^{75, 131, 144, 145} whereas others found greater improvements with DHIT^{74, 135}. Results of our study are in line with previous research showing that CON and DHIT training programs, characterized by equivalent training volume (~ 35 km·w⁻¹; ~ 35% lower respect to training habits) similarly improved 5-km time trial performance in trained master runners.

In order to better understand the influence of each variable collected on 5-km performance, a stepwise regression analysis has been performed. Our data showed that all variables measured were significantly correlated with 5-km performance (Table 3). However, the V_{max} and speed at GET have the higher correlation coefficient (r=-0.856 and r=-0.831, respectively). This result supports previous studies which found that V_{max} and the fractional utilisation of the $\dot{V}O_{2peak}$ (expressed as lactate or ventilatory threshold) are highly correlated with performance in 5-km in young¹⁴⁶⁻¹⁴⁸ and middle-aged runners¹⁴⁹. Moreover, the stepwise regression analysis revealed that only V_{max}, GET and Cr statistically significantly predicted the 5-km performance (adjusted R²=0.855). This finding is in accord with other studies showing that V_{max}, lactate or ventilatory threshold and RE may be better predictors of endurance performance than $\dot{V}O_{2peak}$ in a homogeneous group of trained endurance athletes^{49, 50, 149-151}.

After training a significant improvement of at least one of the variables selected for prediction of 5-km performance has been observed. Both CON and DHIT significantly decreased Cr by 4.6% and 3.9%, respectively reflecting an improvement in running economy. RE is likely influenced by metabolic, biomechanical factors and/or neuromuscular efficiency^{151, 152}. In our study we observed a significant reduction of the overall $\dot{V}O_2$ at steady state during exercise at 60% $\dot{V}O_{2peak}$ together with a significant reduction of $\dot{V}E$, HR, $\Delta[La^-]_b$, and RER. It has been suggested that training interventions able to reduce $\dot{V}E$, HR and $[La^-]$ during exercise may be beneficial to RE by decreasing the energy demand associated with this parameters^{153, 154}. Moreover, the reduction of the RER after training indicates a greater fat oxidation and a conservation of carbohydrate energy reserves shifting the “start” of glycolytic activity to produce ATP aerobically and/or anaerobically at a higher intensity of running⁶⁰. In addition, it is reasonable to

speculate that training may have also induced an increase in the respiratory capacity of skeletal muscle reducing the ATP demand per mitochondria at given submaximal work rate^{67, 79}.

Beside physiological aspects, neuromuscular factors may also be important determinants of RE^{148, 152}. For example, Piacentini et al.¹⁵⁵ showed that an increased neuromuscular function after concurrent strength and endurance training improves running economy and performance in master runners.

High intensity training may lead to high engagement of the neuromuscular system¹⁵⁶ and improve muscular function especially for older athletes which experience a significant decrease in muscle mass and neural function with ageing. Even if we did not directly measure neuromuscular function, the improvement in Vmax (with no change in $\dot{V}O_2\text{peak}$) for DHIT group after training might be related also to improvements in muscular function¹⁵⁷⁻¹⁵⁹.

These data confirm that both CON and DHIT may positively affect the main factors related to exercise economy in master runners. The present result is compatible with earlier studies showing that RE similarly improved after CON and DHIT in moderately trained subjects^{135, 154}. Franch et al.¹⁵⁴ showed that RE was significantly improved in recreationally men (30.4±4.0 yrs) after 6-weeks continuous-distance training (3.1%) and long interval training (3.0%). Helgerud et al.¹³⁵ found that running economy improved in young university students (24.6±4.0 yrs) after 8 weeks of long-distance training and short- or long-interval training (range, 7.5 to 11.0%).

The more economical runners are able to run at lower percentage of their $\dot{V}O_2\text{peak}$, resulting in lower metabolic perturbations at a given speed. Our results show that CON and DHIT group significantly improved speed at GET by 3.7% and 4.8% for CON and DHIT, respectively. It is known that a rightward shift of the GET to a higher running speed is characteristic of successful endurance training programs. This adaptation allows a higher absolute exercise intensity to be sustained without the accumulation of blood lactate that can be translated in an higher average running speed during competitions⁴⁸.

Our data show that a significant reduction in training volume did not change $\dot{V}O_2\text{peak}$ after both CON and DHIT. Nevertheless, the 5-km running performance improved after both training periods. This findings indicates that when master athletes reached a good level of $\dot{V}O_2\text{peak}$ others factors seem to be important in order to obtain further improvements in running performance. GET expressed as an absolute value ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) has been previously indicate as a significant predictor of 5-km performance. However, when expressed as a percentage $\dot{V}O_2\text{peak}$ subjects in this study had a pretraining GET of ~87% $\dot{V}O_2\text{peak}$.

This represents an already high value and it was unlikely that $\dot{V}O_2$ at GET improved without a further improvements in absolute $\dot{V}O_{2peak}$.

Conclusion

In conclusion, this study indicates that a well-controlled training program may significantly reduce the amount of weekly training volume (with a consistent reduction of time) and significantly improve running performance in masters endurance runners.

V_{max} , fractional utilisation of $\dot{V}O_{2peak}$ and running economy are the best predictor of 5-km performance in master runners suggesting that need to focus training on the improvement of this parameters.

Both continuous moderate intensity training and discontinuous high intensity training, characterized by the same total volume, improve running economy and running performance in master runners. Further research is required in order to better understand the effects of different forms of high-intensity training programs on long term adaptations on cardiopulmonary and skeletal muscle function in master endurance runners.

SECOND STUDY

Time-course changes of oxidative stress response to high-intensity discontinuous training versus moderate-intensity continuous training in masters runners

Introduction

During exercise, the high energy demand required by muscle contraction causes an increase of oxygen delivery/uptake, leading to an increase of O₂ consumption up to 200-fold compared to rest in the muscle fibres¹¹⁸. The high O₂ flux along the mitochondrial electron transport chain, in association with an electron leakage, is correlated with an increased production of free radicals and reactive oxygen and nitrogen species (ROS)^{119, 120, 163}. This phenomenon, usually defined as exercise-induced oxidative stress, has been implicated in the damage of cellular membranes, increased cellular swelling, decreased cell membrane fluidity, and DNA damage⁹⁶⁻⁹⁸. In skeletal muscle fibres, exercise-induced oxidative stress is also linked to fatigue, longer recovery time and increased injury rate^{164, 165}. Indeed ROS can modify sarcoplasmic reticulum calcium handling, acting on calcium-release channels and SERCA, and alter structure and function of myofilaments^{90, 166}.

It has been demonstrated that exercise intensity plays an important role in ROS production by modulating the level of exercise-induced oxidative stress^{116, 117}. During aerobic exercise, the generation of ROS increases according to a higher O₂ consumption and, consequently, a higher electron leakage from the electron transport chain¹¹⁹. If ROS generation exceeds antioxidant defences (i.e. when exercise intensity is greater than 60-70% of maximal oxygen uptake) oxidative damage is observed.

Nevertheless, the association between exercise and oxidative stress is not always negative. The chronic repetition of exercise, i.e. exercise training, may have the capability to develop a compensation to oxidative stress in skeletal muscle fibres¹⁶⁶ by means of an adaptation of the antioxidant and repair systems. This might result in a decreased resting level of oxidative damage and an increased resistance to oxidative stress^{120, 167, 168}. Several studies have demonstrated that antioxidant enzymes adaptation is one of the fundamental changes in response to exercise training within the skeletal muscle¹⁶⁶, as described for mitochondrial oxidative enzymes¹⁶⁹. Indeed, increased levels of ROS and oxidative damage are initiators of specific adaptive responses, such as the activation of antioxidant enzymes and enhanced oxidative damage repair^{94, 167}. The effects of training on oxidative stress depend on training characteristics (i.e., intensity, type, volume, duration)¹⁷⁰⁻¹⁷². Several studies have demonstrated that in humans continuous aerobic training, characterized by a constant sub-maximal intensity, reduces ROS

production and increases antioxidant defences^{166, 173, 174}. Recently, focus has shifted toward training modalities different from the traditional continuous aerobic training, such as high-intensity discontinuous training (HIDT). This training method is characterized by brief intermittent bouts of vigorous activity interspersed by periods of rest or low intensity exercise^{54, 58}. HIDT causes repeated O₂ consumption fluctuations related to changes of exercise intensity as opposed to continuous endurance training where O₂ consumption is nearly constant during the exercise.

HIDT, traditionally used by athletes, it is now increasingly employed in young healthy sedentary individuals as an effective time-efficient alternative to moderate intensity continuous endurance training, inducing similar or even superior changes in a range of physiological parameters, performance and health-related markers⁷¹. Indeed, the benefits of HIDT extend to health promotion and are currently proposed for improving health and reducing fatigue also in middle-aged subjects and in many diseases (COPD and cardiac patients)¹⁷⁵.

Aging is associated with increased free radical generation in the skeletal muscle that can cause oxidative modification of protein, lipid, and DNA. Research evidence indicates that senescent organisms are more susceptible to oxidative stress during exercise because of the age-related ultrastructural and biochemical changes that facilitate formation of reactive oxygen species (ROS). Aging also increases the incidence of muscle injury, and the inflammatory response can subject senescent muscle to further oxidative stress¹⁰⁹⁻¹¹². Furthermore, muscle repair and regeneration capacity is reduced at old age that could potentially enhance the accrual of cellular oxidative damage¹⁷⁶. Nevertheless, the elderly who are physically active benefit from exercise-induced adaptation in cellular antioxidant defence systems¹¹¹. Improved muscle mechanics, strength, and endurance make them less vulnerable to acute injury and chronic inflammation. Indeed, moderate levels of oxidative stress are essential for the organisms to adapt and reach a new level of hormesis even if the balance of oxidants and antioxidants becomes more fragile in advance age¹¹⁵.

Up to date no study has investigated the effects of prolonged (> 1 week) high-intensity discontinuous training on ROS production and exercise-induced oxidative stress in middle-age subjects. These data could be particularly relevant to older subject since it has been reported that both resting and exercise-induced free radical-mediated lipid peroxidation is more pronounced in senescent compared with young human skeletal muscle¹⁷⁷.

The aim of this study was to evaluate the effects of 8-week high-intensity discontinuous training (HIDT) on resting level and time-course changes of several indexes of oxidative stress in masters runners. Since HIDT is characterized by repeated variations of intensity associated with changes of redox potential, ATP/ADP

ratio and, consequently, disturbances of cellular homeostasis¹⁷⁸, we hypothesised that HIDT might cause a higher level of exercise-induced oxidative stress compared to a workload-matched, moderate-intensity continuous training (MOD).

Materials and methods

Participants

Twenty healthy masters runners volunteered to participate in this study. The physical and physiological characteristics of the participants are shown in Table 5. They were all male athletes, competing at national level, with several years (21 ± 4 years) of training experience and training habits of about 45 km.wk⁻¹. Participants were matched on PRE gas exchange (GET) value (see above for further details) before being stratified into two groups completing 8 weeks (3 times non consecutively per week) of moderate-intensity continuous (MOD, n = 10) or high-intensity discontinuous training (HIDT, n = 10) (see training intervention for further details). All participants signed a written consent after being informed of all risks, discomforts and benefits associated with the study. All tests were conducted in the laboratories of the Institute of Bioimaging and Molecular Physiology of the National Research Council under close medical supervision and subjects were continuously monitored by 12-lead electrocardiography (ECG). Procedures were in accordance with the Declaration of Helsinki, and institutional review board (Comitato Etico Indipendente ASL Milano Due) approval was received for this study.

