J Physiol Biochem (2014) 70:107–115
DOI 10.1007/s13105-013-0285-0

ORIGINAL PAPER

Increase of pro-oxidants with no evidence of lipid peroxidation in exhaled breath condensate after a 10-km race in non-athletes

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Received: 24 February 2013 / Accepted: 12 August 2013 / Published online: 27 August 2013

Abstract It is a well-established fact that exercise increases pro-oxidants and favors oxidative stress; however, this phenomenon has been poorly studied in human lungs. Pro-oxidative generation (H_2O_2, NO_2^-), lipid peroxidation markers (MDA), and inflammation (pH) in exhaled breath condensate (EBC) have been determined through data from 10 active subjects who ran 10 km; samples were obtained immediately before, at 20, and at 80 min post-exertion. In EBC, the concentration of H_2O_2 at 80 min post-exertion was increased. NO_2^- concentration showed a tendency to increase at 80 min post-exertion, with no variations in MDA and pH. No variations of NO_2^- were found in plasma, while there was an increase of NO_2^- at 80 min post-exertion in the relation between EBC and plasma. NO_2^- in EBC did not correlate to plasmatic NO_2^-, while it did correlate directly with H_2O_2 in EBC, suggesting a localized origin for the exercise-related NO_2^- increase in EBC. MDA in plasma did not increase nor correlate with MDA in EBC. In conclusion, high-intensity exercise increases lung-originated pro-oxidants in non-athlete subjects with no evidence of early lipid peroxidation and changes in the pH value in EBC.

Keywords Exhaled breath condensate · Runners · Lung oxidative stress · Lung inflammation

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Introduction

It is a well-documented fact that exercise favors the increase of pro-oxidants and that in some situations it produces oxidative stress [21, 30]. A reduced group of studies on animals have been focused on the impact of exercise on pulmonary redox equilibrium state, reporting evidence of oxidative stress [4, 36].

Exercise increases lung ventilation and favors higher contact with cold air, air pollutants, and chlorine in swimming pools [22, 43]; at the same time, it favors immune system activation [28]. The aforementioned may be particularly important in subjects who have regimes of long training hours. Consequently, previous studies in humans have demonstrated inflammation and redox state changes in the lungs of athletes such as swimmers [14], skiers [41], and runners [3, 11].

The study of redox state changes in the lungs resulting from exercise is difficult because sampling involves some risks to the participants; for this reason, the use of exhaled breath condensate (EBC), extensively studied in lung diseases and which has been proposed for the evaluation of various tissue processes (oxidative damage, cancer, remodeling, and inflammation) located in this organ [18, 20], can be a useful tool in the characterization of this phenomenon in athletes. Using EBC samples, no changes in the concentration of H_2O_2 ($[H_2O_2]_{EBC}$) were found during exercise [32], but changes in their flow [25] were found. It has also been reported that there was an increase in $[H_2O_2]_{EBC}$ on climbers exposed to altitudes of 6,125 m [1] and in biathletes who trained for 6 weeks at an altitude of 2,800 m [17]. In both protocols, an increase and a trend to the increase of lipid peroxidation measured as malondialdehyde (MDA) and 8-isoprostane, respectively, was shown. Recently, our research group compared amateur long distance runners who trained 50 km a week in 10-, 21.1-, and 42.2-km races, reporting increases in $[H_2O_2]_{EBC}$ and NO_2^- concentration in EBC ($[NO_2^-]_{EBC}$) for the 21.1- and 42.2-km races with no modifications in the participants of the 10-km race. In the three evaluated distances, no increase in lipid peroxidation, measured as the MDA concentration in EBC ($[MDA]_{EBC}$), was shown [3]. In this paper, we extend the description of redox state changes which occur in EBC from participants of long distance races to physically active but non-athlete subjects. We hypothesized that, in this group, a 10-km race could generate an increase in pro-oxidants and favor lung lipid peroxidation since they are not chronically exposed to the pulmonary effects (irritation, dryness, inflammation, cell damage) of the distance runners' training regime. A second objective was to advance in the characterization of EBC markers as originated either locally or from the systemic environment; for this purpose, we compared the concentrations of NO_2^- and MDA in both EBC and plasma.

