

# Erythrocyte membrane fluidity and indices of plasmatic oxidative damage after acute physical exercise in humans

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**Abstract** Optimal levels of membrane fluidity are essential for numerous cell functions including cell growth, solute transport and signal transduction. Since exercise enhances free radical production, our aim was to evaluate in healthy male subjects the effects of an acute bout of maximal and submaximal exercise on the erythrocyte membrane fluidity and its possible relation to the oxidative damage overproduction due to exercise. Subjects ( $n = 34$ ) performed three cycloergometric tests: a continuous progressive exercise, a strenuous exercise until exhaustion and an acute bout of exercise at an intensity corresponding to 70% of maximal work capacity for 30 min. Venous blood samples were collected before and immediately after these exercises. Erythrocyte membrane fluidity was assessed by fluorescence spectroscopy. Plasma malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations and carbonyl content of plasmatic proteins were used as an index of lipid and protein oxidation, respectively. Exercise produced a dramatic drop in the erythrocyte membrane fluidity as compared to resting time, but this was not accompanied by significant changes in the plasmatic MDA and 4-HDA concentrations. The highest erythrocyte

membrane rigidity was detected immediately after strenuous exercise until exhaustion was performed. Protein carbonyl levels were higher after exhaustive exercises than at rest. Continuous progressive and strenuous exercises until exhaustion, but not submaximal workload, resulted in a significant enhanced accumulation of carbonylated proteins in the plasma. These findings are consistent with the idea that exercise exaggerates oxidative damage, which may contribute, at least partially, to explain the rigidity in the membrane of the erythrocytes due to acute exercise.

**Keywords** Acute exercise · Membrane fluidity · Lipid peroxidation · Protein oxidation · Erythrocyte

## Introduction

Numerous processes in or among cells, including signal transduction, solute transport and cell–cell interaction, are membrane mediated (Derby and Gleeson 2007; Sudhakar et al. 2008). Cell membranes are complex bilayer structures compiled of a variety of lipids and proteins. According to the fluid-mosaic model proposed by Singer and Nicolson (1972), membrane lipids form a fluid bilayer where the incorporated proteins as well as the lipids themselves are free to diffuse laterally. Today, we are aware that both membrane structural intactness and adequate dynamics of the lipid bilayer are indispensable requirements for optimal functioning of cells (Stubbs and Smith 1984). Thus, measurements of the membrane fluidity reflect the biophysical and biochemical characteristics of the biological membranes and are an indicator of membrane function (Li et al. 1999).

Over the last few decades, intensive research in the field of oxidative stress indicates that exercise exacerbates free

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radical and other reactive oxygen species generation (Bloomer and Fisher-Wellman 2008; Powers and Jackson 2008). A free radical is any species capable of existence containing one or more unpaired electrons (Halliwell 1991). Aerobic organisms produce free radicals as a consequence of oxygen metabolism and, obviously, exercise causes an increase in oxygen consumption in the mitochondria. During intense exercise, the whole body oxygen uptake increases 20-fold above resting levels and 100-fold in active muscles (Asmussen et al. 1939). Although the relative role of mitochondria to the pro-oxidative stress during exercise is now under discussion, several potential alternative sources of free radicals, such as oxidase systems associated with membranes, nitric oxide production and phagocytic processes, have been proposed and contribute significantly to the overproduction of free radicals (Powers and Jackson 2008).

There is a general consensus that free radical generation during exercise occurs predominantly in skeletal and heart muscles; however, the invasive nature of obtaining biopsies from exercising humans limits their access. Therefore, some reports have claimed that several oxidative damage markers increase their plasmatic concentrations immediately after acute exercise (Ashton et al. 1998; Pialoux et al. 2009). Basically, the mature erythrocytes (RBC) are considered a simple biological bag enveloping a solution of hemoglobin, the main function of which is to act as a vehicle for oxygen transport. In terms of cell membrane structure and function, erythrocyte cell membranes are comparable to those of the plasmatic membranes of most eukaryotic cells (Ponizovsky et al. 2003). Thus, cell membranes isolated from RBC may reflect, at least partially, processes ongoing at various sites in the organism such as skeletal muscles. For this reason and because of its ready availability, membranes isolated from RBC have been used to perform experimental studies on the membrane function in animals (Motta et al. 2009; Portier et al. 2006) and in humans (Brzeszczynska et al. 2008). None of these studies have compared different types of exercise and correlated the membrane function with the plasma oxidative damage.