Table 5. Physical and physiological characteristics of the participants

	MOD (n=10)	HIDT (n=10)
Age (years)	50.6±6.3	45.1±8.5
Body mass (kg)	69.6±10.1	72.2±9.1
Height (m)	1.74±0.07	1.76±0.06
BMI (kg·m ⁻¹)	22.8±1.9	23.1±2.3
$\dot{V}O_{2peak}$ (L·min ⁻¹)	3.24±0.33	3.30±0.34

MOD, moderate-intensity continuous training; HIDT, high-intensity discontinuous training $\dot{V}O_{2peak}$, maximal oxygen consumption; BMI, body mass index

Experimental Design

Participants underwent medical examination and were carefully instructed about the experimental procedures in a preliminary session. In the same occasion, anthropometric measures were collected and familiarisation with the testing procedures and equipment was requested. After that, subjects visited the laboratory twice (DAY1 and DAY2) both before (PRE) and at the end (POST) of training. In DAY1, participants performed an incremental test up to voluntary exhaustion (IE). In DAY2, at least 48 hours after, participants underwent two constant-load submaximal exercises (CLE). Blood and urine samples were collected: at rest (REST) in DAY1 and DAY2; and, in DAY2, immediately at the end (END), after 1 (1H) and 2 (2H) hours of CLE. Blood samples at rest were also collected after 4 weeks (4WK). During all the experimental period was recommended to keep unchanged dietary habits, in particular oxidant and antioxidant food (diet reports were administered throughout the study).

Inclusion criteria. Subjects were included in the study if they: 1) were free of musculoskeletal problems and potentially orthopaedic/neuromuscular limitations; 2) had a resting blood pressure below 140/90 mm Hg (subjects on antihypertensive medications (n=6) maintained their medication throughout the study); 3) had no signs of cardiovascular/respiratory complications (at rest and during testing); 4) reported no tobacco use in the 6 months before the study or during the study; 5) did not assume aspirin, as cyclooxygenase can affect oxidant/antioxidant status, at least 1 week before exercise testing, and 6) were not consuming antioxidant compounds including vitamins, minerals, and medications (i.e., probucol, nebivolol, and anti-inflammatory agents).

Exercise testing procedures. The following exercises were performed on a motorized treadmill (Laufergotest, Jaeger, Germany): a) An incremental exercise (IE) up to voluntary exhaustion (after 6 min warm-up at 10 km·h⁻¹ at 1% grade the speed of the belt was increased by 1 km·h⁻¹ every minute). Voluntary exhaustion was defined as maximal levels of self-perceived exertion using the validated Borg scale¹⁷⁹. Peak oxygen uptake ($\dot{V}O_{2peak}$) was determined as the average of the last 20 s values; b) Two 6-min constant-load exercises (CLE) of moderate (< gas exchange threshold, GET) and heavy (> GET) intensity respectively, separated by a 20-min recovery period. Pulmonary ventilation ($\dot{V}E$, expressed in BTPS - body temperature, pressure, and saturated), O₂ uptake ($\dot{V}O_2$), and CO₂ output ($\dot{V}CO_2$), both expressed in STPD (standard temperature, pressure, and dry), were determined breath-by-breath by a computerized metabolic cart (SensorMedics Vmax29c, Bilthoven, The Netherlands). Expiratory flow measurements were performed by a mass flow sensor (hot wire anemometer), calibrated before each

experiment by a 3 litres syringe at three different flow rates. Tidal volume and $\dot{V}E$ were calculated by integration of the flow tracings recorded at the mouth. $\dot{V}O_2$ and $\dot{V}CO_2$ were determined by continuously monitoring PO_2 and PCO_2 at the mouth throughout the respiratory cycle and from established mass balance equations, after alignment of the expiratory volume and expiratory gases tracings and A/D conversion. Calibration of O_2 and CO_2 analyzers was performed before each experiment by utilizing gas mixtures of known composition. Digital data were transmitted to a personal computer and stored on disk. Gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2$. Heart rate (HR) was determined by ECG. Blood pressure (BP) was measured using a standard cuff sphygmomanometer. Severe hypertension (systolic BP value > 250 mmHg) or falling BP during exercise were considered criteria for the termination of the test.

Blood Sampling and Analyses. Each subject reported to the laboratory at 9:00 a.m. after an overnight fast for blood sampling. Subjects abstained from alcohol and caffeine consumption for at least 24 h, and did not perform physical exercise for the 48 h before testing. Approximately 3 mL of blood was drawn from an antecubital vein, with subjects remaining supine. The blood samples were collected in heparinised Vacutainer® tubes, and plasma was separated by centrifuge (5702R, Eppendorf, Germany) at 1000 g for 10 min at 4 °C. The plasma samples were then stored in multiple aliquots at -80 °C until assayed. Samples were thawed only once before analyses, which were performed within two weeks from collection.

Thiobarbituric acid-reactive substances (TBARS). A TBARS assay kit (Cayman Chemical, U.S.), which allows a rapid photometric detection of the thiobarbituric acid malondialdehyde (TBAMDA) adduct at 532 nm, was used. Samples were read by a microplate reader spectrophotometer (Infinite M200, Tecan, Austria). A linear calibration curve was computed from pure MDA-containing reactions.

Protein Carbonyls (PC). Reactive species produced directly or indirectly through lipid peroxidation intermediates also may oxidatively modify proteins. The accumulation of oxidized proteins was measured by content of reactive carbonyls. A Protein Carbonyl assay kit (Cayman Chemical, U.S.) was used to evaluate colorimetrically-oxidized proteins. The samples were read at 370 nm, by a microplate reader spectrophotometer (Infinite M200, Tecan, Austria), as described in detail by the manufacturer. Oxidized proteins values obtained were normalized to the total protein concentration in the final pellet (absorbance reading at 280 nm), in order to consider protein loss during the washing steps, as suggested in the kit's user manual.

Total antioxidant capacity (TAC). Plasma TAC was measured by an enzymatic assay kit (Cayman Chemical, U.S.) using a microplate reader spectrophotometer (Infinite M200, Tecan, Austria). This assay is based on the ability of antioxidants in the plasma to inhibit the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline) sulfonic acid (ABTS, Sigma) to the radical cation ABTS⁺ by a peroxidase. The amount of the produced ABTS⁺ has been assessed by measuring the absorbance signals at 705 nm. The antioxidants concentration is proportional to the suppression of the absorbance signal. TAC was evaluated by a trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich) standard curve, and was expressed as trolox-equivalent antioxidant capacity concentration (mM).

Urine Sampling and Analysis. Each subject reported to the laboratory at 9:00 a.m. after an overnight fast for urine sampling. All samples were collected by voluntary voiding in a sterile container provided to the subject. Aliquots of the urine were stored at -80 °C until the analyses were performed.

8-hydroxy-2-deoxy Guanosine (8-OH-dG). 8-hydroxy-2-deoxy guanosine (8-OH-dG) has been established as a marker of oxidative DNA damage. A commercially-available enzyme immunoassay EIA kit (Cayman Chemical, U.S.) was utilized. The EIA employs an anti-mouse IgG-coated plate and a tracer consisting of an 8-OH-dG-enzyme conjugate. This format has the advantage of providing low variability and increased sensitivity compared to assays that use antigen-coated plates. This assay is based on the competition between 8-hydroxy-2-deoxy guanosine and a 8-OH-dG acetylcholinesterase (AChE) conjugate (8-OH-dG Tracer) for a limited amount of 8-OH-dG. Because the concentration of the 8-OH-dG Tracer is held constant while the sample concentration of 8-OH-dG varies, the amount of 8-OH-dG Tracer that is able to bind to the 8-OH-dG monoclonal antibody will be inversely proportional to the concentration of 8-OH-dG in the sample. This antibody-8-OH-dG complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction absorbs at 412 nm. The sample 8-OH-dG concentration is determined using a 8-OH-dG standard curve. Urinary concentrations of 8-OH-dG, as any urinary marker, vary considerably, therefore the urinary parameters are usually standardized based on the amount of creatinine excreted in the urine when the collection of the 24 h urine is not possible.

Creatinine. In the absence of renal disease, the excretion rate of creatinine in an individual is relatively constant. Thus, urinary creatinine levels may be used as an index of standardization for 8-OH-dG. A

creatinine assay kit (Cayman Chemical, U.S.) was used to measure creatinine levels in urine samples. Samples were read by a microplate reader spectrophotometer (Infinite M200, Tecan, Austria). Creatinine concentration was determined using a creatinine standard curve.

Training Intervention. On the basis of the individual values of GET (expressed as % of the $\dot{V}O_{2peak}$) obtained at PRE, participants were matched and assigned to either moderate-intensity continuous training group (MOD, n = 10) or high-intensity discontinuous training group (HIDT, n = 10). Each group undertook 8 weeks of training, three times a week. Three different types of training sessions were scheduled, with the total distance covered in each session being matched between the groups, in order to control for the training volume performed. For MOD, the sessions were as follows: a) 64.5 min at 70 % GET, b) 58.5 min at 80 % GET, and c) 54 min at 90 % GET. For HIDT, the work-matched sessions were: a) 18 x (1 min at 120 % GET, 2 min at 65 %), b) 18 x (1 min at 130 % GET, 2 min at 65 %), and c) 18 x (1 min at 140 % GET, 2 min at 65 %). In week 1 and 4, the participants performed only the session type “a” and “b”, while in week 8, the volume of session type “c” was reduced by decreasing the exercise duration (for MOD, 27 min at 90 % GET; for HIDT, 9 x 1 min at 140 % GET, 2 min at 65 %).

Statistical analysis. Data are expressed as Mean \pm Standard Deviation. All results were tested for normal distribution using a Shapiro-Wilk test, and when the assumption of normality was not met, a natural log transformation was applied to reduce the bias due to non-uniformity of the error. Data from the resting oxidative stress measurements were analysed using a Two-Way ANOVA with repeated measures (group x training). Data from the oxidative stress kinetics were analysed using a Three-Way ANOVA with repeated measures (group x training x time). When statistical significance ($p < 0.05$) was obtained for a main factor, a Bonferroni post hoc test was performed. The test-retest variability of the oxidative stress measures was analysed on the resting data in PRE and POST. In our hands the inter- and intra-assay coefficients of variation of the above-mentioned analyses were as follows: TBARS, 5.4% and 7.6%; PC, 4.8% and 11.8%; TAC, 8.5% and 7.7%, respectively.

Results

Resting values

The resting plasma TBARS and PC concentrations are shown in Fig. 6. The upper panels show TBARS values before (PRE), after four weeks (4WK) and at the end (POST) of training in both MOD and HIDT group. In MOD, TBARS concentration declined significantly from PRE ($7.53 \pm 0.30 \mu\text{M}$) to 4WK ($6.50 \pm 0.25 \mu\text{M}$) and remained low in POST ($6.46 \pm 0.27 \mu\text{M}$). Also in HIDT, TBARS concentration declined from PRE ($7.21 \pm 0.32 \mu\text{M}$) to 4WK ($6.78 \pm 0.25 \mu\text{M}$), reaching a statistical significance at POST ($5.85 \pm 0.46 \mu\text{M}$). No significant differences were observed in TBARS concentration between MOD and HIDT in all conditions. The lower panels show PC values in PRE, 4WK and POST for both MOD and HIDT. Training did not significantly modify the PC concentration both in MOD (0.74 ± 0.04 , 0.73 ± 0.04 and $0.73 \pm 0.05 \text{ nmol} \cdot \text{mg}^{-1}$ protein in PRE, 4WK and POST respectively) and HIDT (0.78 ± 0.08 , 0.78 ± 0.04 and $0.76 \pm 0.06 \text{ nmol} \cdot \text{mg}^{-1}$ in PRE, 4WK and POST respectively). No significant differences were observed in the resting concentrations of PC between MOD and HIDT.