Materials and methods

Subjects

Ten non-smoking students of Physical Education (see Table 1) with no history of high or low respiratory tract inflammation during the month previous to the study were made subjects of this study. They also had no history of chronic respiratory diseases (asthma or allergic rhinitis) and did not consume nutritional supplements, antioxidants, or anti-inflammatory medicaments. They practiced 9.2±3.3 h/week of moderate to intense exercise. The distribution of total exercise time, expressed in hours per week, is presented as mean±standard deviation, and the percentage of the total sample performed in this activity is shown in parentheses: running 2.5±0.5 (80 %), swimming 1.2±1.4 (40 %), football 1.5±1.7 (30 %), mountain bike 1.5±6.3 (20 %), tennis 0.9±2.1 (20 %), handball 0.6±1.4(20 %), volleyball 0.5±0.7(20 %), and basketball 0.5±0.7 (20 %). Participants were informed orally and in writing, before signing an informed consent. This study was approved by the Ethics Research Committee of the Universidad de los Andes.

Table 1 General description of participants

	Values
Men/Woman	9/1
Age (years)	20.50±1.60
Weight (kg)	62.64±6.8
Height (cm)	172.4±5.3
VO_2 max (ml kg^{-1} min^{-1})	47.37±6.0
Time of race (min)	50.65±4.63

Values are shown as mean ± SD

Protocol

After being evaluated at rest, they went through a 10-min warm up before running 10 km at maximum effort in an open 330-m racetrack. On each complete turn, the cardiac frequency was determined (Polar, model T31) in order to quantify the intensity of the exercise. All subjects performed this test simultaneously. EBC samples were taken using the previously described device [1, 2]. Subjects were at rest, wearing a nasal clip, and having previously washed their mouths with distilled water. Sampling time was between approximately 10 to 15 min or until 1.5 mL of EBC was obtained. Also, venous blood was drawn, heparinized, and then centrifuged at 3,000 rpm to obtain plasma. Once samples were obtained, they were stored in liquid nitrogen and later at −80 °C until they were analyzed. EBC or plasma samples were taken before (pre) exercise, 20 min after exercise completion (20-post), and 80 min after exercise completion (80-post).

Malondialdehyde in EBC and plasma

MDA concentration was measured according to Larstad et al. [23]. EBC at 300 μL or 50 μL of plasma was mixed with 100 μL of 25 mM thiobarbituric acid. The mixture was incubated for 1 h at 95 °C. After cooling, first in ice for 5 min and then for 40 min at room temperature, the mixture was submitted for high-performance liquid chromatography (Shimadzu LC10AD, Corporation), where a C-18 column 150-mm long and 4.6-mm I.D. (Supelcosil LC-18, Supelco) was used. The mobile phase (1 mL/min) was a 20:80 (*v*/*v*) mixture of acetonitrile in 20 mM potassium phosphate buffer (pH 6.8). Measurements were performed with a fluorescence detector (RF-551, Shimadzu), excitation and emission wavelengths, being at 532 and 553 mm, respectively. Malondialdehyde bis (diethyl acetal) from Merck was applied as standard.

Hydrogen peroxide in EBC

It was measured using FOX2 [31] reagent. This reagent contains Fe^{+2} (250 μM), which in an acidic medium ($HClO_4$, 110 mM), and is oxidized to Fe^{+3} by the presence of H_2O_2. The amount of H_2O_2 is monitored through the reaction between the ferric ion and the xylenol orange indicator (250 μM). Sorbitol (100 mM) was added to the original reagent according to Gay and Gebicki [15]; this method has been previously used by our research group [1–3]. For measurements, 350 μL of EBC and 150 μL of modified FOX2 were taken, then the sample was incubated for 1 h at room temperature, and absorbance was read at 560 nm (Jenway 6405). Three calibration curves were performed for each group's measurements using H_2O_2 (Merck) as standard.

pH in EBC

It was measured using the protocol of Paget-Brown et al. [33]. EBC at 100 μL was bubbled with argon for 8 min at a flow rate of 350 mL/min, and pH was later measured using a 3×38 mm (Diameter×Length) microelectrode (Cole and Palmer) connected to a pH meter (Oakton® Acorn pH 6).

Nitrites in EBC and plasma

Nitrite concentration was measured using spectrophotometric test based on the Griess reaction [16]. Griess reagent at 300 μL (0.1 % naphthylethylenediamine–dihydrochloride, 1 % sulphanilamide, 3 % H_3PO_4) was added to 300 μL of EBC or plasma deproteinized with $NaOH/ZnSO_4$. The mixture was incubated for 10 min, and absorbance was measured at 550 nm. Three calibration curves were performed for each group's measurements using sodium nitrite (Merck) as standard.

