

## HYDROGEN PEROXIDE RELEASE AND ACID-BASE STATUS IN EXHALED BREATH CONDENSATE AT REST AND AFTER MAXIMAL EXERCISE IN YOUNG, HEALTHY SUBJECTS

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### Abstract

**Objective:** Exhaled breath condensate (EBC) contains among a large number of mediators hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as a marker of airway inflammation and oxidative stress. Similarly EBC pH also changes in respiratory diseases. It was the aim of our investigation to prove if hydrogen peroxide release and changes in pH of EBC changes with exercise.

**Methods:** EBC was collected from 100 litres exhaled air along with samples of arterialized blood of 16 healthy subjects (9 males, 7 females, age 23 ± 1 years). EBC hydrogen peroxide was analyzed with EcoCheck amperometer (FILT, Berlin). The rate of H<sub>2</sub>O<sub>2</sub> release was calculated from the concentration and collection time. pH and PCO<sub>2</sub> in blood and in EBC were measured with the Radiometer blood gas analyzer, EBC was equilibrated with a gas mixture (5% CO<sub>2</sub> in O<sub>2</sub>). The bicarbonate concentration was calculated according to the law of mass action for CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> (pK = 6.1). **Results:** H<sub>2</sub>O<sub>2</sub> concentration in EBC was 190 ± 109 nmol/l, and H<sub>2</sub>O<sub>2</sub> release at rest was 31.0 ± 18.3 pmol/min. At maximal exercise, the H<sub>2</sub>O<sub>2</sub> concentration in EBC increased to 250 ± 120 nmol/l, and H<sub>2</sub>O<sub>2</sub> release significantly increased at maximal exercise to 84.4 ± 39.9 pmol/min (P<0.01). At rest pH of the CO<sub>2</sub> equilibrated EBC was at 6.08 ± 0.23 and the [HCO<sub>3</sub><sup>-</sup>] was 1.03 ± 0.40 mmol/l. At maximum exercise, pH 6.18 ± 0.17 and [HCO<sub>3</sub><sup>-</sup>] 1.23 ± 0.30 mmol/l remained almost unaltered.

**Conclusions:** The rate of H<sub>2</sub>O<sub>2</sub> release in EBC increased during exhausting exercise (external load: 300 Watt) by a factor of 2, whereas the pH and the bicarbonate concentration of the EBC, equilibrated with 5% CO<sub>2</sub> at 37°C were not significantly altered. It has to be proven by further experiments whether there is a linear relationship between the rates of H<sub>2</sub>O<sub>2</sub> release in EBC in graded submaximal exercise.

**Key words:** reference values, exhaled breath condensate, oxidative stress, hydrogen peroxide, respiration, spirometry

### INTRODUCTION

Exhaled breath condensate (EBC) contains a large number of mediators, which are influenced by airway

infections and other lung diseases and modulated by therapeutic intervention. Among others there is hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which can be measured by currently developed micro enzyme detectors of high sensitivity.

H<sub>2</sub>O<sub>2</sub> is released by neutrophils and eosinophils and by macrophages and epithelial airway cells; it provides one line of defence should infection occur and is therefore the most important marker of airway infection. It is synthesised by superoxide dismutase induced reaction of O<sup>-</sup> radicals and H<sup>+</sup> ions. A peroxidase is secreted by airway epithelial cells, which converts hydrogen peroxide into hypothiocyanous acid, a toxic compound that kills pathogens.

Similarly exhaled breath condensate (EBC) pH also changes in respiratory diseases. Like in other body fluids pH homeostasis is maintained by the interacting acid-base- and buffer systems, mainly influenced by the CO<sub>2</sub>-bicarbonate reaction of the extracellular fluid. Therefore the pH of EBC is found to be unstable outside the airway tract, due to the volatility of CO<sub>2</sub> molecules leaving the fluid. This results in a decreased CO<sub>2</sub> and bicarbonate with an increasing pH of the EBC.

