Regular physical exercise prevents nitrosative stress caused by ageing in elderly athletes

JSA 2015;1:2-7

Jonathan Fusi, Emanuele Guidotti, Augusto Innocenti, Leonardo Tocchini, Emiliano Ricciardi, Marco Rossi, Fabio Galetta, Gino Santoro, Ferdinando Franzoni

Department of Clinical and Experimental Medicine and Department of Surgical, Medical and Molecular Pathology and Critical Care at the University of Pisa

Regular physical exercise prevents nitrosative stress caused by ageing in elderly athletes

Abstract

Ageing is associated with an increased susceptibility to free radical-induced tissue damage. One of the most important classes of free radicals generated in living systems is represented by reactive nitrogen species (RNS), responsible for the so-called nitrosative stress. It has been shown that physical activity is able to induce up-regulation of antioxidant systems contributing to prevent oxidative stress. The aim of the present study was to assess whether regular physical activity is able to counteract age-induced nitrosative stress. Thirty male endurance athletes (average age 70.8 ± 6.1 years, VO2max 59.07 ± 8.5 ml/kg/min) and thirty age-sex-matched sedentary controls (average age 71.5 ± 4.3 years, VO2max 25.6 ± 8.2 ml/kg/min) were studied. Plasma free radicals antioxidant capacity against peroxynitrite, one of the most important RNS, was evaluated as Total Oxyradical Scavenging Capacity (TOSC) units. Results. Plasma TOSC values against peroxynitrite were higher in athletes than in sedentary controls (22.94 ± 1.85 vs 15.41 ± 1.24 units/ml, p < 0.001). In the athletes group, TOSC values were related to VO2max (r = 0.44, p < 0.05). In conclusion, these results suggest that regular physical activity is associated with increased antioxidant defences in elderly athletes. In athletes, a direct correlation between the scavenger scavenger of the plasma and the VO2max (r = 0.44, p <0.05) was also observed. These results confirm that regular physical activity practised for many years can determine the best response to nitrosative stress induced by peroxynitrite.

Key words: physical activity - oxidative stress - free radicals – peroxynitrite

Riassunto

L'invecchiamento è associato a una maggiore suscettibilità al danno tissutale mediato da radicali liberi. Una delle più importanti classi di radicali generati nei sistemi viventi è rappresentata dai radicali liberi dell'azoto (RNS), responsabili del danno cellulare definito come stress nitrosativo. È stato dimostrato che l'attività fisica regolare migliori i sistemi antiossidanti dell'organismo, contribuendo a prevenire e contrastare lo stress ossidativo. L'obiettivo del presente studio è quello di valutare se l'attività fisica sia in grado di contrastare lo stress nitrosativo indotto dall'invecchiamento. A tale scopo sono stati reclutati 30 atleti master di sesso maschile (età media 70,8 ± 6,1 anni, VO_2max 59,07 ± 8,5 ml/kg/min) e 30 soggetti di controllo (età media 71,5 ± 4,3 anni, VO_2max 25,6 ± 8,2 ml/kg/min) sani, ma con stile di vita sedentario. La capacità antiossidante plasmatica nei confronti del perossinitrito, uno dei principali RNS, è stata valutata mediante tecnica gascromatografica Total Oxyradical Scavenging Capacity Assay *(TOSCA). I risultati ottenuti dimostrano che gli atleti anziani presentano una più alta attività scavenger plasmatica nei confronti del perossinitrito rispetto ai soggetti di controllo (22,94 ± 1,85 vs 15,41 ± 1,24 units/ml, p < 0,001). Negli atleti è stata inoltre osservata una correlazione diretta tra la capacità scavenger del plasma e il VO_2max (r = 0,44, p < 0,05). Tali risultati confermano che l'attività fisica regolare condotta per molti anni è in grado di determinare una miglior risposta allo stress nitrosativo indotto dal perossinitrito.*

Parole chiave: attività fisica - stress ossidativo - radicali liberi - perossinitrito

Introduction

Ageing is one of the most important independent risk factors for cardiovascular diseases. It is associated with increased susceptibility to free radical-induced tissue damage caused by a progressive loss of natural antioxidants capacities, resulting in increased oxidative stress (1).