Statistics

Using the Shapiro–Wilk normality test, it was observed that the samples did not come from a Gaussian distribution; therefore, non-parametric tests were applied. The Friedman test was used for repeated samples, and Dunn's test was used as a further test for all the measured parameters. Correlations were determined by the Spearman correlation coefficient. The significance level used was of $p<0.05$. For statistical analysis, GraphPad Prism, USA software was used.

Results

Exercise intensity estimated as the percentage of cardiac reserve was at 91.2±4.7 %. The race time was 50.6±4.6 min. Both variables are expressed as mean and standard deviation. An increase in $[H_2O_2]_{EBC}$ (Fig. 1) as compared with the pre-value at 80-post ($p<0.05$) was seen. $[NO_2^-]_{EBC}$ showed a trend to significance; it had

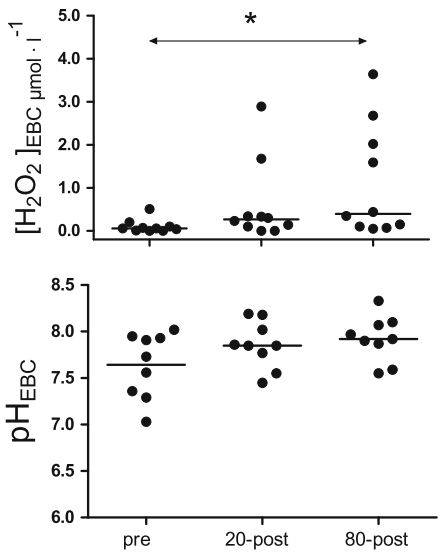

Fig. 1 $[H_2O_2]_{EBC}$ and pH_{EBC} in participants of a 10-km race. The *line* represents the median value. *$p<0.05$ is different from the pre-value

a value of p=0.045 in the Friedmann test, with no differences between groups in the posteriori test. No changes in nitrites in EBC and plasma ($[NO_2^-]_P$) after the race (p=0.97) were seen. The relation $[NO_2^-]_{EBC}/[NO_2^-]_P$ showed increases (Fig. 2) on the pre-value in the 80-post (p<0.05). No differences in $[MDA]_{EBC}$ (p=0.60), in the values of malondialdehyde in EBC and plasma ($[MDA]_P$; p=0.83), or in the relation between $[MDA]_{EBC}/[MDA]_P$ (p=0.60) as shown in Fig. 3 were observed. The pH in EBC (pH_{EBC}; p=0.39) showed no post-race differences (Fig. 1).

Correlations were made between absolute values and their absolute changes (deltas). A first group of absolute deltas was obtained from the difference between the absolute values of the 20-post-stages minus pre-stages. The second group of deltas was obtained from the differences between the absolute values of the 80-post-stages minus 20-post-stages; in the performed delta correlations, both sets of data were considered together.

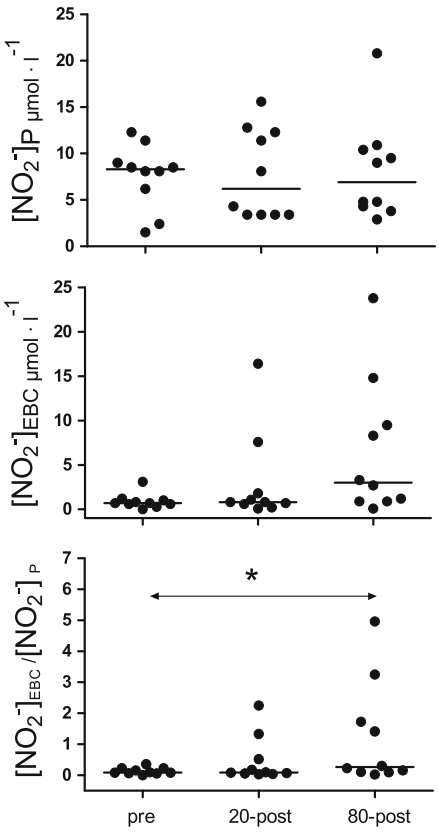

Fig. 2 $[NO_2^-]_P$, $[NO_2^-]_{EBC}$, and $[NO_2^-]_{EBC}/[NO_2^-]_P$ in participants in a 10-km race. The *line* represents the median value.*p<0.05 different from pre-value