Argon deaeration was suggested as a method to keep the pH constant. This increases the reproducibility of the measurements. Deaerated samples turned out to be stable, not influenced by hypo- or hyperventilation and independent from environmental temperature time of storage. In our study, we preferred the method of keeping the CO<sub>2</sub> partial pressure of the EBC constant by equilibration with a 5% CO<sub>2</sub> gas mixture thus simulating a mean CO<sub>2</sub>-fraction within the lung. Furthermore the experiments were designed to determine bicarbonate, the main buffer within the pH range of EBC in vivo.

The aim of the investigation was to prove, if maximal exercise results in an increased rate of hydrogen peroxide release and an altered acid-base status of EBC. In a group of young and healthy subjects we measured H<sub>2</sub>O<sub>2</sub> and pH of the equilibrated EBC (with a 5% CO<sub>2</sub> gas mixture) before and after exhausting exercise.

## MATERIAL AND METHODS

### ANTHROPOMETRICAL DATA OF SUBJECTS INVESTIGATED

The investigation was carried out on 16 healthy sporting subjects (9 males, 7 females), age  $23 \pm 1$  years (range 22–26 years),  $175 \pm 8.4$  cm height, and normal BMI  $22.2 \pm 1.7$  kg/m<sup>2</sup> (range 19.7–24.8 kg/m<sup>2</sup>). The subjects were free from acute airway infections and had, according to the ECCS references [1], highly normal values for the forced vital capacity (males  $113 \pm 11.4$  %pred and females  $108 \pm 10.3$  %pred). Mean values for FEV1 were highly normal,  $105.7 \pm 8.8$  %pred in females and  $119.1 \pm 10.5$  %pred in males. Tiffeneau Index another marker for airway obstruction was also highly normal in all subjects (males  $107 \pm 4.5$  %pred and  $102 \pm 5.9$  %pred).

### PROTOCOL

The subjects performed bicycle exercise (Ergoline E900), preferentially in the morning hours in a air conditioned room, following a protocol used in performance evaluation of athletes, starting with 5 minutes at 50 Watt and increasing external load by 50 Watt every 3 min. Respiratory parameters, using a medium sized face mask and heart rate (Polar belt) were continuously recorded by a ZAN 600USB CPA spirometer (ZAN Oberthulba, Germany). Before each test appropriate calibrations of the air-flow sensor and O<sub>2</sub>- and CO<sub>2</sub>-sensors were performed, with calibration gases from cylinders (5% CO<sub>2</sub>, 16% O<sub>2</sub>, 79% N<sub>2</sub>) and ambient air. During rest and immediately after the exercise 100 litres exhaled air and samples of arterialized blood of the ear lobe were collected. 100 l exhaled air at 37°C saturated with water vapour should contain 4.3 ml water [2].

EBC was obtained by cooling 100 l expired to -20°C (ECoScreen I, FILT, Berlin). At rest 100 l exhaled air were collected in  $8.4 \pm 2.0$  min and 1.68 ± 0.39 ml EBC obtained. After exhausting exercise at  $220 \pm 23.5$  Watt 100 l of exhaled air were collected in  $3.9 \pm 1.8$  min and  $1.20 \pm 0.44$  ml EBC collected. The volumes represent 39.1% and 27.9% of the theoretical water vapour content of 100 l exhaled air. From the EBC sample, three aliquots were taken for lactate, H<sub>2</sub>O<sub>2</sub>, and pH and acid base measurements. The samples were stored on ice, deep frozen to -12°C.

H<sub>2</sub>O<sub>2</sub> analysis was performed amperometrically after conversion of H<sub>2</sub>O<sub>2</sub> by peroxidase (H<sub>2</sub>O<sub>2</sub> → 2H<sup>+</sup> + O<sub>2</sub> + 2e<sup>-</sup>) using miniaturized enzyme detectors (ECoCheck, FILT, Berlin). Measurements can be performed in an effective range between 30–3000 nmol/l. Calibrations were performed daily. The method was described in detail in previous papers [3, 4]. Acid-base parameters were measured in crude EBC samples. H<sub>2</sub>O<sub>2</sub> was measured in 0.3 ml EBC diluted in 0.3 ml buffer solution. The rate of release from the lung (mol/min) is calculated from the concentration of hydrogen peroxide and the collection time of EBC. pH and PCO<sub>2</sub> measurements of EBC (equilibrated with a gas mixture of 5% CO<sub>2</sub> in oxygen at 37°C) and of blood taken from the ear lobe are performed with a