Free radicals are highly reactive and unstable molecules in organic systems produced by oxidative phosphorylation or as a response to inflammations.
Although a multitude of free radicals exists, those deriving from oxygen or nitrogen, together defined as RONS (Reactive Oxygen and Nitrogen Species), represent the largest class of radicals generated in living systems (2).
Normally, a delicate balance exists between the production of oxidant factors, represented by reactive oxygen species (ROS), and their elimination through an elaborate system of antioxidant defenses, made of enzymes being responsible for the conversion of free radicals and by antioxidant molecules able to neutralize them, the so-called scavengers. When this balance is altered in favour of free radical expression, a condition called oxidative stress is established which alters the structure and function of proteins, lipids and nucleic acids, thus inducing cell damage (3)(4). Similarly, reactive nitrogen species (RNS) as well are in equilibrium with a buffer system made of scavengers and, as is the case for ROS, when this balance no longer exists a condition of cell damage emerges which, given the type of species involved, is described as nitrosative stress. The alteration of the RNS / scavenger balance in vivo has been associated with inflammatory processes, neurotoxicity and ischemia. In addition, nitrosative stress frequently coexists with oxidative stress and the two conditions overlap (5). Among the RNS, the radical being most associated with neurodegenerative and cardiovascular diseases was identified in peroxynitrite ($ONOO^-$) (6-8).
It has been shown that although physical activity causes on one hand an increase in the production of RONS, especially by **increasing** the mitochondrial oxidative processes, on the other hand it stimulates adaptive phenomena of up-regulation of the body's antioxidant systems. This phenomenon contributes to maintaining the balance between the production of RONS and scavenger systems by helping to prevent and counteract oxidative stress (9) (10). The fact that physical activity can improve the scavenger response of the human body to oxidative stress has been highlighted in a previous work against two species of ROS: peroxyl (ROO-) and hydroxyl (OH-) radicals (11). The aim of this study was to evaluate how physical activity is able to counteract nitrosative stress caused by ageing, comparing the antioxidant activity of plasma to peroxynitrite in a group of elderly sports amateurs and in an age-matched sedentary control group.

Materials and Methods

Subjects

Thirty masters runners (males, average age 70.8 ± 6.1) belonging to the Marathon Club of Pisa and practicing endurance activities for many years (average age 28.4 ± 10.5) were enrolled in the study. They attended at least 5 training sessions per week for a total of about 7 hours per week, plus a competitive marathon or half marathon at least one Sunday a month. Thirty sedentary healthy volunteers (average age 71.5 ± 4.3) were selected by the cardiology clinic of the Department of Clinical and Experimental Medicine at the University of Pisa to form the control group. All subjects were healthy and free of the main cardiovascular risk factors on the basis of an accurate medical history, a complete physical examination as well as a baseline and stress ECG. They were non-smokers and no one of them was taking medications or vitamin supplements of any kind. Athletes and controls showed a maximum oxygen uptake (VO2max), assessed through a cardiopulmonary exercise testing (CPET) (QUARK PFT ERGO, Cosmed, ITALY) respectively higher than 50 ml/kg /min and lower than 35 ml/kg/min. The study was approved by the Ethics Committee 'Comitato Etico di Area Vasta Nord Ovest' (CEAVNO) for Clinical Trials, and all the parties signed a written consent to the study.