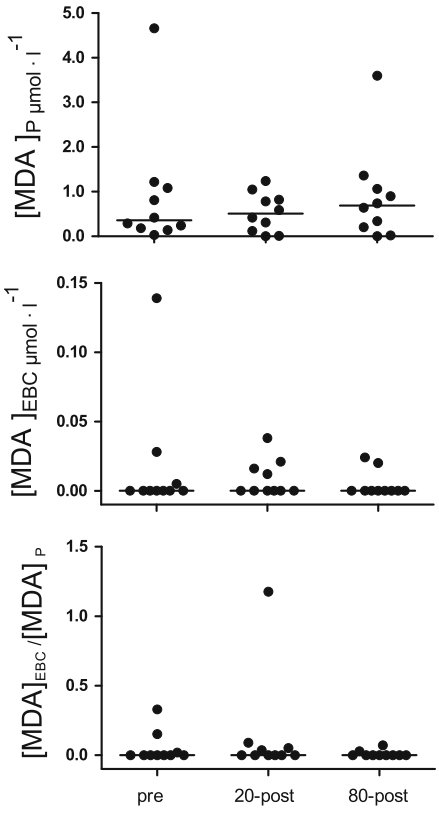

Fig. 3 $[MDA]_P$, $[MDA]_{EBC}$, and $[MDA]_{EBC}/[MDA]_P$ in participants in a 10-km race. The *line* represents the median value

Regarding nitrite, no significant correlations between absolute values of $[NO_2^-]_P$ versus $[NO_2^-]_{EBC}$ (r=0.21, n=30, p=0.26) and for absolute changes between these same variables (r=0.18, n=20, p=0.46) were observed. A similar result was found for $[MDA]_P$ versus $[MDA]_{EBC}$ for absolute values (r=−0.22, n=30, p=0.24) and between absolute changes (r=−0.16, n=20, p=0.49). Both the relation between absolute values of $[H_2O_2]_{EBC}$ versus $[NO_2^-]_{EBC}$ (r=0.69, n=30, p<0.0001) and absolute changes (r=0.73, n=20, p<0.0002) showed a significant association as shown in Fig. 4. No significant correlations between absolute values of $[H_2O_2]_{EBC}$ and $[NO_2^-]_{EBC}$ with pH_{EBC} were found. Correlations between absolute changes of $[H_2O_2]_{EBC}$ and pH_{EBC} showed a trend to significance (r=−0.45, n=18, p=0.06), while absolute changes between $[NO_2^-]_{EBC}$ versus pH_{EBC} were significant (r=−0.61, n=18, p=0.007; see Fig. 5). No significant correlations between the race time and intensity (measured as the percentage of cardiac reserve) with the studied variables in EBC and plasma in 20-post and 80-post were found.

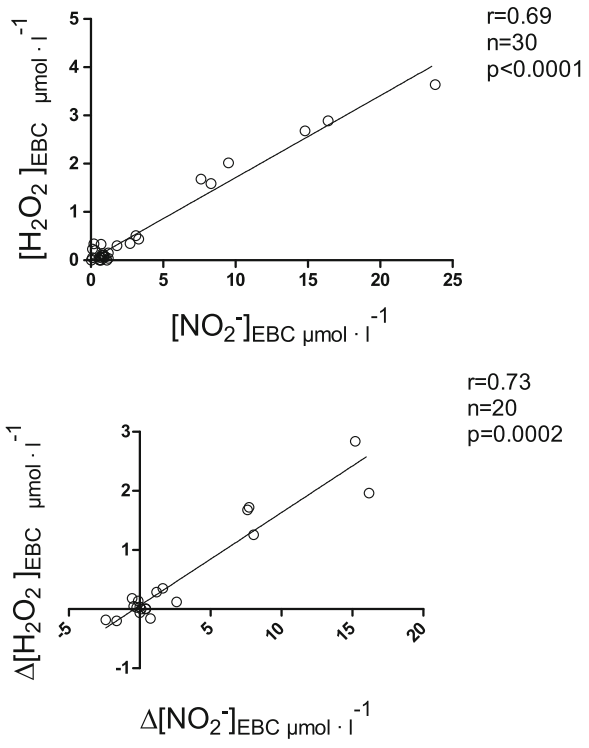

Fig. 4 Relationship between $[NO_2^-]_{EBC}$ versus $[H_2O_2]_{EBC}$ (top) and $\Delta[NO_2^-]_{EBC}$ versus $\Delta[H_2O_2]_{EBC}$ (bottom) in participants of a 10-km race