The purpose of this study was to primarily explore the impact of three different protocols of acute exercise on the erythrocyte membrane fluidity in healthy humans, estimated by changes in the fluorescence polarization of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene sulfonate (TMA-DPH). Since the *in vitro* association between membrane rigidity and oxidative damage has been widely reported (García et al. 1997), in this study we also relate this physiological occurrence in the erythrocyte membrane to the fluctuations in the plasma of the protein carbonyls contents and the concentrations of malondialdehyde (MDA) plus 4-hydroxyalkenals (4-HDA),

two frequently used measurements that reflect protein and lipid oxidation, respectively.

## Materials and methods

### Subjects

A total of 34 healthy untrained male subject volunteers aged 19–29 years ( $23.0 \pm 0.41$ ) were involved in this study. Only males were included to avoid any distortion in the hormonal response to physical exercise caused by sex differences (Bloomer and Fisher-Wellman 2008). The anthropometric characteristics of these subjects are summarized in Table 1. None of them participated regularly in any kind of sportive competition and did not engage in any form of vigorous exercise or take medications for 24 h before the study was carried out. All subjects underwent an extensive previous medical evaluation that included a history and physical examination, electrocardiogram and biochemical profile to discard possible pathologies. Subjects gave their informed written consent to participate in the study, and it was designed according to the principles of the Declaration of Helsinki. The experimental protocol was approved by Aragón Ethical Committee for Clinical Research (reference CP02/2010).

### Experimental procedures

Exercise was performed on an electronically braked cycle ergometer between 8:00 and 10:30 a.m. after an overnight fast. All subjects pedaled at an established cadence of 60 rounds per minute. Three ergometric tests were performed on each subject at random order at intervals of at least 1 week. Firstly, the maximal oxygen consumption ( $VO_{2max}$ ) and the maximal working capacity (MWC) were determined using a continuous progressive exercise test where the work load was increased by 10 W every minute until exhaustion (Carta et al. 1991). The oxygen uptake was

**Table 1** Age, anthropometric data such as height, weight and body mass index (BMI), and ergometric parameters such as maximal oxygen consumption ( $VO_{2max}$ ) and maximal working capacity (MWC) during resting time

<i>n</i> = 34	Values
Age (years)	23 ± 0.41
Height (cm)	177.59 ± 1.11
Weight (kg)	75.25 ± 2.84
BMI (kg m <sup>-2</sup> )	23.72 ± 0.69
$VO_{2max}$ (mL kg <sup>-1</sup> min <sup>-1</sup> )	43.8 ± 1.58
MWC (W)	239.8 ± 7.4

Results are means ± SE of 34 male healthy subjects

determined using a “breath by breath” gas exchange analyzer, CPX Express (MedGraphics®). Secondly, all subjects performed a strenuous test until exhaustion. The initial load was 100 W less than the MWC determined according to the first test (Caputo and Denadai 2004). Finally, the subjects pedaled for 30 min at a submaximal workload chosen at about 70% of the expected maximum for each individual (Bloomer et al. 2006).

Peripheral venous blood samples obtained before exercise (A) and immediately post-exercise trials (B, C and D, respectively) were drawn by antecubital veinpuncture and collected into lithium heparin-containing tubes; 10 mL of blood was collected at each sampling time. Blood samples were immediately centrifuged at  $1,000\times g$  for 10 min in a Beckman Allegra 64R refrigerated centrifuge (Fullerton, USA). Plasma was stored in 250  $\mu\text{L}$  aliquots at  $-80^\circ\text{C}$  until carbonyl content in the proteins and MDA + 4-HDA concentrations were measured. The RBC phase at the bottom was washed three times with 10 mL of saline solution (0.9% NaCl) and was finally dissolved in 1:6 w/v in 10 mM hypotonic TRIS–HCl buffer (pH = 7.4) and, thus, hemolyzed to isolate the membranes from the RBC.

#### Analytical procedures

All the chemicals and solvents, of the highest grade available, were acquired from Sigma (Madrid, Spain). TMA-DPH was obtained from Molecular Probes (Eugene, USA).