Experimental Design

After cannulation of the antecubital vein, the subjects underwent a blood test (50 ml) under controlled environmental conditions (temperature 22-24 °C) at least 48 hours after their last sports activity. The sample obtained and collected in test tubes containing dipotassium-ethylenediaminetetraacetic acid (EDTA, 10 l µl/ CC) was immediately centrifuged at 3,000 g for 10 min to obtain plasma samples divided into 500 µl aliquots and stored in Eppendorf at -80 °C for subsequent analyses. The evaluation of plasma antioxidant capacity was performed through TOSCA assay (Total Oxyradical Scavenging Capacity Assay) (12).
In short, the paper is based on the artificial genesis of peroxynitrite derivatives at 35 °C from the decomposition of SIN-1 (3-morpholinosydnonimine N-ethylcarbamide) in a potassium phosphate buffer 100 mM (pH 7.4) with 0.1 mM of DTPA (diethylene-triamine-pentaacetic acid) **(8).** Reactions with KMBA (α-keto-γ- (methylthiol) butyric acid) 0.2 mM were performed in 10-ml vials sealed with Mininert® gas tight valves (Supelco, Bellefonte, PA) in a final volume of 1 ml (16). The plasma analyzed was diluted 1/100 into potassium phosphate buffer 100 mM (pH 7.4) in a final volume of 1 ml (10 µl plasma + 990 µl potassium phosphate buffer). The production of ethylene was measured by gas chromatography analysis of a 200 µl aliquot taken from the vials' headspace at regular time intervals throughout the entire duration of the race. The analysis was carried out with a Hewlett-Packard Gas Chromatograph (HP 7820A Series, Andoven, MA) equipped with a Supelco DB-1 capillary column (30 × 0.32 × 0.25 mm) and a flame ionization detector (FID). The temperatures of the oven, of the injector and of the FID were respectively 35, 160 and 220 °C. Hydrogen was used as a gas carrier (at a flow of 1 ml/min). The TOSCA

values were calculated with the equation: TOSCA = 100 - (∫SA / ∫CA × 100), where ∫SA and ∫CA represent the integrals of the areas for the sample and the control reaction, respectively. The results were expressed in TOSCA units per ml of plasma. A TOSCA value of 0 corresponds to a sample without scavenger capacity (no inhibition of the formation of ethylene compared to the control reaction, ∫SA / ∫CA = 1), while a TOSCA value of 100 corresponds to the maximum efficiency of the sample analyzed.
The linearity of the dose-response curve between the plasma (in ml) and the antioxidant response (TOSCA value) was tested, and good correlation coefficients were obtained (generally greater than 0.9) for the different doses of plasma which were used to test the validity of our experiments. Each experiment was performed twice. The coefficient of variation (CV) of the method is <5%.

Statistical Analysis

The results were expressed as mean ± standard deviation. The differences between the two populations were measured using the Student t-test. The differences were considered statistically significant when $p < 0.05$. To assess any correlation between variables, the univariate and multivariate linear regression was used.

Results

The two study groups proved similar in terms of age, weight, height, BMI, systolic and diastolic blood pressure, blood sugar, total cholesterol and LDL cholesterol (Tab. I). As expected, the athletes had a significantly lower heart rate (53.9 ± 5.2 vs 65.3 ± 9.2, $p < 0.001$) and a significantly higher VO2max (59.07 ± 8.5 vs 25.6 ± 8.2, $p < 0.001$) compared to sedentary controls. Furthermore, it was found that the plasma concentration of HDL cholesterol in the group of athletes was significantly higher than that in the control group (59.7 ± 11.3 vs 43.4 ± 8.7, $p < 0.01$) (Tab. I). As shown in Figure 1, the antioxidant capacity against peroxynitrite measured in the group of athletes was significantly higher than that in the control group (TOSCA value: 22.94 ± 1.85 vs 15.41 ± 1.24 units / ml, $p < 0.001$).
The analysis of the correlation between variables also highlighted a direct correlation in the whole population studied between the antioxidant capacity against peroxynitrite and the VO2max ($r = 0.44$, $p < 0.05$) (Fig. 2). No correlation was found between the other variables measured.