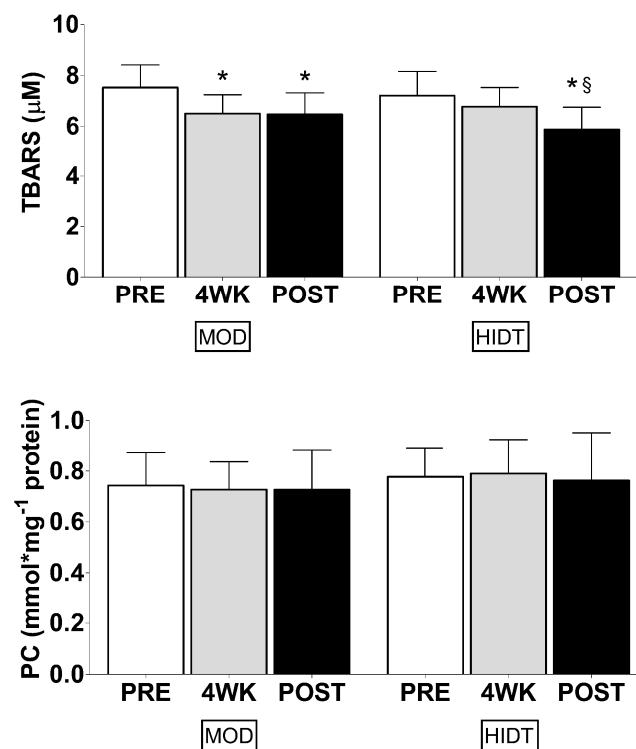


Figure 2. Effect of continuous moderate-intensity training (MOD) and high-intensity discontinuous training (HIDT) on thiobarbituric acid-reactive substances (TBARS) and protein carbonyls (PC). White bars represent pre-training (PRE) values, grey bars 4 weeks (4W) of training values and black bars post-training (POST) values. Values are expressed as means \pm SD. * Significantly different from PRE ($P < 0.05$); § Significantly different from 4WK ($P < 0.05$)

In Fig. 7, resting plasma TAC values are shown. In MOD, TAC values resulted significantly reduced in 4WK ($1.84 \pm 0.12 \text{ mM}$) respect to PRE ($2.40 \pm 0.20 \text{ mM}$), without any other significant change in the last four weeks of training ($1.87 \pm 0.11 \text{ mM}$, POST). In HIDT, TAC values were unaffected by training (1.95 ± 0.15 ,

1.79±0.12 and 1.98±0.13 mM in PRE, 4WK and POST, respectively). No significant differences were observed in TAC between MOD e HIDT.

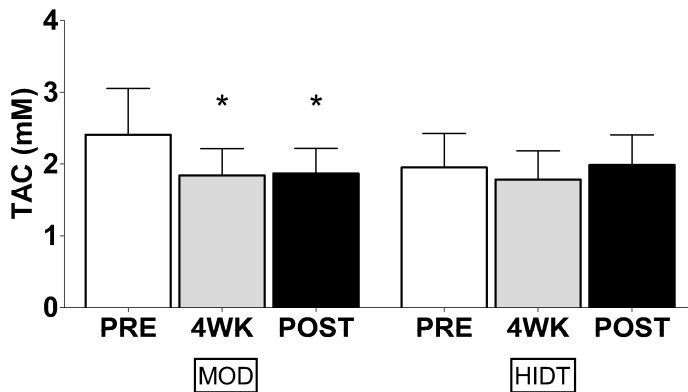


Figure 3. Effect of continuous moderate-intensity training (MOD) and high-intensity discontinuous training (HIDT) on total antioxidant capacity (TAC). White bars represent pre-training (PRE) values, grey bars 4 weeks (4W) of training values and black bars post-training (POST) values. Values are expressed as means ± SD. * Significantly different from PRE (P<0.05)

In Fig. 8 individual TAC values are shown. A large individual difference in resting TAC values among the subjects was observed at PRE both for MOD and HIDT. At POST, TAC values distribution was less scattered both for MOD and HIDT.

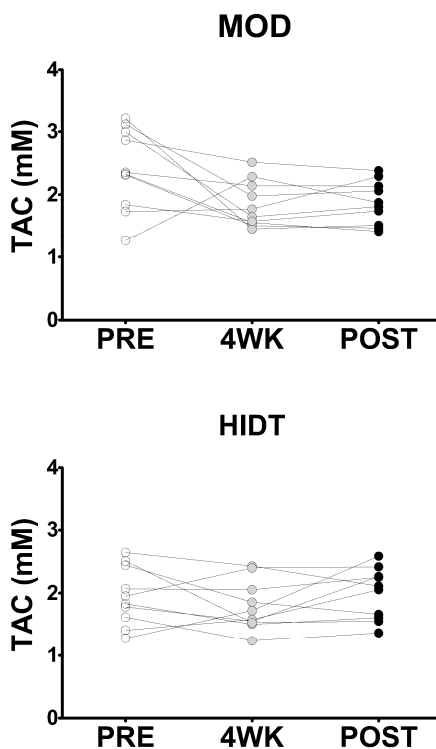


Figure 4. Individual changes in TAC value in MOD and HIDT. White squares represent pre-training (PRE) values and black squares represent post-training (POST) values.

The urinary levels of 8-OH-dG, biomarker of in vivo oxidative DNA base modifications, are shown in Fig. 9. The 8-OH-dG concentration significantly decreased from PRE (5.50 ± 0.66 and 4.52 ± 0.50 ng . mg⁻¹ creatinine in both MOD and HIDT, respectively) to POST (4.16 ± 0.40 and 3.18 ± 0.34 ng . mg⁻¹ creatinine in both MOD and HIDT, respectively). No significant differences in 8-OH-dG concentration were observed between HIDT and MOD.

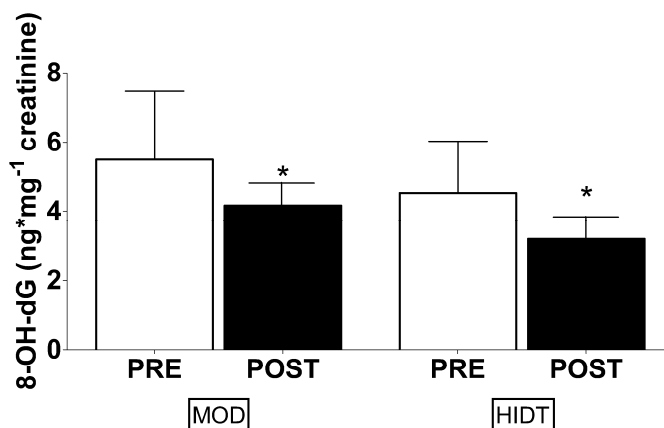


Figure 5. Effect of continuous moderate-intensity training (MOD) and high-intensity discontinuous training (HIDT) on oxidative damage of DNA measured by 8- hydroxy -2-deoxy guanosine (8-OH-dG). White bars represent pre-training (PRE) values and black bars are post-training (POST) values. Values are expressed as means \pm SD. * Significantly different from PRE ($P < 0.05$)

Kinetics of adjustment

The time course of TBARS and PC concentration changes obtained before, immediately after and at 1 and 2 hours of recovery from CLE carried out PRE and POST are shown in Fig. 10. In both groups and in all conditions TBARS concentration significantly increased immediately after exercise and returned toward resting levels thereafter. In MOD (Fig. 10a), as for PRE, TBARS concentration increased significantly in END (9.90 ± 0.68 μ M) and returned toward resting levels thereafter (8.28 ± 0.57 μ M and 7.62 ± 0.63 μ M in 1H and 2H respectively). As for POST, time course of TBARS concentration was similar but TBARS values were always significantly lower than PRE. In HIDT (Fig. 10b), the time course changes of TBARS were similar to those described for MOD. No significant differences were observed between MOD and HIDT.

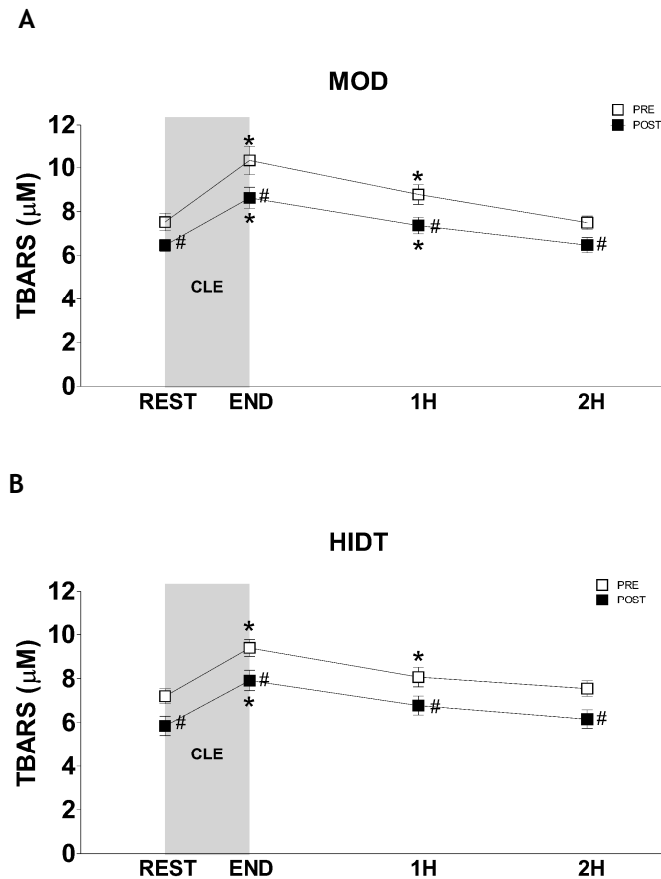


Figure 6. Time course changes of TBARS concentration recorded before (REST) and after (END, 1H and 2H) constant-load submaximal exercise trials (CLE). White squares indicate pre training (PRE) values and black squares post-training (POST) values. Values are expressed as means \pm SD. * $P < 0.05$ compared to REST; # $P < 0.05$ compared to PRE

The time course changes of PC concentration are shown in Fig. 11. In PRE, PC increased progressively after CLE, reaching the significantly highest value at 1H (1.33 ± 0.22 and 1.32 ± 0.30 $\text{nmol} \cdot \text{mg}^{-1}$ protein in both MOD and HIDT, respectively), and returning to resting values at 2H (0.87 ± 0.07 and 0.90 ± 0.09 $\text{nmol} \cdot \text{mg}^{-1}$ protein in both MOD and HIDT, respectively). In POST, the time course of PC concentration was very similar but, as for MOD (Fig. 11a), the peak value reached at 1H (1.04 ± 0.07 $\text{nmol} \cdot \text{mg}^{-1}$ protein) was significantly lower than in PRE.

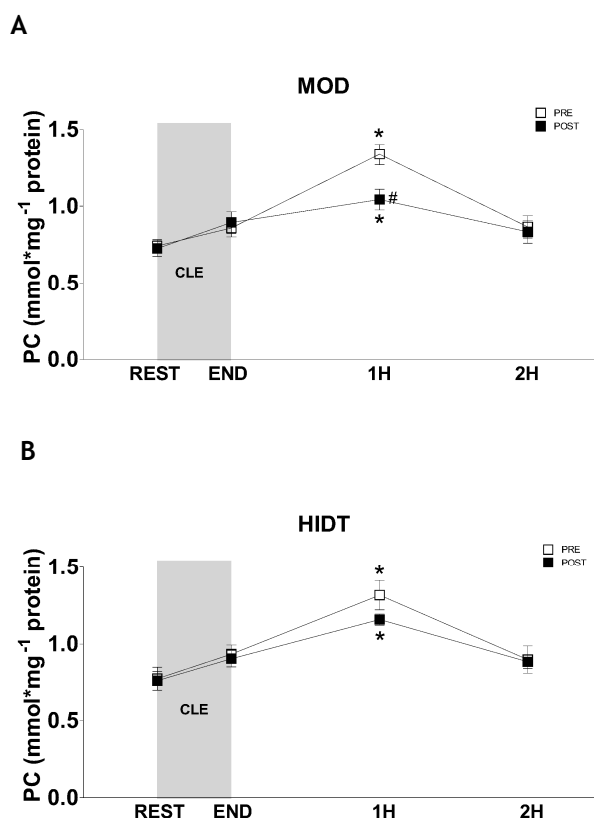


Figure 7. Time course changes of PC concentration recorded before (REST) and after (END, 1H and 2H) constant-load submaximal exercise trials (CLE). White squares indicate pre training (PRE) values and black squares post-training (POST) values. Values are expressed as means \pm SD. * $P < 0.05$ compared to basal value; # $P < 0.05$ compared to PRE

Discussion

This study was designed to evaluate the oxidative stress response to high-intensity discontinuous training versus moderate-intensity continuous training in masters runners. The main findings are listed hereafter.