The isolation of RBC membranes was carried out according to the protocol of Hanahan and Ekholm (1974) with minor modifications. To sum up, the hemolyzed RBC was centrifuged at  $30,000\times g$  for 20 min. The pellet was resuspended 1:5 w/v in 10 mM TRIS buffer and washed twice more. The final pellet was resuspended in 1:1 w/v and kept at  $-80^\circ\text{C}$  until membrane fluidity assay.

Fluidity was monitored from triplicate determinations using TMA-DPH as fluorescent probe. Its incorporation into the RBC membrane and the determination of membrane fluidity were carried out according to that described elsewhere (Yu et al. 1992). RBC membranes (0.5 mg protein/mL) were re-suspended in 50 mM TRIS (3 mL final volume) and mixed with TMA-DPH (66.7 nM). After stirring vigorously on a vortex for 1 min, the preparation was incubated for 30 min at  $37^\circ\text{C}$ . Fluorescence measurements were performed in a Perkin-Elmer LS-55 Luminescence Spectrometer equipped with a circulatory water bath to maintain the temperature at  $22 \pm 0.1^\circ\text{C}$ . Excitation and emission wavelengths of 360 and 430 nm were used, respectively. The emission intensity of vertically polarized light was detected by an analyzer oriented parallel ( $I_{V_V}$ ) or perpendicular ( $I_{V_H}$ ) to the excitation plane. A correction factor for the optical

system,  $G$ , was used. Polarization ( $P$ ) was calculated by the equation:

$$P = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + GI_{V_H}}$$

An inverse relationship exists between membrane fluidity and polarization (Yu et al. 1992); thus, membrane fluidity is expressed as  $1/P$ . Protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford 1976).

The content of protein carbonyls was measured according to the method of Levine et al. (1990). To the plasma, 100  $\mu\text{L}$  of 50 mM TRIS buffer and 200  $\mu\text{L}$  of 10 mM 2,4-dinitrophenylhydrazine (DNPH) solution was added and the mixture was vortexed followed by incubation at  $37^\circ\text{C}$  for 1 h. Ice-cold trichloroacetic acid (325  $\mu\text{L}$ ) was added to the mixture. The pellet obtained after centrifugation at  $3,000\times g$  for 10 min was washed three times with 1 mL ethanol/ethyl acetate (1:1, v:v). The last pellet was dissolved in 6 M guanidine (700  $\mu\text{L}$ ) and incubated again at  $37^\circ\text{C}$  for 15 min. After centrifugation at  $12,000\times g$  for 10 min, the absorbance of the supernatant was measured spectrophotometrically at 375 nm, and its concentration was expressed as  $\mu\text{M}$  carbonyl groups. Guanidine was used as a blank.

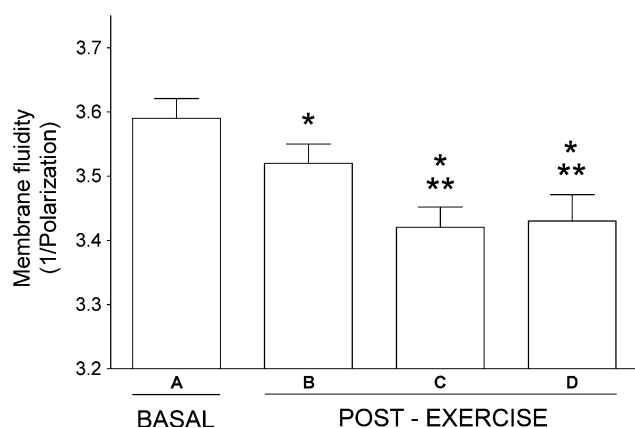
MDA + 4-HDA concentrations ( $\mu\text{M}$ ) were used as an index of the oxidative breakdown of lipids in the plasma (Janero 1990). In the assay, MDA + 4-HDA react with *N*-methyl-2-phenylindole, yielding a stable chromophore with a maximum peak absorbance at 586 nm; 1,1,3,3-tetramethoxypropane was used as a standard.

#### Statistical analysis

The data were expressed as arithmetic mean and standard error (SE) values. Mean differences were determined using a paired *t* test, with a level of significance at  $p \leq 0.05$ .