blood gas analyzer (Radiometer ABL520, Copenhagen) [5]. HCO<sub>3</sub><sup>-</sup> is calculated according to the mass action law of the CO<sub>2</sub>-bicarbonate reaction (pK=6.1; solubility of CO<sub>2</sub>=0.03 mmol/l Torr). For CO<sub>2</sub> equilibration a small PVC capillary was made for bubbling the carbon through the EBC sample, filled into a 2 ml Eppendorf vial.

### STATISTICAL ANALYSIS

Data were compiled in tables (Microsoft, Excel 2003). The final statistical analysis was performed using the SPSS 11.5 statistic program. After testing the data for homogeneity and normal distribution, means and standard deviations were calculated and presented in tables and Box-plots. Using Fisher's t-test for paired data, respiratory and cardiovascular data were tested for significant alterations (P<0.05).

## RESULTS

### RESPIRATORY AND CARDIAC RESPONSES

At maximum exercise ventilation increased nearly 10-fold to  $123 \pm 21.3$  and  $97.7 \pm 10.7$  l/min in males and females, respectively. At maximum load, females reached a mean heart rate of  $186 \pm 9.5$  beats/min at  $200 \pm 23.6$  Watt and males  $180 \pm 6.3$  beats/min at  $241 \pm 24.9$  Watt. Oxygen consumption increased to  $44.9 \pm 3.3$  and  $49.6 \pm 6.6$  ml/min/kg in females and males, respectively. In all subjects, respiratory exchange rate (RER) exceeded the value of 1.00, and reached  $1.15 \pm 0.05$  at break-off point in females and  $1.14 \pm 0.07$  in males.

### EXHALED BREATH CONDENSATE

Under resting conditions, 100 l of exhaled air was collected within  $8.4 \pm 2.0$  min, which delivered  $1.68 \pm 0.39$  ml EBC. This volume of fluid is 39.1% of the theoretical water content of 100 l of air saturated with water at 37°C. After maximal exercise, the collection time was  $3.9 \pm 1.8$  min (P<0.05), EBC volume was  $1.20 \pm 0.44$  ml, which is 27.9% of the theoretical water content.

### HYDROXYGEN PEROXIDE RELEASE IN EBC

H<sub>2</sub>O<sub>2</sub> concentration in EBC was  $190 \pm 109$  nmol/l. At rest H<sub>2</sub>O<sub>2</sub> release in the collected EBC was  $31.0 \pm 18.3$  pmol/min (Figs. 1 and 2). At maximal exercise (external load  $220 \pm 20$  Watt), the H<sub>2</sub>O<sub>2</sub> concentration in EBC increased to  $250 \pm 120$  nmol/l, and H<sub>2</sub>O<sub>2</sub> release significantly increased at maximal exercise to  $84.4 \pm 39.9$  pmol/min (P<0.01) (Figs. 1 and 2). Taking the theoretical water volumes of EBC into account the following maximal rates of release of hydrogen peroxide are calculated:  $79.5 \pm 46.8$  pmol/min at rest,  $301 \pm 143$  pmol/min (P<0.01) at maximal exercise.