- TBARS resting values were significantly reduced after training both in MOD and HIDT.

It is known that moderate intensity aerobic training such as those adopted by Fatouros et al.¹⁷³, i.e. 50-80% of HRmax for 16 weeks, or by Leeuwenburgh et al.¹¹⁵ i.e. 75% of $\dot{V}O_2$ max for 6 weeks, decreases resting lipid peroxidation levels. Our data are in agreement with these results since we observed in MOD a reduction of TBARS resting values. As for HIDT, the effects on lipid peroxidation levels are not well understood. A trend towards a reduction of resting plasma TBARS levels was shown in young subjects performing three sessions of HIDT within 1 week¹⁸⁰. Our data confirm and extend these findings. We observed in masters runners a significant reduction in TBARS resting values only after 8 weeks of HIDT, but not after 4 weeks. Thus, the training duration seems to be an important variable affecting this adaptation. It is plausible that free radicals production and, consequently, lipid peroxidation induced by every single

session of HIDT could be progressively reduced as observed within 1 week by Fisher et al.¹⁸⁰. Moreover, it has been suggested that exercise training lowers resting lipid peroxidation by up-regulating antioxidant enzyme levels in tissues engaged in systematic exercise¹⁸¹. So, the 8 weeks of training could have allowed enough time for the antioxidant systems to reduce the acute damage of each single session of high-intensity training.

- No significant change in PC resting values was observed in both MOD and HIDT.

It is known that aging is associated with increased free radical generation in the skeletal muscle and increased oxidative modification of protein, lipid, and DNA⁹⁶⁻⁹⁸. Moreover, some studies show that long-term training increases the macromolecular oxidative damage in elderly men. For example, Gonzalo-Calvo et al.¹⁸² recently demonstrated that the level of carbonyl protein content in plasma and erythrocytes, are higher in a group of older men (>65 years) undergoing long-term training than in one group of sedentary subjects. Our data showed no significant changes in PC resting values after training in both MOD and HIDT, confirming previous reports on sedentary individuals undergoing 12 weeks of resistance training¹⁸³. Now, oxidative modifications of protein (as accumulation of reactive carbonyl derivatives) can serve as a tag to indicate which proteins need to be replaced¹⁶⁸. Proteins are usually replaced by proteasome complex and an increased activity of proteasome could be an important factor that affects the rate of protein turnover and the remodeling of skeletal muscle¹⁸⁴. Since it is known that exercise can induce the activity of proteasome complex and increase the rate of protein turnover, it is plausible that MOD and HIDT induced both the accumulation of reactive carbonyl derivatives and the increase of damaged proteins proteolysis, leading to no significant changes in PC resting values. Therefore the unchanged PC resting values recorded in our study may be seen as a positive effect of both training protocols adopted.

- The accumulation of 8-OH-dG in urine was significantly reduced in MOD and HIDT.

Several studies conducted submaximal aerobic exercise protocols under laboratory conditions to investigate DNA effects. DNA damage was neither seen after intense treadmill running in male subjects of different training status¹⁸⁵ nor in well-trained endurance athletes¹⁸⁶. However, conflicting findings were reported when maximal exercise protocols, i.e. tests until exhaustion, were conducted under laboratory conditions. Increased levels of DNA strand breaks were observed after exhaustive treadmill running in subjects of different training status¹⁷¹. Moller et al.¹⁸⁷ demonstrated DNA strand breaks and oxidative DNA damage after an maximal cycle ergometer test under high altitude hypoxia, but not normal

(normoxic) conditions. Furthermore, there were no differences in urinary 8-OHdG concentrations before and after supplementation with β -carotene within the 3 d following a cycle ergometer test to exhaustion¹⁸⁸.

As for training, a few studies have examined whether periods of intensified training affect genome stability. Increased urinary 8-OHdG levels were observed in 23 healthy males in response to a vigorous physical training programme (about 10 h of exercise for 30 d)¹⁸⁹ and in male long-distance runners throughout a training period for 8 d compared to a sedentary period¹⁹⁰. However, in a longitudinal study no differences in urinary excretion of 8-OHdG between a group of long-distance runners and a sedentary control group were observed¹⁹¹. Our data showed a decrease (~25%) in urinary 8-OH-dG excretion in both MOD and HDT groups. These results could be explained by less DNA damage but also by activation of DNA repair processes. In fact, the activities of DNA damage-repairing enzymes are up-regulated by training¹⁹². To our knowledge this is the first study to evaluate oxidative DNA damage in humans following high intensity training. Contrary to our hypothesis the disturbances of cellular homeostasis caused by repeated variations of intensity in HDT did not determine DNA damage significantly different from MOD. Therefore the beneficial adaptation observed may be independent from the intensity of training.

- The defences against oxidative damage were lowered only in MOD, not in HDT.

Skeletal muscle is a remarkably adaptive tissue that is capable of changing its morphological, physiological, and biochemical properties in response to various perturbations. The adaptations are accomplished by various signal transduction pathways that relay external stimuli to changes in intracellular enzyme activity and/or gene expression. Exercise-induced oxidative stress serves as an important signal to stimulate muscle adaptation of antioxidant systems via activation of the redox-sensitive signalling pathways^{88, 94}. While an acute bout of muscular contraction is sufficient to activate these pathways, up-regulation of enzyme protein synthesis requires cumulative effects from repeated bouts of exercise, that is, exercise training.

The effect of chronic exercise on redox status and antioxidant defence is a much-debated question. Chronic exercise training has been suggested to induce an increase of the activity of the antioxidant defence systems by animals¹⁹³ and humans studies^{173, 194}. However, other studies have shown no change in sedentary individuals^{94, 193, 194}, or even a decrease in antioxidant capacity with training^{115, 195-197}. Results of the present study showed a significant decrease of the resting TAC values in MOD but not in the

HIDT group. Even though it cannot be excluded that the different intensity of the training programmes could be responsible for this finding, an alternative explanation could be proposed.

TAC value can be considered a reliable biomarker of antioxidant defence, although it should be interpreted with some caution. It is well known that oxidative stress biomarkers are influenced by sex, age, lifestyle (i.e. smoking), dietary intake, previous strenuous exercise and/or training status. To overcome this inconvenience a “theoretically” homogeneous experimental group (males, no smokers, masters athletes) was chosen in present study. Nevertheless, large individual differences in resting TAC values among the subjects were observed at PRE (Fig. 7), resulting in a higher starting antioxidant defence level in MOD than in HIDT. Therefore, we believe that the significant training-induced decrease of TAC value observed in MOD might be attributed to a higher baseline. If we compare the participants’ individual data before, during and after training it is easy to notice that training has induced a converging of TAC values towards an optimal level, especially in MOD (Fig. 7). In fact, participants who were characterized by low pre-training TAC values showed an increase of these, while subjects with high pre-training values showed a decrease. It is becoming increasingly clear that reactive species act in a hormetic manner¹⁹⁸ since an optimal ROS level is beneficial for the cell survival, whereas too little or too much ROS result in impaired physiological function. Therefore, excessive attenuation of ROS production, caused by high total antioxidant capacity values, if on one hand reduces oxidative damage on the other might be considered detrimental for cellular functionality.

- Kinetics of adjustment of oxidative stress biomarkers to acute exercise.

There is an abundance of literature indicating that exercise increases the production of reactive oxygen species to a point that can exceed antioxidant defenses and thus cause oxidative stress^{163, 197, 199-201}. Few studies, however, have investigated with an adequate sampling time, the kinetics of adjustment of oxidative stress biomarkers after exercise. Michailidis et al.²⁰⁰ after a specific aerobic exercise protocol have observed the highest value of TBARS and PC at 1 h and 4 h after exercise, respectively. In the present study the highest value of TBARS and PC was measured immediately at the end and 1 h after exercise, respectively. This shorter-lived response of PC and TBARS could be attributed, at least in part, to the lower intensity and shorter duration of the exercise protocol used in our study. More generally, the findings of the present study provide further evidence to the notion that non-muscle-damaging exercise induces alterations in redox homeostasis that last only few hours post exercise¹²¹.

Moreover, our study also evaluated the effect of training protocols to the exercise-induced oxidative damage kinetics. According to Nikolaidis et al. ¹²¹, it becomes clear that the resting levels of many redox biomarkers return limited information compared to the ones modified by an acute exercise session. In other words, it may be easier to find an existing effect of a redox agent on body fluids redox status after exercise than at rest, simply because the stimulus of exercise may extend the magnitude and the duration of change in redox homeostasis. Both MOD and HIDT did not affect the time-course of plasma oxidative stress biomarkers. However, TBARS values at any time resulted significantly lower after training and PC peak value decreased after both HIDT and MOD resulting statistically different only for the latter. The decrease in the peak PC value observed might be a consequence of the activation of mechanisms induced by training procedures that more efficiently remove the oxidatively modified proteins from circulation.

Limitations of the study

This manuscript attempts to evaluate the effects of high-intensity discontinuous training on oxidative damage. Many approaches allow evaluation and demonstration of the participation of ROS in biochemical events. Indeed, the literature is replete with descriptions of different methodologies and approaches for these purposes. The only technique for direct detection of radicals is electron spin resonance, which allows the detection of relatively stable radicals. The indirect detection of ROS intervention is based on the dosage of specific end products resulting from the interaction of the ROS with biological macromolecules, such as DNA, proteins and lipid. The appearance of these end products serves as proof of the prior existence of ROS that left their footprints in the cell. The authors are aware that neither thiobarbituric acid reactive substances nor protein carbonyls or 8-hydroxy-2deoxy guanosine represent specific biomarkers of lipid peroxidation, protein oxidation or DNA base modifications. Nevertheless, we believe that our array of biomarkers is well able to characterize the oxidative status during the post-exercise period (and clearly equals or exceeds that of many similar investigations see for example ¹²⁰ and ¹⁶⁷). It is possible that oxidative stress may have occurred in tissues aside from blood, such as skeletal muscle, which may be the ideal tissue when studying exercise stress. Of course, biopsies are required for obtaining samples for analyses, which is likely the reason why so few human investigations include the analysis of oxidative stress biomarkers in skeletal muscle.

Conclusion

In conclusion, high-intensity discontinuous and continuous moderate-intensity training induced similar beneficial effects in masters runners, reducing the resting levels of oxidative stress biomarkers in plasma and urine. In addition, we provide further evidence that aerobic exercise induces alterations in redox homeostasis that last only few hours post exercise and are attenuated by training.

Therefore our hypothesis that HIRT might cause a higher level of exercise-induced oxidative stress compared to a workload-matched, moderate-intensity continuous training appears to be incorrect. It is also important to underline that these training adaptive responses appear effective even in middle-aged subjects.

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THIRD STUDY

Effects of manipulating volume and intensity training in masters swimmers

Introduction

An intense exercise event is generally considered to be one lasting between 1 and 8 min, where there is a mix of ATP-derived energy from both aerobic and anaerobic energy systems⁵⁵. It has been claimed that successful training programs for athletes who compete in events like olympic rowing, kayak and canoa, most swimming races, running up to 3000 m and track cycling depend from an appropriate blend of both high-volume and high-intensity training components^{55, 202}. Whereas high-volume low-intensity training has positive long-term effect on several physiological variables²⁰³, high-intensity low-volume training plays a key role for short-term improvements in performance⁵⁵. Indeed, when a period of high-intensity training is supplemented into the already high training volume of well-trained athletes further enhancements in both intense and prolonged performance are possible⁷².