#### Results

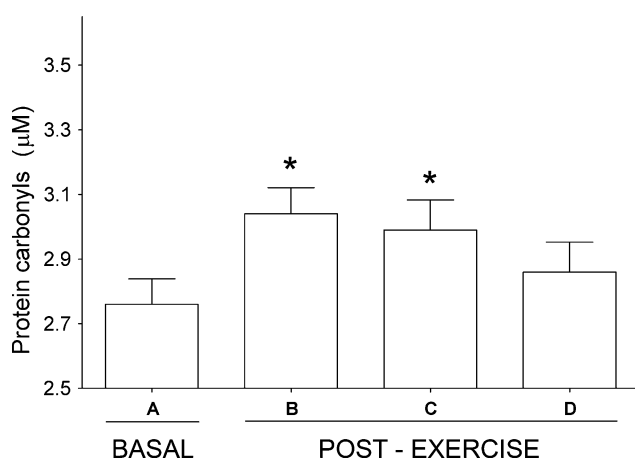
The effects of RBC membrane fluidity of a single bout of maximal and submaximal exercises, performed according to our study design, are illustrated in Fig. 1. Continuous progressive exercise was followed by a decrease of the erythrocyte membrane fluidity levels ( $3.52 \pm 0.30$ ) in comparison to pre-exercise values ( $3.59 \pm 0.31$ ). The strenuous exercise until exhaustion and the submaximal workload (70% MWC) for 30 min produced a dramatic drop in the RBC membrane fluidity as compared to the resting period ( $3.42 \pm 0.32$  and  $3.43 \pm 0.42$ , respectively). No significant changes were observed after strenuous and moderate exercise.



**Fig. 1** Membrane fluidity in erythrocytes isolated from 34 male healthy subjects during basal (A) and immediately after a continuous progressive exercise test (B), a strenuous test performed until exhaustion (C) and a submaximal exercise (70% of the expected maximum workload) for 30 min (D). Data (mean  $\pm$  SE) are expressed as the inverse of polarization. \*Statistical differences ( $p \leq 0.05$ ) versus pre-exercise, \*\* $p < 0.05$  versus continuous progressive exercise

With regard to protein carbonyl concentrations in plasma (Fig. 2), immediately following the continuous progressive exercise and the strenuous exercise until exhaustion (B:  $2.76 \pm 0.08 \mu\text{M}$ , and C:  $2.99 \pm 0.09 \mu\text{M}$ , respectively), protein carbonylation increased significantly as compared to carbonyl concentrations during resting time (A:  $2.76 \pm 0.08 \mu\text{M}$ ). However, there were no significant changes just after the submaximal one ( $2.86 \pm 0.09$  vs.  $2.76 \pm 0.08 \mu\text{M}$ ).

Combined MDA + 4-HDA concentrations have been used as an index of lipid peroxidation in biological samples. Statistical analysis of plasmatic MDA + 4-HDA levels for oxidative damage to lipids did not reveal

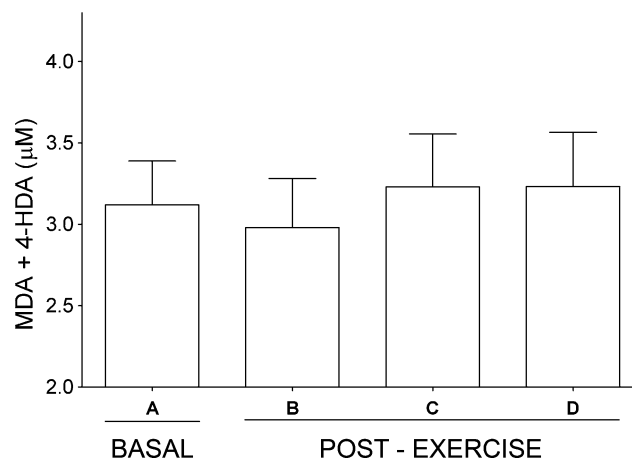


**Fig. 2** Effect of acute exercise on plasmatic protein carbonylation ( $\mu\text{M}$ ) in 34 male healthy subjects at resting time (A) and immediately post-exercise (B, C and D). Data is expressed as mean  $\pm$  SE. \*Statistical differences ( $p < 0.05$ ) versus pre-exercise

significant effects for either maximal or submaximal exercise protocols (A =  $3.09 \pm 0.26 \mu\text{M}$ , B =  $2.97 \pm 0.29 \mu\text{M}$ , C =  $3.19 \pm 0.32 \mu\text{M}$ , D =  $3.23 \pm 0.33 \mu\text{M}$ ) (Fig. 3). The apparent rise in plasma MDA + 4-HDA levels at the end of the strenuous exercise performed until exhaustion and the submaximal cycloergometric tests compared to the basal were not significant ( $p = 0.646$  and  $0.621$ , respectively).