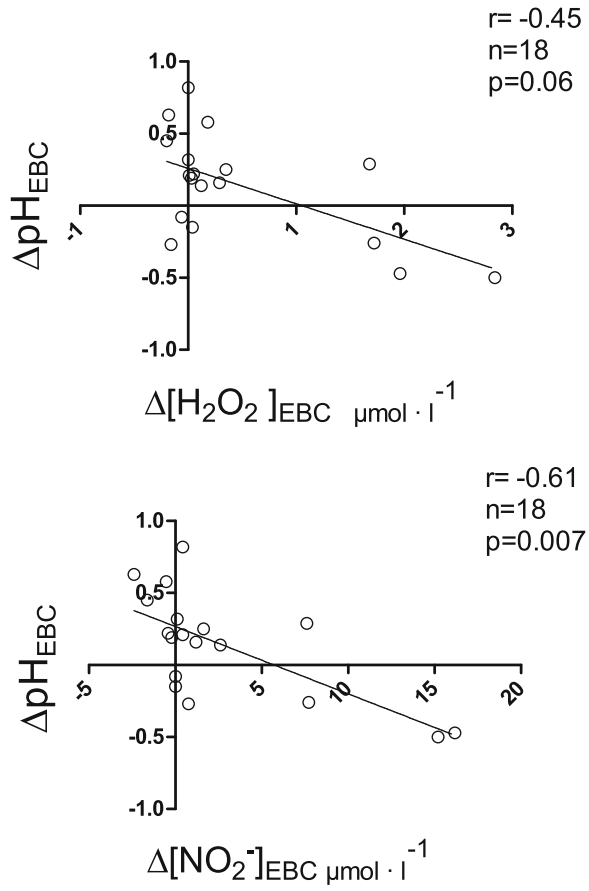

Fig. 5 Relationship between $\Delta[H_2O_2]_{EBC}$ versus $\Delta[pH]_{EBC}$ (top) and $\Delta[NO_2^-]_{EBC}$ versus $\Delta[pH]_{EBC}$ (bottom) in participants of a 10-km race

Discussion

Exercise increases lung ventilation and the speed with which the surrounding air reaches our lungs. High-intensity and prolonged exercising, typical of endurance races, inflame the airways [6, 42] and increase its pro-oxidants [3] as it has been previously described. One of the factors that have an influence on oxidative stress produced by exercise is the fitness degree of the participants; Brooks et al. [7] showed higher NO and superoxide anion formation because of acute exercise in sedentary rats' perfused/infused muscle versus muscles of trained rats. In patients with chronic obstructive pulmonary disease, there was less oxidative stress induced by acute exercise after their participation in a physical training program [34]. In this sense, it is possible that subjects, such as those from the present study, physically active but not subjected to high endurance athletes' regime (long sessions of prolonged aerobic exercise), are more prone to increase in pro-oxidative formation since the intensity of the performed exercise reached 90 % of the cardiac reserve (see the "Results" section). In this study, the main result is the increase in $[H_2O_2]_{EBC}$ and $[NO_2^-]_{EBC}/[NO_2^-]_P$; both parameters show a tendency at 20 min that becomes significant at 80 min post-race. At the same time, despite the increase of these species, there is no lipid peroxidation increase, as it has been previously described in the lungs of animals [38], nor pH_{EBC} decrease as an indicator of tissue inflammation, as it has been described in pathologies such as asthma, bronchiectasis, and adult respiratory distress [20].

Regarding $[H_2O_2]_{EBC}$ results, there are few similar experiences in the literature to the one presented here, especially because of the increased exercise time (over 30 min) of our protocol, which makes it difficult to compare; Nowak et al. [32], Marek et al. [25], and Mercken et al. [26] conducted submaximal and maximal

exercises finding no differences in $[H_2O_2]_{EBC}$, but they used a protocol with only 6- and 15-min exercising times, respectively. Data presented herein are comparable to our previous report on amateur long distance runners [3]. In that report, no changes in 10-km runners were observed and absolute changes were minor in this distance in comparison to 21.1- and 42.2-km races. In this report, we found that in a group of non-runner subjects, $[H_2O_2]_{EBC}$ does increase after the race, as measured on the same times as of the aforementioned work. The fact that trained subjects do not show any increase in this pro-oxidant in this distance may be partly explained by the induction of anti-oxidant defenses [37] and by a lower inflammatory response as a result of chronic exercise [47]. This finding shall be later reevaluated in subjects with different levels of training directly compared under the same conditions.