As far as swimming is concerned, most events last less than 8 minutes and thus can be classified as intense exercises⁵⁵. Nevertheless, in swimming practice the relationship between training volume and intensity is still debated and training programs are usually characterized by high-volume sessions (~ 4-10 km·d⁻¹) mainly performed at low-intensity^{68, 204-208}. In literature, a limited number of studies have investigated the influence of manipulating training volume and/or intensity in swimmers^{68, 205, 206, 209}. For example, Costill et al.^{68, 205} showed that a consistent increase in training volume (from 4.3 to 9.0 km·d⁻¹ and from 5.0 to 9.3 km·d⁻¹, respectively), without changing the training intensity, did not lead to further performance improvements in trained athletes. More recently, it has been found that 4 weeks of either high-volume (with higher contribution of low-intensity) or low-volume (with higher contribution of high-intensity) training program induced the same effects in highly-trained, and untrained subjects²⁰⁹. In these studies, however, the difference between high-intensity and high-volume training was not clear. For example, Faude et al.²⁰⁶ observed that high-training volumes are not advantageous compared to a high-intensity training of lower volume in ten young swimmers (nationally ranked 42th or better). Nevertheless, more than ~45% of training volume during high-intensity training period was performed below individual anaerobic threshold (i.e. low intensity). Similarly, Souttanakis et al.²⁰⁹, evaluating if 4-week sprint training alone would create equally favourable adaptations to performance compared with a combined endurance and sprint training (i.e. "high-volume" training) in collegiate swimmers, concluded that both training programs provide similar improvements in swimming performance. However, in the daily

endurance training program they included about 1.0 km (47%) of high-intensity (i.e. at and above lactate threshold) swimming.

To the best of our knowledge, previous studies were focused on young subjects and the difference between high-volume and high-intensity training was blurred whereas there are no data on the effects of training program with different intensity/volume characteristics in older swimmers. Thus, the aim of this study was to investigate the effects of a substantial manipulation of training volume and intensity in a group of trained “masters” swimmers. Our hypothesis was that an increase in training volume performed at low-intensity did not lead to improvement of indexes of aerobic capacity and performance. Conversely, 6 weeks of high-intensity and low volume training could positively affect physiological variables and performance, underling the importance of training intensity on performance also in masters swimmers. Since the number and the level of performance of masters swimmers has been continuously increasing (at a much greater rate than young athletes) ^{9, 15}, these data should be also interesting for coaches and athletes in order to design training program.

Materials and methods

Participants

10 male masters swimmers (age: 32.3 ± 5.1 years, height: 1.81 ± 0.04 m, body mass: 77.0 ± 6.5 kg) participated in the study. Athletes had a training experience of 11 ± 4 years and their average training volume was ~ 3 km·d⁻¹, three times a week. They were specialized in freestyle on distances between 50-400m and they competed in the “14th FINA World Masters Championship” two weeks after the end of the study. Before the start of the study, subjects were screened medically (history, physical examination, and resting ECG) to ensure that there were no contraindications to study participation. No subject participated in any study prior to this experiment. Each athlete was fully informed about the aims, methods and risks associated with participation and gave his written informed consent before the start of the study. All procedures were in accordance with the Declaration of Helsinki and the study was approved by the local Ethics Committee.

Design

The training protocol was conducted at the beginning of the competitive season, after a period of early preparation including water and dry-land training. All swimmers were tested before (PRE) and at the end of two different 6-week training periods. Each testing session was preceded by a week of tapering (Figure

12). Between the two training periods 14 days of low-intensity training of short duration were performed. The first training period was characterized by an increase in training volume and a decrease in training intensity (high-volume low-intensity training, HvLi) in relation to training habits. The second training period was characterized by a reduction of total training volume and an increase of training intensity (low-volume high-intensity training, LvHi). See below for further details.

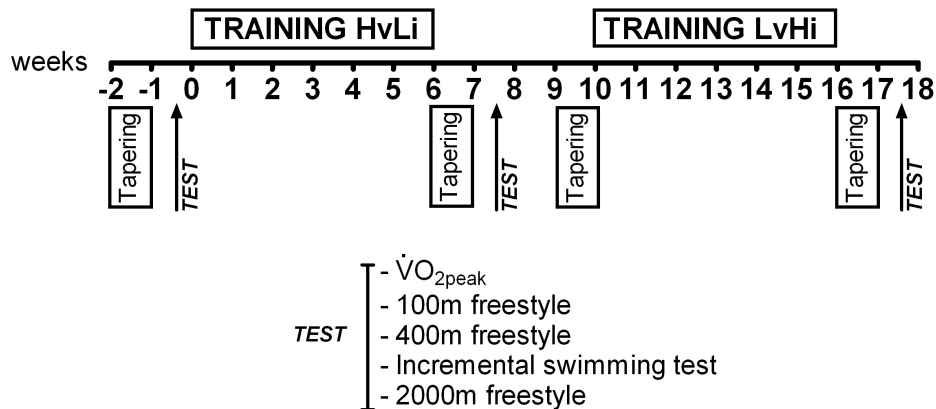


Figure 8. Study design. Each subject performed the HvLi training period first then the LvHi training period. HvLi, high volume-low intensity; LvHi, low volume-high intensity.

Methodology

Prior to the beginning of the study all athletes were fully familiarized with testing procedures. Athletes were invited to keep a food diary for the two days before and during the first testing session and encouraged to follow the same diet before and during all subsequent testing sessions. Tests were performed in an indoor 25-m swimming pool. Subjects were tested after a week of tapering (PRE), one week after the HvLi training period and one week after HiLv training. During the weeks of tapering only low-intensity training of short duration was performed to ensure that subjects were similarly recovered at the start of the testing period. Tests were carried out at the same time of the day and in the same order. Monday morning subjects performed an incremental test to exhaustion to assess $\dot{V}O_{2peak}$. Wednesday morning a 100 and 400m freestyle test were performed with at least 2 h rest between trials. In the evening, at least 4 h after morning testing session, subjects performed an incremental swimming test to assess individual anaerobic threshold (IAT). Friday morning a re-test on 100 and 400m freestyle was performed. In the evening, at least 4 h after morning testing session, subjects performed a 2000m freestyle test.

$\dot{V}O_{2peak}$. Tests were carried out under medical supervision and subjects were monitored by 12-lead ECG. A mechanical braked arm ergometer (Cardio Rehab 891E, Monark, Sweden) was used. The subject was seated in front of the ergometer; chair height was adjusted so that the crankshaft of the ergometer was at approximately shoulder height and the arms were fully extended horizontally during cranking. Arm cranking was digitally displayed to the subjects and maintained constant at 60 rpm. The subject began arm cranking at 15 W for three minutes. Resistance was then increased of 15 W every minute up to exhaustion. Values of cardiovascular, ventilator and gas exchange variables determined during the last 30 seconds of the exhausting load were considered “peak” values. Pulmonary ventilation ($\dot{V}E$, in BTPS), O_2 consumption ($\dot{V}O_2$), and CO_2 output ($\dot{V}CO_2$), both in STPD, were determined breath-by-breath by a metabolic cart (Vmax229, SensorMedics, The Netherlands). Gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2$. HR was determined from the ECG signal. At rest and at various times (1, 3, and 5 min) during recovery, 20 μ L of capillary blood was obtained from a preheated earlobe for the determination of blood lactate concentration ([La]b) by an enzymatic method (Biosen 5030; EKF, Germany).

Performance tests. All test sessions took place in an indoor 25-m swimming pool, 1.90 m deep, with a water temperature of 26 °C. A standardized warm-up, consisting primarily of 1000 m of aerobic swimming of low- to moderate intensity, was conducted before each protocol. Athletes reported at the poolside in a fasted state and performed the test at the same time (± 1 hour). Swimming performance was evaluated by 100m, 400m and 2000m freestyle trials. They started from inside the water in order to avoid the influence of the dive. Time was taken with a manual stopwatch. The 100m and 400m tests were performed to evaluate possible training-induced changes during standardized race distances. The 2000m maximal test was chosen because it represents a distance with a very-high component of aerobic energy production.¹⁶

Individual anaerobic threshold (IAT). An incremental swimming test (IST) was used to assess IAT^{206, 210}. The IST consisted of 7 x 200 m crawl bouts which were swum with increasing intensity. The predefined speed of the last step was chosen according to the personal best time in the 400m freestyle distance that each swimmer was able to accomplish at that time. The mean target speed for each step of the incremental protocol was successively determined by subtracting 0.05 $m \cdot s^{-1}$. Swimming speeds were precisely given by a light emitting diode system (LEDs) positioned on the bottom of the pool and

controlled wirelessly through PC. (Virtual Trainer 2.0, Aqvatech engineering, Italy). Rest between stages was standardized at 45 s to allow an adequate blood sampling. Capillary blood samples (20 µl) were taken from the earlobe before start, at the end of each stage and 1 and 3 min after cessation of exercise and analyzed for lactate concentrations. These data allowed assessing IAT through the [La]b vs. speed curve modelling method assumed to be the interception point of the best fit of a combined linear and exponential pair of regressions used to determine the exact point for the beginning of an exponential rise in [La]b, also known as lactate inflexion point ²¹¹.

Training characteristics. The swimmers trained 3 times per week during the two 6-week periods in an indoor 25-m swimming pool. The athletes' coach collaborated in the schedule of training programs and conducted all training sessions. Training contents were classified in four intensity zones based on the individual anaerobic threshold (IAT): zone 1, 80-90% IAT; zone 2, 100-105% IAT; zone 3, 110-120% IAT; zone 4, >130% IAT. Weekly training volume and percentage of training at different intensity zones during the HvLi and LvHi are presented in Table 6. HvLi was characterized by a nearly 30% increase in total volume as compared to their previous training habits, whereas in LvHi percentage of training volume was decreased by 50% in relation to HvLi. At the start and at the end of each training session swimmers performed controlled warm-up (500 m per session) and cool-down (300 m per session) respectively. All athletes received a detailed training plan before the start of the study and they filled in a daily training log book. Dry-land training (resistance, athletics, cross training) was not performed. Competitions were not allowed during both training periods.

Table 6. Total week training volume and training amount at different intensity zones during HvLi and LvHi

Training contents	Example training sets	HvLi		HiLv	
		Distance (m)	% of total	Distance (m)	% of total
Zone 1 (80-90% IAT)	4x400m, rest 60s / 16x100m, rest 20s / 2x800m, rest 20s	4000	33.3	0	0
Zone 2 (100-105% IAT)	8x250m, rest 30s / 4x100m, rest 15s / 5x400m, rest 40s	6800	56.7	600	10
Zone 3 (110-120% IAT)	4x50m, rest 15s / 2x100m, rest 15s	600	5	2700	45
Zone 4 (>130% IAT)	8x25m, rest 10s / 4x50m, rest 10s	600	5	2700	45
Total amount		12000	100	6000	100

HvLi, high volume-low intensity; LvHi, low volume-high intensity; IAT, individual anaerobic threshold

Statistical Analysis

Data are expressed as mean \pm standard deviation (SD) and as mean change (%) \pm 90% confidence limits (CL). All results were tested for normal distribution using a Shapiro-Wilk test, and when the assumption of normality was not met, a natural log transformation was applied to reduce the bias due to non-uniformity of the error. Data were analysed using a One-Way ANOVA with repeated measures. When statistical significance ($p < 0.05$) was obtained, a Bonferroni post hoc test was performed (Prism 5, GraphPad). Race-to-race variability on 100m and 400m was calculated over the last five competition performed by participants and expressed as %CV (coefficient of variation). CV was 1.2% and 1.8% for 100m and 400m, respectively. Reliability of 2000m performance test was assessed before the study by two trials separated each one by 48h of rest. Typical error expressed as %CV was 1.8%. Intraclass correlation coefficients (ICC) were calculated to assess the reproducibility of performance tests. ICC values of 0.97, 0.98, 0.96 were found for 100m, 400m and 2000m respectively. The magnitude of changes was assessed with a spreadsheet²¹². The qualitative probabilistic terms were defined by the following scale: 20 <0.5%, almost certainly not; 0.5-5%, very unlikely; 5-25%, unlikely; 25-75%, possibly; 75-95%, likely or probably; 95-99.5%, very likely; >99.5%, most likely or almost certainly. The effect was deemed unclear if its CL overlapped the thresholds for small positive and negative effects²¹³. We used a smallest worthwhile change (SWC) (0.3 of CV) of 0.4% and 0.6% for 100m and 400m respectively. As for $\dot{V}O_{2\text{peak}}$, speed at IAT and 2000m, the SWC (0.2xSD) was 0.67 ml·kg⁻¹·min⁻¹, 0.03 m·s⁻¹ and 26.7 s, respectively.