## Discussion

Our study demonstrates that an acute period of exhausting or submaximal controlled exercise results in a decrease in the RBC membrane fluidity. To the best of our knowledge, this is the first report showing that acute physical exercise affects the membrane fluidity of erythrocytes in healthy human males using measurements of the membrane fluidity assessed by fluorescence spectroscopy. The principle of the fluorescence spectroscopy studies is based on the intercalation into the membrane of a fluorescent molecule, in this case TMA-DPH, which, when illuminated by polarized light, emits a fluorescence signal. The degree of polarization depends on the state of mobility of the TMA-DPH, which reflects motion in the membrane lipid environment (Yu et al. 1992). Our results are in agreement with the rigidity detected in mitochondrial membranes isolated from skeletal muscle immediately after exhausting exercise in Sprague–Dawley rats (Li et al. 1999). These authors labeled the membranes with DPH. TMA-DPH is a DPH derivative, which incorporates a trimethylammonium substituent to improve its localization in the membrane. Therefore, TMA-DPH is intercalated parallel to the long molecular axis of the phospholipids



**Fig. 3** Plasmatic concentrations ( $\mu\text{M}$ ) of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) in 34 male healthy subjects during rest (A) and immediately post-exercise (B, C and D). Data is expressed as mean  $\pm$  SE

with the cationic residue oriented at the surface (Prendergast et al. 1981).

Previous observations have been reported using another technical procedure to measure membrane fluidity, electron paramagnetic resonance spectroscopy. Membrane fluidity of RBC obtained from competition horses decreased immediately after a standardized exercise, which included a final step at maximal velocity performed on a race track (De Moffarts et al. 2007). Similar observations, rigidity in erythrocyte membranes, were found in four sedentary dogs after submaximal exercise in a study attempting to elucidate the role of vitamin E supplementation in preventing the effects of submaximal exercise-induced oxidative damage over a long period of time (Motta et al. 2009). Finally, Brzeczynska et al. (2008) detected erythrocyte rigidity 1 h after an exhaustive incremental cycling test performed in 11 healthy untrained males. These authors employed three spin labels, 5-, 12- or 16-doxyloctanoic acids to provide information of changes in the microenvironment in the nearest region of different depths of membrane bilayers. Since an increase in the order was detected only for the 5-doxyloctanoic acid spin label, they concluded that exercise-induced decreases in erythrocyte membrane fluidity were located in the polar region of the membranes (Brzeczynska et al. 2008). To measure fluidity changes in this specific region of the membrane, we chose TMA-DPH because the polar region of this probe is anchored at the lipid–water interface, while the hydrocarbon moiety enters the lipid part of the membrane. The length of the hydrophobic part of the TMA-DPH molecule is approximately equivalent to that of a 10-carbon aliphatic chain (Kuhry et al. 1983) and will thus provide information on this more superficial region including the surface and glycerol side chain region of the plasma membrane (Prendergast et al. 1981).

On the other hand, several studies suggested that regular exercise increased fluidity and improved the membrane viscosity of human RBC (Cazzola et al. 2003; Kamada et al. 1993; Tsuda et al. 2003). Since erythrocytes are essential for rapid and homogeneous perfusion of oxygen in the microcirculation (Dutta-Roy et al. 1985), increasing membrane fluidity and lowering the microviscosity, both provoked by aerobic training, may play an important role in erythrocyte diffusion in the microcirculation (Tsuda et al. 2003).