During physical exercise, in the lungs, nitric oxide is involved in both dilatation of airways to increase mobilized airflow and vasodilatation in order to avoid excessive increase in pulmonary artery pressure [45]. In the pathological context, nitric oxide participates in lung redox imbalance that occurred in inflammatory processes [44]. Nitric oxide has a short half-life; therefore, in many experimental models, more stable metabolites such as nitrite and nitrate are determined [46]. Nitric oxide and its related compounds have a complex metabolism; thus, it is not yet fully clarified. This happens, among other aspects, because of its multiple origins; it can be formed from typical lung cells (epithelial cells, endothelial cells, smooth muscle cells) as well as in leukocytes and erythrocytes [8].

Regarding the effect of a long distance race on $[NO_2^-]_{EBC}$, to our knowledge, this parameter has been previously reported only by our research group, and no changes in $[NO_2^-]_{EBC}$ after a 10-km race were observed, while changes in 21.1- and 42.2-km races were observed [11]. Unlike this previous work, we currently report a trend to increased $[NO_2^-]_{EBC}$ and an increase in the relation between $[NO_2^-]_{EBC}/[NO_2^-]_P$, which means an increase in this pro-oxidant in the lungs by exercise in subjects who are not accustomed to this physical effort unlike usual runners. In the particular case of the time in which the increase of the relationship, $[NO_2^-]_{EBC}/[NO_2^-]_P$ (80-post-stage) is potentially observed; it can result from nitric oxide increases that occurred during exercising, since NO_2^- may remain without being completely debugged, in the increase of the lung endothelial nitric oxide synthase activity as seen in animal models and/or in the increased activity of this enzyme as described in human leukocytes after exercise [24, 29].

Contrary to the few measurement reports of $[NO_2^-]_{EBC}$, nitrite has been previously measured in plasma during exercise. In this regard, some reports have observed increases in plasma levels of nitrite+ nitrate combination after 10 min of exercise at 75 % of maximal oxygen consumption [10]; however, this finding is not systematic for acute exercise. Bloomer et al. [5] found no nitrite increases in plasma after 30 min of treadmill exercise. In both athletes and sedentary people, Poveda et al. [35] found no changes in $[NO_2^-]_P$ after maximum exercise on a treadmill. In our study, $[NO_2^-]_P$ determination was done to evaluate if the eventual $[NO_2^-]_{EBC}$ increase could be explained by simultaneous increases in plasma (something that was not observed). This lack of NO_2^- increase in plasma and the lack of correlation between individual values and $[NO_2^-]_{EBC}$ and $[NO_2^-]_P$ absolute changes indicate that it is likely that the increase of EBC, because of exercise in these species, may be a localized phenomenon. This idea is also supported by the increase in $[NO_2^-]_{EBC}/[NO_2^-]_P$ relation and by the fact that $[NO_2^-]_{EBC}$ correlates with another exhaled air marker such as $[H_2O_2]_{EBC}$ (see Fig. 4). The finding of this statistically significant association is consistent with our previous report on long distance runners [3]. We believe that our collected data, as a whole, strongly supports the idea that intense prolonged exercise in this population—under the described conditions—alters the redox state of the pulmonary microenvironment.

The increase of the described pro-oxidants was not concomitant with $[MDA]_{EBC}$ increases (lipid peroxidation/oxidative damage indicator) and pH_{EBC} decreases (indicator of tissue inflammation) that were expected to occur. Regarding pH, Riediker and Danuser [39] reported a pH_{EBC} increase immediately and until 60 min post-exercise (30 min of fast walking at 60 % of maximal cardiac frequency).