Results

In relation to PRE, $\dot{V}O_{2\text{peak}}$ was significantly higher after HvLi (35.6 \pm 3.3 vs 40.1 \pm 5.1 ml·kg⁻¹·min⁻¹, $P=0.002$) and LvHi (35.6 \pm 3.3 vs 39.8 \pm 6.3 ml·kg⁻¹·min⁻¹, $P=0.002$) (Figure 13, upper panel). No differences were found between HvLi and LvHi. $\dot{V}E$ values were 116.4 \pm 22.7, 128.7 \pm 21.9 and 118.2 \pm 25.5 L·min⁻¹ in PRE, HvLi and LvHi respectively ($P>0.05$). Subjects attained peak HR values (174 \pm 7, 174 \pm 9 and 171 \pm 7 b·min⁻¹ in PRE, HvLi, and LvHi respectively, $P>0.05$), around 95% of the age predicted maximum. R (1.3 \pm 0.1, 1.3 \pm 0.1 and 1.2 \pm 0.1 in PRE, HvLi, and LvHi respectively, $P>0.05$) and [La]b peak (11.8 \pm 2.0, 12.1 \pm 2.6 and 12.1 \pm 3.0 mM in PRE, HvLi, and LvHi respectively, $P>0.05$) values indicated that all subjects reached maximum exercise capacity in testing session.

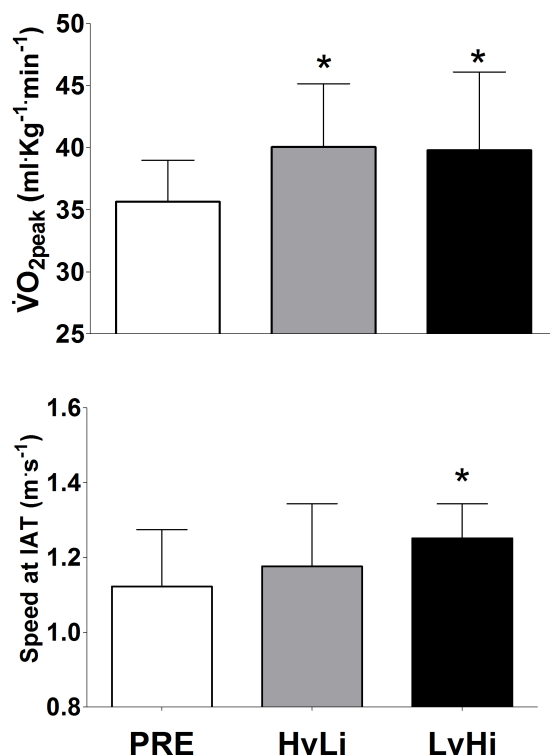


Figure 9. Upper panel: $\dot{V}O_{2peak}$ (ml·kg⁻¹·min⁻¹) values (mean ± SD) before intervention (PRE), after 6 weeks of high volume-low intensity training (HvLi) and after 6 weeks of low volume-high intensity training (LvHi) (n=10). * significantly different from PRE (P<0.05). Lower panel: Speed (m·s⁻¹) at individual anaerobic threshold (IAT) (mean ± SD) before intervention (PRE), after 6 weeks of high volume-low intensity training (HvLi) and after 6 weeks of low volume-high intensity training (LvHi) (n=10). * significantly different from PRE (P<0.05).

Best performance on 100m, 400m and 2000m is shown in Table 7. After HvLi, swimmers significantly improved performances on 400m (P=0.002) and 2000m (P=0.025). No differences were found on 100m. In relation to HvLi, 100m time performance significantly improved (P=0.001). As for 400m and 2000m, time performance did not change. In whole, all performance tests after LvHi were significantly better than PRE. After LvHi, speed at IAT was significantly higher than PRE (P=0.004); no significant differences were observed between PRE and HvLi.

Table 7. Performance results before and after the two training periods (n=10)

	PRE	HvLi	LvHi
100m (s)	62.8 ± 4.5 %WR (81 ± 9)	63.1 ± 4.9 %WR (81 ± 10)	62.0 ± 4.7 * # %WR (82 ± 10)
400m (s)	312.1 ± 24.4 %WR (76 ± 10)	303.2 ± 22.0 * %WR (79 ± 9)	303.3 ± 24.0 * %WR (79 ± 10)
2000m (s)	1743.6 ± 133.5	1683.9 ± 116.0 *	1686.5 ± 135.0 *

Data are presented as mean ± SD. %WR, percentage of the FINA masters world record; HvLi, high volume-low intensity; LvHi, low volume-high intensity; * significantly different from PRE; # significantly different from HvLi. (P<0.05)

Training effects expressed as mean changes (\pm 90% CL) are shown in Table 8. After HvLi, beneficial effects were found on 400m ($-2.8 \pm 1.8\%$), 2000m ($-3.4 \pm 2.9\%$), speed at IAT (4.9 ± 4.7) and $\dot{V}O_{2peak}$ ($11.9 \pm 4.9\%$). Moreover, a possible negative effect was found on 100m time performance ($0.5 \pm 0.7\%$). After LvHi, qualitative analysis showed beneficial effects for $\dot{V}O_{2peak}$ ($10.8 \pm 4.1\%$), 100m ($-1.2 \pm 0.8\%$), 400m ($-2.8 \pm 1.8\%$), 2000m ($-3.3 \pm 1.3\%$) and speed at IAT ($12.4 \pm 5.3\%$), in relation to PRE. Additional practical effects were found after LvHi for 100m ($-1.7 \pm 0.6\%$) and speed at IAT ($7.1 \pm 6.3\%$), in relation to HvLi.

Table 8. Effects of training on peak oxygen uptake, individual anaerobic threshold and swimming performance (n=10)

	PRE vs HvLi		PRE vs LvHi		LvHi vs HvLi	
	change (%) \pm 90% CL	qualitative descriptor	change (%) \pm 90% CL	qualitative descriptor	change (%) \pm 90% CL	qualitative descriptor
$\dot{V}O_{2peak}$	11.9 ± 4.9	Most Likely	10.8 ± 4.1	Very likely	-1.0 ± 5.1	Unclear
Speed at IAT	4.9 ± 4.7	Likely	12.4 ± 5.3	Most likely	7.1 ± 6.3	Likely
100m	0.5 ± 0.7	Possibly	-1.2 ± 0.8	Very likely	-1.7 ± 0.6	Very likely
400m	-2.8 ± 1.8	Very likely	-2.8 ± 1.8	Very likely	-0.1 ± 0.5	Trivial
2000m	-3.4 ± 2.9	Likely	-3.3 ± 1.3	Very likely	0.1 ± 2.8	Unclear

HvLi, high volume-low intensity; LvHi, low volume-high intensity; CL, confidence limits; IAT, individual anaerobic threshold.

Discussion

The main results of this study show that in masters swimmers an increase of training volume performed at low intensity lead to an improvement of $\dot{V}O_{2peak}$ and enhances performance on middle (400m) and long distance (2000m) swimming events whereas short distance performance (100m) may be impaired. A subsequent period of high-intensity low-volume training results in an improvement of short distance performance (100m) and speed at IAT without impairing $\dot{V}O_{2peak}$ and middle-long distance performance. The present data underline the role of intensity of training stimuli as a key factor for athletes engaged in short distance events.

Effects of high-volume low-intensity training

High-volume training represents an essential part of training content in well-trained and elite athletes^{56, 214}. It is recognized that high-volume low-intensity training leads to positive adaptations on key parameters of aerobic fitness^{48, 69}, contributes to the high muscle energy status of athletes²¹⁵, and increases the ability to sustain high muscular power output for long durations²¹⁶. So far, few investigations have examined the effects of an increased training volume in swimming performance. Costill et al.⁶⁸ showed that in highly-trained swimmers (training volume, ~ 4.2 km·d⁻¹) a 10 day training period characterized by doubled volume and similar intensity, did not enhance sprinting (22.86 m freestyle) and endurance (365.8 m freestyle) performance. Some years later Costill et al.²⁰⁵ reached similar results when comparing two groups of collegiate swimmers who trained with quite different volumes (5.0 vs. 9.0 km per day for 6 weeks) and concluded that a considerable increase in training volume does not lead to further swimming performance (45.8 m and 91.4 m sprint freestyle) enhancements. However, in this study the authors, comparing different training protocol in two separate groups of swimmers, followed a case-control design and training intensity was not modified. More recently, Faude et al.²⁰⁶ reported that in young competitive swimmers 4 weeks of high volume training (6.6 km·d⁻¹; about 30% increase in relation to their usual training), lead to only a modest increase on IAT without any change in 100 and 400m performance. Moreover, in a retrospective study of high-level swimmers Mujika et al.²⁰⁷ found no correlation between seasonal training volume and performance. Conversely, our study shows that in masters swimmers (training volume ~ 3 km·d⁻¹) a ~ 33% increase in weekly volume compared with training habits, mostly (90%) performed at intensity around IAT, significantly improves aerobic capacity, as indicated by the increase in $\dot{V}O_{2peak}$ (about 12%), and by test performances on 400 and 2000m freestyle test. Speed at IAT, an index of aerobic capacity, did not significantly changed after HvLi but a worthwhile effect was observed (likely). The discrepancy between our data and those obtained in the above studies could be ascribed to the different performance level and training habits of the athletes investigated. It is known that an increase of low intensity training volume, especially in untrained or moderate-trained subjects, leads to an improved delivery of oxygen to the exercising muscles coupled with increased utilization of oxygen by the working muscles, resulting in an increase of physical work capacity⁵⁴. However, it has been suggested that in an already high-volume training program, an increase in training volume, especially if performed at low-intensity, do not lead to further improvement of endurance performance or associated variables⁵⁴. Compared to the athletes of the previous studies, our masters swimmers performed a significantly lower volume of training. As a

result, the increase of training volume (by 30%) over this period of training coupled with the level of our masters swimmers may explain the improved indexes of aerobic capacity and 400m/2000m performance. As for 100m, our subjects did not show an increased performance in accordance with results of previous studies^{205, 206}. This is not surprising, since for an intense exercise lasting ~ 60s energy contribution arises mostly from anaerobic sources²¹⁷. Thus, an increase of training volume, mostly performed at low-intensity, does not improve performance in short intense events, suggesting the necessity of a different stimulus.