Several structural reasons have been proposed as the cause of the RBC membrane rigidity. Firstly, these may be disturbances in the lipid membrane composition, e.g., a drop in the polyunsaturated/saturated fatty acid ratio in the membrane (Curtis et al. 1984; Shinitzky 1984). It is well documented that exercise results in free radical and other reactive oxygen species overproduction (Bloomer and Fisher-Wellman 2008; Powers and Jackson 2008). Since

the polyunsaturated fatty acids are particularly sensitive to the aggressive behavior of free radicals (Gutteridge 1995), it seems reasonable that oxidative damage to the erythrocyte lipid bilayer may be involved in the RBC rigidity due to acute exercise. Secondly, oxidative stress in a biological membrane induces the formation of cross-linking of lipid–lipid and lipid–protein moieties (Chen and Yu 1994). Thirdly, membrane proteins may be modulators of membrane fluidity (Shinitzky 1984). The thiol groups of these proteins are easily oxidized by free radicals (Nakazawa et al. 1996), and it has even been suggested that acute physical exercise may result in aggregation of the spectrin–actin cytoskeletal protein complex, which leads to a decrease in the membrane dynamics (Brzeczynska et al. 2008).

Membrane fluidity is a physicochemical feature of cell membranes that plays an important role in modulating RBC functions such as permeability, transport of ions or oxygen, membrane-associated enzyme activities and osmotic fragility (Derby and Gleeson 2007; Shinitzky 1984; Sudhakar et al. 2008). Thus, maintaining adequate erythrocyte membrane fluidity levels is essential for optimal cell physiology. During exercise, RBC are highly exposed to mechanical and oxidative stress, even more than other cells, especially considering that RBC continuously produce free radicals and also because of their limited repair mechanisms (Cimen 2008; Petibois and Deleris 2005). Several studies have claimed that vigorous exercise acutely impairs RBC deformability (Eichner 1985; Szygula 1990), and oxidative stress results in lipid and protein oxidation in the membrane, which may destabilize the lipid bilayer and the cytoskeleton, thereby compromising cell survival (Dumaswala et al. 1999). The RBC membrane rigidity due to acute exercise detected during our study is consistent with these findings.

In addition to RBC membrane rigidity, we detected increased oxidative damage in the plasma due to acute exercise, although these effects were detected in a biomolecule-specific manner; that is, plasmatic protein oxidation increased following exercise, whereas plasmatic lipid oxidation was minimally affected.

Protein oxidation due to oxidative stress results in a loss of its biological function and structural modifications, e.g., the addition of carbonyl groups to its amino acid residues (Davies and Goldberg 1987; Levine and Stadtman 2001). In this way, plasmatic protein carbonylation increased acutely in resistance trained men following a single set of anaerobic exercise (Bloomer et al. 2007; Bloomer and Fisher-Wellman 2008). However, the magnitude of protein damage resulting from aerobic exercise depends on its intensity. It has recently been proposed that a single bout of exercise for 40 min elevated protein carbonyl groups in plasma of trained men in a workload-dependent manner

(Lamprecht et al. 2008). Thereby, these authors observed that oxidative damage to the plasmatic proteins occurs after exercises performed at 75 and 80% of  $VO_{2max}$ ; however, these alterations were only statistically significant when reading 80% of  $VO_{2max}$ . In agreement with these previous studies, we found significant rises in protein carbonylation when the untrained volunteers performed maximal exercises (B and C); however, we were unable to notice a significant change in the oxidation of plasmatic protein at submaximal workloads of 70% of the expected maximum  $VO_2$  for 30 min.

Finally, acute exercise has been proved to elevate MDA concentrations or several reactive products of lipid peroxidation in the active muscle (Alessio et al. 2000; Davies et al. 1982; Ji and Fu 1992; Li et al. 1999) as well as other tissues and plasma (Brzezczynska et al. 2008; Motta et al. 2009). In contrast to these observations, but in accordance with our results, other researchers have found that exercise minimally modifies or even reduces the degree of lipid peroxidation, presumably by increasing the capacity of the antioxidant system (Bloomer et al. 2007; Groussard et al. 2003; Kim et al. 1996) or due to increased clearance or distribution throughout the body (Leaf et al. 1997).

In conclusion, our study shows that erythrocyte membranes in untrained healthy males were less fluid after an acute period of maximal or submaximal exercise. Based on the elevation of protein carbonylation in the plasma, a marker of systemic oxidative damage, as well as the previously reported pro-oxidative effects of physical exercise, it seems reasonable that the disturbances in the dynamics of the RBC lipid bilayers may be attributed, to some extent, to the overproduction of free radicals.

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**Conflict of interest** None.

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