A later report by Marek et al. did not report any changes in pH_{EBC} that was measured immediately after performing a maximal exercise to exhaustion in cycling (time is not reported) [25]. Ferdinands et al. [13] reported the absence of pH_{EBC} changes after acute exercise; however, they found lower pH_{EBC} values in regular runners. In a recent report on racehorses, Cathcart et al. found increased pH_{EBC} 20 to 30 min after running 1.6 km at a moderate to high intensity [9]. The tendency to maintain pH_{EBC} values after exercise, herein reported or

alkalinization reported by other groups, has no explanation yet; however, some authors have suggested the hypothesis that this phenomenon is due to the increase in ammonium secretion (buffer) of the airway epithelium in response to exercise [9]. In the pathological context, subjects with chronically inflamed airways, like asthmatic patients, have lower ammonium levels [19]. Similarly, Mickleborough et al. [27] did not find any changes in the pH_{EBC} in asthmatic subjects after hyperventilation at 85 % of maximal voluntary ventilation for 6 min. These patients decreased their levels of airway inflammation after ingesting a preparation rich in omega three fatty acids for 3 weeks. So, a decrease of exhaled nitric oxide and an increase in the basal value of pH_{EBC} was evidenced, and in contrast to that previously observed in the first hyperventilation test, an alkalinization of pH_{EBC} after the said test was evidenced, which can be interpreted as a better response to acidosis of the airway [27].

In another aspect, our previous data obtained on amateur runners showed the same trend to the increase (proved herein) of pH value in the 10-km runners' group, while there is a trend to a decrease in the groups of 21.1- and 42.2-km races [3]. The difference in the pH_{EBC} response between 10-km races and longer distances may be related to the greater intensity of the inflammatory response against the increased stimulus (distance of the race) and the time necessary to establish an inflammatory process in the tissue; thus, the time in a 10-km race is about 1 h, while a marathon of amateurs takes about 4 h. In this regard, it will be a great contribution, in the future, to extend the follow-up time of this parameter after the race and to include specific markers of inflammation such as cytokines. Furthermore, the different acids and bases found in the EBC samples should be more specifically analyzed.

Although there was no decrease in pH_{EBC} or inverse correlations between the absolute values of the pro-oxidants as we found in our report [3], in this work, we found inverse associations between the absolute changes of pro-oxidants (trend to H_2O_2 and significance for NO_2^-) studied in EBC and the absolute changes of pH_{EBC} (see Fig. 5), which supports the hypothesis that pro-oxidant changes are related to inflammation at this level.

Regarding $[MDA]_{EBC}$, previous results showed no differences at low heights (670 m) after high-intensity cycloergometric exercise [1]. Similar results were found in a 120-W cycloergometric protocol [32]; both findings are equivalent to those reported here but with protocols dissimilar to ours. Failure to find an expected relation between the increase in pro-oxidants and the increase in lipid peroxidation may take place because changes in this parameter occur at a later time as compared to our measuring. Senturk et al. [40] found MDA increases in plasma 12 h after 10 min of extenuating exercise; Fatouros et al. [12] found the highest $[MDA]_P$ at 24 h after a soccer match. Another possibility is that this pro-oxidative increase may be part of a physiological process in the described groups and conditions and is not associated to tissue damage in this organ. In this work, $[MDA]_P$ is also determined (see Fig. 3) in order to advance in the elucidation of the localized or systemic origin of this marker in EBC. In this respect, similar to that observed in $[MDA]_{EBC}$, we did not find any changes in neither $[MDA]_P$ nor changes in the relation between $[MDA]_{EBC}/[MDA]_P$ (see Fig. 3), so the interpretation of our findings becomes difficult. However, the lack of correlation between absolute values as well as between their absolute changes support the hypothesis that $[MDA]_{EBC}$ is not related to $[MDA]_P$; this was also observed in a previous work in which cyclists performed a maximal exercise at 2,160 m of altitude, showing increases in $[MDA]_{EBC}$ with no changes in MDA measured in serum. In the aforementioned work, no significant correlations between the said parameters were found [1].

In conclusion, unlike the previous results obtained in amateur runners, in physically active subjects, 50 min of high-intensity race (10 km) produces an increase in oxygen- and nitrogen-derived pro-oxidative species. Probably, this could be related to a stronger reaction response regarding the formation of pro-oxidant/inflammatory factors which are common in subjects less adapted to high-intensity and prolonged exercise. Despite the increase of pro-oxidants, we did not find any early modifications in lung lipid peroxidation and pH value in EBC. Nitrites in EBC most likely originated from a localized process in lungs.

Acknowledgments We acknowledge Mr. Luis Pizarro Zúñiga for his technical assistance in sample collection and chemical analysis of the samples.

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