Effects of low-volume high-intensity training

Effectiveness of high intensity training on performance and physiological factors in trained and untrained subjects is now largely recognized^{54, 71, 72}. There is a general consensus that for well-trained athletes a short period of high intensity training supplemented into an already high volume training program can elicit improvements in both intense and prolonged endurance exercise performance. One of the most interesting aspects of high intensity training is that performance can be improved, or at least maintained, under conditions of reduced weekly volume. For example, Iaia et al.^{136, 218} demonstrated that in runners 4 weeks of consistent reduction in training volume (from 45 to 15 km·wk⁻¹), with concomitant increase in training intensity (175% of aerobic power), did not impair 10 km performance, $\dot{V}O_{2peak}$, skeletal muscle oxidative enzyme activity and muscle capillarization. The importance of training intensity on performance has been recognized also in swimmers. Mujika et al.²⁰⁷ found that performance improvements in high-level swimmers were correlated with the mean training intensity of the preceding season (R=0.69), but not with training volume. Soultanakis et al.²⁰⁹ reported in recreational swimmers that 4 weeks high intensity (above lactate threshold) training (training volume, ~ 5.5 km·wk⁻¹) improved performance on 50 m freestyle. In the present study, our masters swimmers performed, after a period of high volume and low intensity training, 6-week of high-intensity (~90% of training contents above the IAT) and low volume (50% reduction in respect of previous one). This change in training did not affect middle (400m) and long (2000m) distance performance, significantly increased speed at individual anaerobic threshold and reduced 100m freestyle time. Moreover, a classical physiological variable related to aerobic performance ($\dot{V}O_{2peak}$) did not change after high-intensity training (compared to high-volume). These results confirm our hypothesis that high-intensity and low volume training could positively affect physiological variables and performance but the lack of improvements in middle-long distance performance and $\dot{V}O_{2peak}$ were

unexpected. Even if we do not have an exhaustive explanation, the different physiological determinants of aerobic performance may be responsible. Indeed, it has been hypothesized that both central (cardiovascular) and peripheral (muscular) changes are associated with high-volume low-intensity training⁶⁰ whereas performance improvements reported after low-volume high-intensity training seem primarily due to peripheral adaptations⁷³. Thus, it is possible that cardiovascular adaptations induced by the first period of high-volume low-intensity training may be responsible for the improvements in middle-long distance performance and $\dot{V}O_{2\text{peak}}$, and a significant reduction of training volume even performed at high-intensity did not determine further changes. Our results are in accordance with other studies showing that typical determinants of aerobic metabolism (muscle oxidative capacity and capillarization), $\dot{V}O_{2\text{max}}$ and performance were maintained despite a significant reduction in the total training volume¹³⁶. In addition, these data seem to support the hypothesis that an extraordinary high-aerobic capacity does not seem necessary prerequisite for maximal performance in short intense swimming events²⁰⁶. Moreover, the results of this study confirm the importance of training intensity for maximal swimming performance in competitions lasting between 20 s and 5 min (50 m to 400m events, > 80% of swimming competitions). Adaptations induced by intensity- or volume- training should be addressed in future studies in order to better understand the relevance in swimming of more intense training periods in high-training volumes.

Limitations of the study

To our knowledge, only three studies^{206, 209, 219} investigated the interactions between training intensity and training volume by comparing one swimming training regimen with another. In these studies the difference between high-volume and high-intensity training was blurred. In our opinion, the present study is the first that compares two completely opposite training programs, high-volume low-intensity and high-intensity low-volume. Nevertheless we cannot exclude the influence of the first training period on the second one. Therefore, although our results seem to show that high intensity training is at least as effective as high volume training, further studies are needed to provide a definitive answer on this topic. Secondly, our swimmers were “masters” athletes competing primarily at regional/national level and not directly comparable with other swimmers of different age and/or performance level. Thus, our results need to be considered in the context of the subjects evaluated and it is not possible to directly extend our conclusions to younger and/or elite swimmers.

Practical applications and Conclusions

In conclusion, this study indicates that in masters swimmers an increase of training volume may lead to an improvement of indexes of aerobic capacity and middle-long distance performance. A subsequent period of high-intensity low-volume training, besides maintaining previous improvements, may positively affect also short distance performance. Thus, it seems possible to attain the same effects on aerobic capacity with a consistent reduction of time that could be reserved for other relevant training contents. The present data also suggest that intensity and not volume is the key factor for athletes engaged in short distance events. From a practical point of view, as the number of masters swimmers is increasing, our study can provide new insights into mechanisms of training effects in masters and contribute to the effectiveness of training programs specifically designed for the large public of masters swimmers.

FOURTH STUDY

Training effects on ROS production determined by electron paramagnetic resonance (EPR) in master swimmers

Introduction

Cells are exposed to a large variety of Reactive Oxygen Species (ROS) from both exogenous and endogenous sources. At appropriate concentration, ROS are known to act as important signaling molecules essential to cell function, playing various regulatory roles in cells⁸⁸. Nevertheless the effects of ROS are dose dependent and when ROS generation exceeds antioxidant defenses oxidative damage is observed¹⁰². Exercise is associated with an increase in oxygen uptake by whole body and particularly by skeletal muscle, utilized, among others, into mitochondria for substrate metabolism and ATP production¹¹⁸. As reported, an increase of 10-fold in the rate of whole body oxygen consumption and an increase of more than 100-fold in the oxygen flux in active muscles, during whole-body exercise, results in increased ROS formation, shifting the cellular environment from a reduced to an oxidized state, independently of physical activity types (aerobic, anaerobic or resistance)¹⁶⁶. Many factors might contribute to the oxidative stress induced by exercise also influencing the oxidative rate, such as recruited muscle groups, types of contraction, exercise frequency and intensity and exercising population. Physical exercise is one of the most characteristic examples demonstrating that ROS are not necessarily harmful, considering that the well-known benefits of regular exercise on muscle function and health are accompanied by repeated episodes of oxidative stress¹²¹. The promoting effects of regular exercise on different cellular functions include the up-regulation of antioxidant and oxidative damage repairing systems and induction of trophic factors¹²⁸. Finally, training can play positive or negative effects on oxidative stress, depending on training load and specificity²²⁰.

Previously it was demonstrated that high-intensity discontinuous and continuous moderate-intensity training induced similar beneficial effects in master runners, reducing the resting levels of oxidative stress biomarkers and inducing changes in total antioxidant capacity level²²¹.

Many investigators have assumed that skeletal muscle provides the major source of ROS generation during exercise⁹⁰. Nevertheless, other tissues such as heart, lungs, or blood may also contribute to total body ROS generation during exercise¹²¹. Recent reports have indicated the potential role that blood may play at rest or during exercise on ROS production¹²². The whole blood or parts of it: plasma¹²⁴, erythrocytes¹²⁵, neutrophils^{124, 126}, lymphocytes¹¹⁷, platelets¹²⁷ have reported an increased production of various

reactive species after exercise. However, the majority of the relevant human studies measured the redox status by using plasma. This probably can be ascribed to two reasons: 1) the assumption that plasma better reflects tissue redox status¹²³ and 2) the easiness of plasma collection. During exercise, ROS are generated by both blood and muscle and it is reasonable to assume that a corresponding systemic steady state level is reached in blood. The same may hold true for exchanges among blood constituents¹²³ once that certain basic assumptions are met: reactive species with adequate half-life have the ability to cross membranes and generate new reactive species at the vicinity of the considered compartments.

Usually, direct measurements of free radical and reactive species production are very difficult due to their high reactivity and low steady-state concentration²²². Consequently, for the assessment of oxidative stress, indirect methods are mainly used. Indeed, Electronic Paramagnetic Resonance (EPR) spectroscopy is the only technique available to directly detect the 'instantaneous' presence and to quantitate ROS concentration in biological samples. Nevertheless ROS half-life ($t_{1/2}$ (s): superoxide $[O_2^{\cdot-}] 10^{-4}$; Nitric oxide $[NO^{\cdot}] 4 \cdot 10^{-1}$, at room temperature) is too short when compared to the EPR time scale so they are EPR-invisible. This is only when 'trapped' and transformed in a more stable radical species that they become EPR detectable. Moreover, in EPR spectra, signal areas are proportional to the number of the excited electron spins, leading to absolute concentration levels, when a stable radical compound is adopted as reference.

The present study aimed at examining the effects of High-Intensity Discontinuous Training exercise on ROS production and on antioxidant capacity in master swimmers by applying reliable, rapid, and micro-invasive EPR measurement of the instantaneous concentration of ROS and antioxidant power using a novel redox sensor to measure the levels of reducing species directly in human peripheral blood^{223, 224}.

Materials and Methods

Participants

10 male masters swimmers (age: 32.3 ± 5.1 years, height: 1.81 ± 0.04 m, body mass: 77.0 ± 6.5 kg) participated in the study. Athletes had a training experience of 11 ± 4 years and their average training volume was $\sim 3 \text{ km} \cdot \text{d}^{-1}$, three times a week. They were specialized in freestyle on distances between 50-400m. All athletes belonged to the Master swimmer category as established by both Fédération Internationale de Natation Amateur (FINA: <http://www.fina.org>). No special diet, minerals, vitamins or other kind of supplementation were administered to swimmers. During the experimental phase of the study antioxidant supply was excluded and participants sustained only the programmed training protocol.

Furthermore, participants abstained from food (6h) and physical activity, alcohol and caffeine consumption (24h) prior to testing and were not currently taking any medications or supplements.

Before the start of the study, subjects were screened medically (history, physical examination, and resting ECG) to ensure that there were no contraindications to study participation. No subject participated in any study prior to this experiment. Each athlete was fully informed about the aims, methods and risks associated with participation and gave his written informed consent before the start of the study. All procedures were in accordance with the Declaration of Helsinki and the study was approved by the local Ethics Committee

Experimental Protocol

All subjects visited the laboratory two times: before (PRE Trg (Trg = Training)) and after (POST Trg) 6-weeks of High-Intensity Discontinuous Training (HIDT). On the experimental day, the subjects arrived at the laboratory 2.5 h after consuming a standardized breakfast. All tests were performed under close medical supervision and subjects were continuously monitored by 12-lead electrocardiography (ECG). Participants sat at the arm crank ergometer (Monark 891E, Stockholm, Sweden) with the crankshaft in line with the shoulder joint²²⁵. All subjects were instructed to remain seated during the test. Subjects performed an incremental exercise (IE) up to voluntary exhaustion. The subject began arm cranking at 15 W for three minutes. Resistance was then increased of 15 W every minute up to exhaustion. Values of cardiovascular, ventilator and gas exchange variables determined during the last 30 seconds of the exhausting load were considered “peak” values. Pulmonary ventilation ($\dot{V}E$, in BTPS), O₂ consumption ($\dot{V}O_2$), and CO₂ output ($\dot{V}CO_2$), both in STPD, were determined breath-by-breath by a metabolic cart (Vmax229, SensorMedics, The Netherlands). Gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2$. HR was determined from the ECG signal. At rest and at various times (1, 3, and 5 min) during recovery, 20 μ L of capillary blood was obtained from a preheated earlobe for the determination of blood lactate concentration ([La]b) by an enzymatic method (Biosen 5030; EKF, Germany).

EPR measurements

At rest, at the end of IE and after 10 minutes of recovery, ROS production rate was determined in 50 μ L capillary blood by means of a recently developed EPR micro-invasive method^{223, 224}. The capillary blood samples were collected at both PRE and POST Trg periods.

In summary, EPR experiments were carried out by using e-scan spectrometer (Bruker, Germany), operating at the common X-Band microwave frequency (~9.8 GHz). Acquisition EPR parameters were: microwave frequency: 9.652 GHz; modulation frequency: 86 kHz; modulation amplitude: 2.28 G; sweep width: 60 G; microwave power: 21.90 mW; number of scans: 10; receiver gain: $3.17 \cdot 10^{-1}$. The instrument was interfaced to a temperature and gas controller unit (Bio III, Noxygen Science Transfer & Diagnostics GmbH, Germany) allowing temperature to be kept at the constant chosen level (37°C). Radical signals generated by the reaction of the 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine probe (CMH, Noxygen Science Transfer & Diagnostics, Germany) with the blood radicals, were acquired and the spectra sequentially transformed for about 6 min in order to calculate the ROS production rate. The calculated spectral data were transformed in absolute concentration levels ($\mu\text{mol} \cdot \text{min}^{-1}$) by recording the CP* (3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) stable radical signal adopted as reference (10 μM). All EPR data were handled using the software standardly supplied by Bruker (Win-EPR version 2.11).