Discussion

Our group has previously shown that physical activity can improve the response of the human body against oxidative stress induced by ROS, in particular by peroxyl (ROO-) and hydroxyl (OH-) radicals (11). However, to our knowledge, there are no data on the effectiveness of exercise in improving the capacity of the human body to counteract nitrosative stress. This paper documents the fact that regular physical activity is also effective in reducing nitrosative stress induced by ageing. Peroxynitrite has been linked to various pathologies of the cardiovascular and central nervous system (6-8). In the human body it is mainly generated through a process of controlled diffusion between nitric oxide (NO) and superoxide (O_2^{-}), according to the following reaction:

$$O_2^{\cdot -} + NO \longrightarrow ONOO^-$$

This reaction is one of the fastest known in chemistry, even faster than the dismutation catalyzed by the enzyme

Table I. Clinical characteristics of the study population (mean ± SD).

	Athletes	Controls
Age (anni)	70,8 ± 6,1	71,5 ± 4,3
Height (cm)	172,1 ± 5,8	173,5 ± 6,2
Weight (kg)	75,9 ± 5,4	77,2 ± 6,9
BMI (kg/m^2)	23,8 ± 2,1	25 ± 1,6
HR (bpm)	53,9 ± 5,2*	65,3 ± 9,2
SAP (mmHg)	126,3 ± 4,2	128,2 ± 5,4
DAP (mmHg)	78,2 ± 5,2	79,7 ± 5,8
Blood glucose (mg/dL)	93,0 ± 9,1	92,2 ± 10,0
Total cholesterol (mg/dL)	185,9 ± 21,3	187,4 ± 17,0
HDL cholesterol (mg/dL)	59,7 ± 11,3[1]	43,4 ± 8,7
LDL cholesterol (mg/dL)	111,8 ± 12,8	117,5 ± 10,2
VO_2max (ml/kg/min)	59,07 ± 8,5	25,6 ± 8,2

* $p < 0.001$; [1] $p < 0.01$ *vs* sedentary.

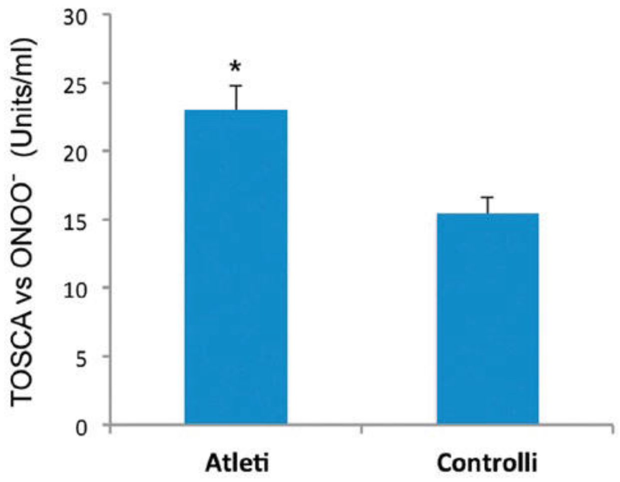

Figure 1. Plasma Antioxidant activity against peroxynitrite in the group of athletes and sedentary controls. * $p < 0.001$.

superoxide dismutase, one of the most represented endogenous antioxidants (13).

For its chemical properties, peroxynitrite is considered an important biological oxidant and a central mediator of many pathological processes affecting both the cardiovascular system and the central nervous system (14). In fact, given its radical nature, it can interact with all cellular components, including DNA, thus altering their structure and consequently their function. At a cellular level, a major production site of peroxynitrite is represented by the mitochondrion (15), the main source of $O_2^{\cdot-}$ which combines with NO given the easy diffusion of the latter from the cytosol to this site (6) (16).

The subsequent reaction of peroxynitrite with mitochondrial components irreversibly alters the activity of complexes I and II of the electron and ATPase transport chain, thus modifying mitochondrial bioenergetics and calcium homeostasis and ultimately promoting the further formation of $O_2^{\cdot-}$ (6) (17)(18).

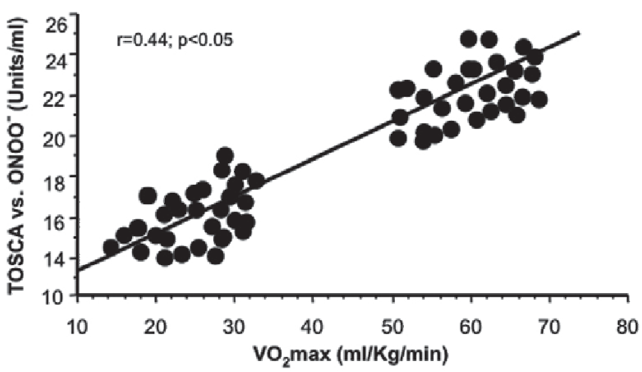

Figure 2. Analysis of the correlation between maximum oxygen consumption and plasma antioxidant activity against peroxynitrite in the study population.

The data collected in this study showed, for the first time, that the antioxidant activity of plasma for peroxynitrite derivatives is significantly higher in a group of elderly people who have done amateur high level running activities for many years compared to that in sedentary controls. Our results can therefore assume that exercise can counteract the damaging effects caused by the nitrosative stress associated with ageing. This evidence is supported by the direct correlation between the scavenging capacity of plasma against peroxynitrite and VO2max, and shows that the level of fitness and training and thus the degree of fitness are the main correlate of the best response to nitrosative stress.

In fact, physical activity is universally recognized as an important factor in primary and secondary prevention. Regular physical exercise, in fact, is not only a fundamental factor which reduces the risk of onset and progression of arterial hypertension, dyslipidemias, diabetes and metabolic disorders in general, but has proven to be extremely important for the direct impact on mortality (19-23).

However it is assumed that these effects may be fully obtained only when RONS are produced in physiological or slightly higher quantities (24). The excessive production of RONS can be the result of a great variety of stressors ranging from exposure to pollutants to excessive or inappropriate nutrient intake (25). More generally, any situation in which an increase in oxygen consumption is found can lead to an acute state of oxidative stress. This condition can then also occur during intense and / or prolonged exercise (26). Thus, on the one hand exercise causes an increased production of RONS, especially through an increase in mitochondrial oxidative processes, but on the other hand it stimulates adaptive phenomena rather than oxidative insults thanks to the increased production of reactive species. A repeated exposure of cellular systems to an increasing production of RONS arising from exercise leads, in fact, to an up-regulation of the body antioxidant systems (9) (10). It is precisely the alteration of the redox balance, associated to a smaller environment, that may seem to entail a protective adaptation to RONS during sequential training sessions, as well as in case of exposure to conditions not associated to exercise. The results obtained with the present work are therefore in favour of the hypothesis that regular exercise improves plasma antioxidant capacity and reduces oxidative and nitrosative stress induced by ageing.

Presumably, the increase in antioxidant activity following physical training is the result of a process of adaptation according to the principles of hormesis: in response to a repeated exposure to toxins or stressors of various origins in limited quantity, the body undergoes a favourable adaptation which results in an improvement of physical performance and of general health (27) (28). The intermediate level of RONS produced, which is therefore optimal, apparently leads to a state of equilibrium and advantage for overall health, whereas a production being too low or too high leads to an alteration of the body's defenses, or to extensive oxidative damage and inflammation. Although the exact understanding of the relationship between RONS and exercise still remains open to interpretation and insights, it is now clear that both aerobic (29) and anaerobic exercise (30) may potentially cause acute oxidative and nitrosative stress through various types of biochemical mechanisms. Different exercise protocols can induce different levels of production of RONS, since the oxidative damage they cause has proved to be dependent on the intensity and duration of the exercise itself (31). It should be taken into account, however, that also other factors, including age (32), degree of training (10) and diet (33) play a crucial role in the possible alteration of the balance between antioxidant defenses and pro-oxidant elements. From a molecular point of view, it is worth considering that the positive effects of regular physical activity may be mediated in part by the increased bioavailability of NO resulting from physical exercise and in part by a RONS-mediated activation of gene transcription pathways that ultimately determine a greater production of antioxidant enzymes. The RONS, in fact, seem to be able to act as an activation signal of a series of molecules that in turn activate nuclear transcription factors being sensitive to the redox state, such as Nuclear Factor kB (NF-kB). The gene regions promoters of several antioxidant enzymes such as superoxide dismutase, iNOS (Inducible Nitric Oxide Synthetase) and glutamylcysteine synthetase contain binding sites for NF-kB and are therefore potential targets for the up-regulation induced by exercise through NF-kB with the RONS as second messengers (26) (34)(35).