Antioxidant capacity

Reducing capacity in blood was measured by a redox sensor in 10 μl of capillary blood. The electrochemical measurements were performed using a commercial EDEL potentiostat electrochemical analyser (Edel Therapeutics, Switzerland) in a three-electrode arrangement. The working electrode (WE) was a screen-printed carbon electrode operating in conjunction with a screen-printed counter and a silver/silver-chloride (Ag/AgCl) reference one. This technique is an electrochemical-based method responding to all water-soluble compounds in biological fluids, which can be oxidized within a defined potential range^{226, 227}. Blood sample was loaded onto a chip and an increasing potential between 0 and 1.2 V at a scan rate of 100 $\text{mV} \cdot \text{s}^{-1}$ (versus Ag/AgCl reference electrode) was applied while the resulting current was measured at the working electrode. The result was then pseudo-titrated to account for the most biologically relevant antioxidants. Data are expressed in nW.

Training intervention

Subjects trained 3 times per week during 6-weeks in an indoor 25-m swimming pool. Training contents were classified in three intensity zones based on the individual anaerobic threshold (zone 1, 100-105% IAT; zone 2, 110-120% IAT; zone 3, >130% IAT). Total training volume and training amount at different intensity zones are presented in Table 6. The athletes' coach participated in the schedule of training programs and conducted all training sessions. Dry-land training (resistance, athletics, cross training) was not performed.

At the start and end of each training session swimmers performed controlled warm-up (500 m per session) and cool-down (300 m per session) respectively.

Table 6. Weekly training contents were classified in three intensity zones based on the individual anaerobic threshold

Training contents	HiLv	
	Distance (m)	% of total
Zone 2 (100-105% IAT)	600	10
Zone 3 (110-120% IAT)	2700	45
Zone 4 (>130% IAT)	2700	45
Total amount	6000	100

HiLv, High-intensity Low-volume; IAT, individual anaerobic threshold

Statistical Analysis

Descriptive statistics such as mean \pm SD were used to summarize continuous variables. Data were analyzed using parametric statistics following mathematical confirmation of a normal distribution using Shapiro-Wilks W test. Experimental data were compared by ANOVA variance analysis followed by Bonferroni's multiple comparison test to further check the among groups' significance (GraphPad Prism 6, Software Inc. San Diego, CA). The relationship between selected dependent variables was assessed using Pearson Correlation coefficients. $P < 0.05$ statistical significance level was accepted.

Results

The kinetics of ROS production data estimated by the EPR spectra recorded at rest, immediately after the IE and at 10 min of recovery are shown in Figure 14.

After IE, ROS production increased significantly with respect to REST ($P < 0.01$) in PRE Trg (2.82 ± 0.66 vs $3.28 \pm 0.66 \mu\text{mol}\cdot\text{min}^{-1}$) while the increase was not significant in POST Trg (2.24 ± 0.14 vs $2.46 \pm 0.12 \mu\text{mol}\cdot\text{min}^{-1}$). Thereafter ROS production attained the resting levels in the time course of recovery, although in PRE Trg ROS level was found still more significantly ($P < 0.05$) higher ($3.13 \pm 0.30 \mu\text{mol}\cdot\text{min}^{-1}$) at 10 minutes of recovery in relation to REST.

HIDT induced a significant ($P < 0.001$) decrease in the ROS production rate at REST in POST Trg compared to PRE Trg (2.24 ± 0.14 vs 2.82 ± 0.66 respectively). Moreover, the attained peak value (END) resulted significantly ($P < 0.001$) lower in POST Trg than in PRE Trg despite a similar trend. Finally, a significant

difference ($P < 0.001$) in the time course of recovery (10 minutes after exercise: 3.13 ± 0.30 vs 2.29 ± 0.11 respectively) between ROS production in PRE Trg and POST Trg was observed.

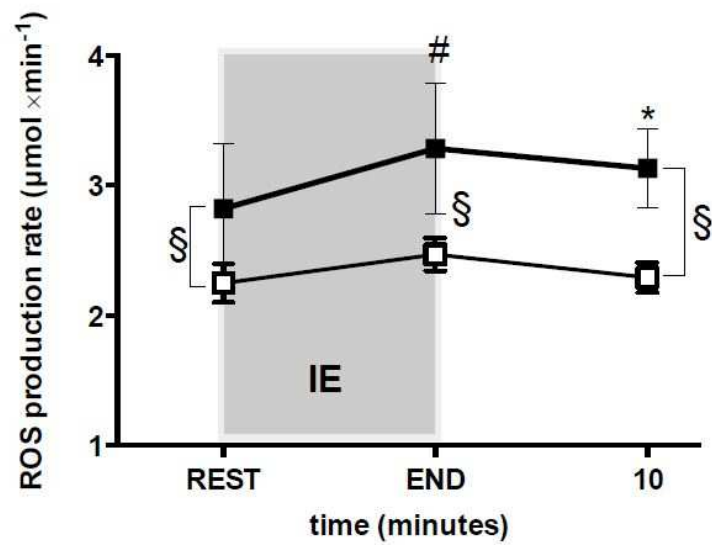


Figura 10. Time course of ROS production rate ($\mu\text{mol} \cdot \text{min}^{-1}$) detected by EPR technique before (REST), immediately after the IE (END) and at 10 minutes of recovery. The data obtained during two sessions of IE are shown: PRE Trg (full squares) and POST Trg (empty squares). Changes over time were significant at: $P < 0.05$ during recovery (10 minutes after exercise) in PRE Trg (* symbol); $P < 0.01$ comparing peak levels in PRE Trg vs REST (# symbol); $P < 0.001$ between PRE Trg and POST Trg at REST, END and 10 minutes of recovery (§ symbol).

Antioxidant capacity changes after IE are displayed in Figure 15 as well. This parameter was found significantly increased respect to the REST at the END, and at 10 minutes of recovery, in both PRE (136.6 ± 11.34 ; 151.1 ± 13.1 ; 165.3 ± 10.9 nW respectively) and POST Trg (154.7 ± 15.1 ; 171.4 ± 12.6 ; 191.5 ± 14.7 nW respectively). HDT induced a significant ($P < 0.01$) increase of antioxidant capacity in POST Trg compared to PRE Trg at REST, END and after 10 minutes of recovery (+13%; +13%; +16% respectively).

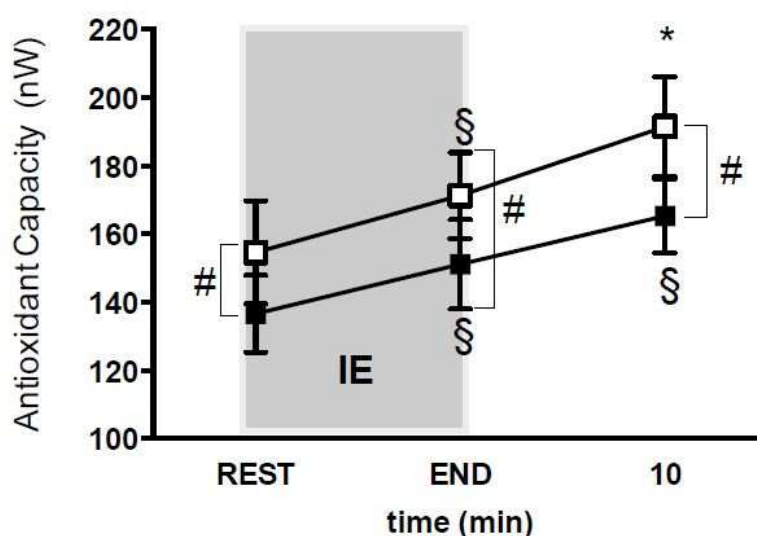


Figura 11. Time course of antioxidant capacity (nW) before (REST), immediately after the IE (END) and at 10 minutes of recovery: PRE Trg (full squares) and POST Trg (empty squares). Changes over time were significant in PRE Trg at: $P < 0.001$ at the END of exercise and during recovery (10 minutes after exercise) (§); in POST Trg at: $P < 0.001$ at the END (§) and $P < 0.05$ during recovery (10 minutes after exercise) (*); $P < 0.01$ between PRE Trg and POST Trg at REST, END and 10 minutes of recovery (# symbol).

Discussion

Many experimental works have analyzed the redox biology of exercise with high relevance to the area of Sport Science²²²: the general benefits of physical exercise are widely known and understood²²⁸ but it is important to emphasize that exercise may generate an excessive production of free radicals¹¹⁹. As well known and widely reported in the literature, compared to enzymatic methods able to measure end point biomarkers of oxidative stress damage (oxidized proteins and membrane lipids), EPR is the only technique allowing the direct detection and quantification of ROS. However despite the great interest in measuring ROS in biology and medicine, EPR technique has not till now been widely used because of several technical and methodological problems²²⁹. The observation that muscular exercise increases ROS production in skeletal muscles was for the first time reported by Davies¹⁶³. In the following years, a lot of studies on animals and humans have showed an increase of free radicals production after aerobic or anaerobic exercise both in sedentary or athletes subjects, according to exercise intensity^{177, 220}. This increase was also observed in this study using an innovative method^{223, 224} that employed EPR technique to attain a rapid and micro-invasive measurement of ROS concentration in human peripheral blood. Compared with other spin trap and/or probe molecules, CMH was considered the spin probe of choice to quantify ROS in a most physiological way. Indeed, it shows greatest efficacy for trapping $O_2\cdot^-$

radicals, the reaction being much faster ($1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) and producing stable CM-nitroxide, thereby enabling the reaction with both extra and intracellular $\text{O}_2\cdot^-$. Moreover CMH detects ROS from all cellular compartments, including mitochondria²³⁰.

During PRE and POST Trg sessions a significant increase of ROS production was found at the end of IE (+16% and +10% respectively); this was followed by a gradual decrease in the magnitude of the ROS production in both sessions, returning toward resting values after 10 min (+11% and +2% respectively). This finding is in agreement with the idea that increased ROS generation caused by physical exercise overwhelms the body capacity to detoxify ROS and that upon chronic training, adaptive responses, including the one of the antioxidant defense system, better controls ROS production both at rest and after IE. Indeed antioxidant capacity significantly improved at REST (+13%) and maintained high levels 10 min after the end of the exercise (+16%).

One of the aims of this study was to investigate, by means of the same mini-invasive measurement method adopted for ROS production levels determination, whether alterations in redox homeostasis can be monitored to assess the fitness of intensively training athletes.

Aiming at minimizing the invasiveness of the method and hence to improve its potential for routine applications, oxidative stress markers (e.g. thiobarbituric acid substances, protein carbonyls) determination, requiring more invasive venous blood samples, was herein avoided. This choice was also supported by the linear correlation between ROS production rate and the above-mentioned biomarkers concentration previously observed at rest^{223, 224}. In addition, the time-course changes of the same oxidative stress biomarkers were found delayed and of longer duration with respect to ROS production kinetics so that no correlation was possible in dynamic conditions²²³.

Finally the obtained results support that such HIDT protocol, characterized by repeated variations of intensity associated with changes of redox potential, ATP/ADP ratio and, consequently, disturbances of cellular homeostasis, can play a positive effect on oxidative stress leading to decrease in lipid peroxidation and DNA damage and on antioxidant capacity reducing ROS production.

Conclusions

The study showed that 6-weeks of HIDT training improves antioxidant (+13%) capacity and significantly ($p < 0.001$) decreases baseline ROS production (-20%). Results also show that after identical exercise trained individuals produced lower levels of ROS related to higher level of antioxidant capacity compared to an

untrained state. The adopted micro-invasive procedure for ROS rate production measurement by EPR appeared to be a reliable method to evaluate oxidative stress adaptation to acute exercise and training.

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APPENDIX I: Scientific Production

Publications peer reviewed

1. Mrakic-Sposta S, Gussoni M, Porcelli S, **Pugliese L**, Pavei G, Bellistri G, Montorsi M, Tacchini P, Vezzoli A. Training effects on ROS production determined by electron paramagnetic resonance (EPR) in master swimmers. *Oxid Med Cell Longev* (Accepted, 2014).
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