In conclusion, given the demonstration of a correlation between physical activity, mechanisms of gene transcription and plasma antioxidant activity, it is desirable that future research in this area lead to identify with greater accuracy and completeness the molecular mechanisms behind the adaptation of the antioxidant activity induced by physical activity, in order to obtain a better quantification of the degree of sporting activity (and consequently of antioxidant activity) needed to generate a beneficial effect for health.

References

1. Beckman K, Ames B. *The free radical theory of aging matures*. Physiol Rev 1998;78:547-81.
2. Halliwell B. *Reactive oxygen species in living systems: source, biochemistry, and role in human disease.* Am J Med 1991;91:14S-22S.
3. Sies H. *Oxidative stress: introductory remarks*. In: Sies H, ed. *Oxidative Stress*. London: Academic Press 1985, pp. 1-7.
4. Darley-Usmar V, Starke-Reed P. *Antioxidants: strategies for interventions in aging and age-related diseases. A workshop sponsored by the National Institute on Aging and by the Office of Dietary Supplements*. Antioxid Redox Signal 2000;2:375-7.
5. Klatt P, Lamas S. *Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress*. Eur J Biochem 2000;267:4928-44.
6. Szabò C, Ischiropoulos H, Radi R. *Peroxynitrite: biochemistry, pathophysiology and development of therapeutics*. Nat Rev Drug Discov 2007;6:662-80.
7. Peluffo G, Radi R. *Biochemistry of protein tyrosine nitration in cardiovascular pathology*. Cardiovasc Res 2007;75:291-302.
8. Levrand S, Vannay-Bouchiche C, Pesse B, et al. *Peroxynitrite is a major trigger of cardiomyocyte apoptosis in vitro and in vivo.* Free Radic Biol Med 2006;41:886-95.
9. Elosua R, Molina L, Fito M, et al. *Response of oxidative stress biomarkers to a 16-week aerobic physical activity program, and to acute physical activity, in healthy young men and women*. Atherosclerosis 2003;167:327-34.
10. Fatouros IG, Jamurtas AZ, Villiotou V, et al. *Oxidative stress responses in older men during endurance training and detraining*. Med Sci Sports Exerc 2004;36:2065-72.
11. Franzoni F, Plantinga Y, Femia FR, et al. *Plasma antioxidant activity and cutaneous microvascular endothelial function in athletes and sedentary controls.* Biomed Pharmacother 2004;58:432-6.
12. Regoli F, Winston GW. *Quantification of total oxidant scavenging capacity (TOSCA) of antioxidants for peroxynitrite, peroxyl radicals and hydroxyl radicals*. Toxicol Appl Pharmacol 1999;156:96-105.
13. Radi R, Cassina A, Hodara R, et al. *Peroxynitrite reactions and formation in mitochondria*. Free Radic Biol Med 2002;33:1451-64.
14. Poderoso JJ, Carreras MC, Lisdero C, et al. *Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles*. Arch Biochem Biophys 1996;328:85-92.
15. Bringold U, Ghafourifar P, Richter C. *Peroxynitrite formed by mitochondrial NO synthase promotes mitochondrial Ca2 release.* Free Radic Biol Med 2000;29:343-8.
16. Radi R, Rodriguez M, Castro L, et al. *Inhibition of mitochondrial electron transport by peroxynitrite*. Arch Biochem Biophys 1994;308:89-95.
17. Radak Z, Chung HY, Goto S. *Exercise and hormesis: oxidative stress-related adaptation for successful aging*. Biogerontology 2005;6:71-5.
18. Ji LL, Gomez-Cabrera MC, Vina J. *Exercise and hormesis: activation of cellular antioxidant signaling pathway*. Ann N Y Acad Sci 2006;1067:425-35.
19. Shephard RJ, Balady GJ. *Exercise as cardiovascular therapy*. Circulation 1999;99:963-72.
20. Boule NG, Haddad E, Kenny GP, et al. *Effects of exercise on glycemic control and body mass in type 2 diabetes mellitus: a meta-analysis of controlled clinical trials*. JAMA 2001;286:1218-27.
21. Williams PT. *Vigorous exercise, fitness and incident hypertension, high cholesterol, and diabetes*. Med Sci Sports Exerc 2008;40:998-1006.
22. Blair SN, Kohl HW III, Barlow CE, et al. *Changes in physical fitness and all-cause mortality. A prospective study of healthy and unhealthy men.* JAMA 1995;273:1093-8.
23. Myers J, Prakash M, Froelicher V, et al. *Exercise capacity and mortality among men referred for exercise testing*. N Engl J Med 2002;346:793-801.
24. Reid MB. *Invited review: redox modulation of skeletal muscle contraction: what we know and what we don't*. J Appl Physiol 2001;90:724-31.
25. Goldstein S, Mere'nyi G. *The chemistry of peroxynitrite: implications for biological activity*. Methods Enzymol 2008;436:49-61.

26 Vollaard NB, Shearman JP, Cooper CE. *Exercise-induced oxidative stress: myths, realities and physiological relevance.* Sports Med 2005;35:1045-62.

27 Beckman JS, Beckman TW, Chen J, et al. *Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide*. Proc Natl Acad Sci USA 1990;87:1620-4.

28 Ferrer-Sueta G, Radi R. *Chemical biology of peroxynitrite: kinetics, diffusion, and radicals*. ACS Chem Biol 2009;4:161-77.

29 Davies KJ, Quintanilha AT, Brooks GA, et al. *Free radicals and tissue damage produced by exercise*. Biochem Biophys Res Commun 1982;107:1198-205.

30 Groussard C, Rannou-Bekono F, Machefer G, et al. *Changes in blood lipid peroxidation markers and antioxidants after a single sprint anaerobic exercise.* Eur J Appl Physiol 2003;89:14-20.

31 Bloomer RJ, Davis PG, Consitt LA, et al. *Plasma protein carbonyl response to increasing exercise duration in aerobically trained men and women*. Int J Sports Med 2007;28:21-5.

32 Roubenoff R. *Age-related loss of associations between acute exercise-induced IL-6 and oxidative stress*. Am J Physiol Endocrino Metab 2006;291:E340-9.

33 Watson TA, Callister R, Taylor RD, et al. *Antioxidant restriction and oxidative stress in short-duration exhaustive exercise*. Med Sci Sports Exerc 2005;37:63-71.

34 Hemmrich K, Suschek CV, Lerzynski G, et al. *iNOS activity is essential for endothelial stress gene expression protecting against oxidative damage.* J Appl Physiol 2003;95:1937-46.

35 Franco AA, Odom RS, Rando TA. *Regulation of antioxidant enzyme gene expression in response to oxidative stress and during differentiation of mouse skeletal muscle*. Free Radic Biol Med 1999;27:1122-32.

CORRESPONDENCE

Ferdinando Franzoni
f.franzoni@int.med.unipi.it