### ACID-BASE PARAMETERS OF EBC

In Fig. 3 and Fig. 4 the pH and HCO<sub>3</sub><sup>-</sup>, respectively, of the EBC, which has been equilibrated with a humidified

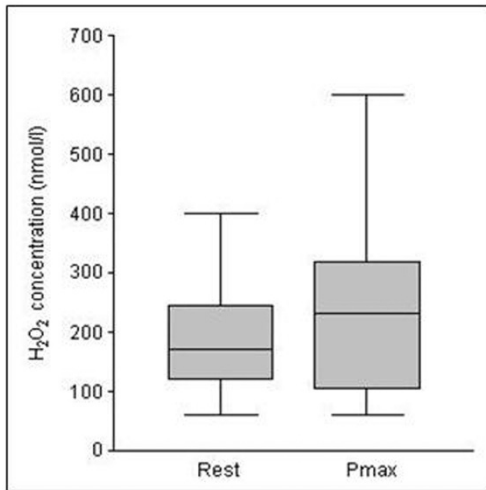


Fig. 1.  $H_2O_2$  concentration in exhaled breath condensate (EBC) (nmol/l EBC) at rest and after maximal load (Pmax).

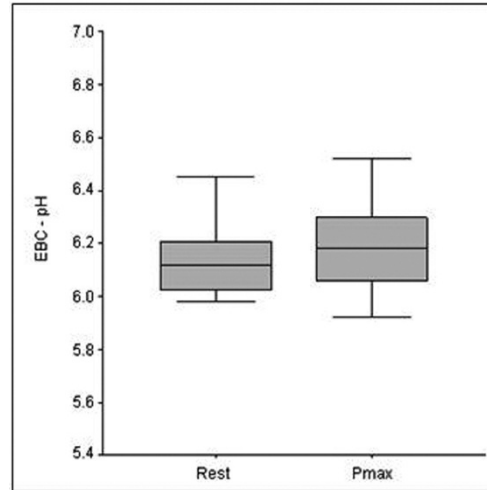


Fig. 3. pH of 5%  $CO_2$ -equilibrated exhaled breath condensate (EBC) at rest and after maximal load (Pmax).

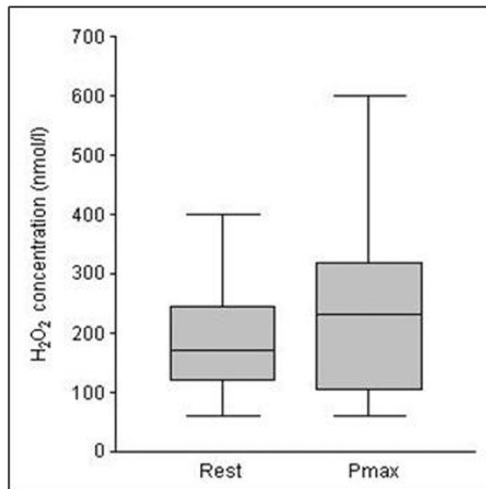


Fig. 2.  $H_2O_2$  release (pmol/min) in exhaled breath condensate (EBC) at rest and after maximal load (Pmax).

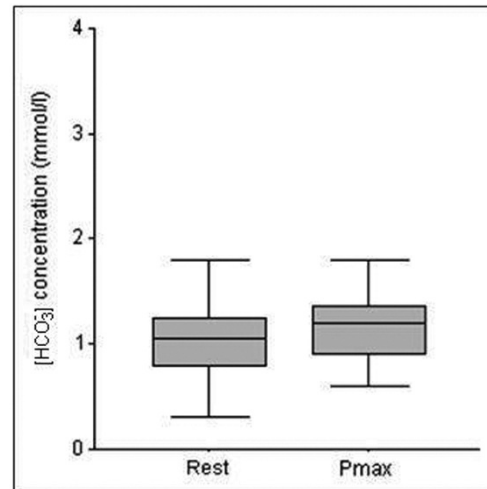


Fig. 4. Bicarbonate concentration (mmol/l EBC) in 5%  $CO_2$ -equilibrated exhaled breath condensate (EBC) at rest and after maximal load (Pmax).

Table 1. Mean values of respiratory and cardio-vascular parameters at rest and after exhausting exercise.

		VE (l/min)	VO <sub>2</sub> (l/min)	VCO <sub>2</sub> (l/min)	RER	VE/VO <sub>2</sub> (l/min)/lO <sub>2</sub>	VO <sub>2</sub> /kg (ml/min/kg)	HR (1/min)	O <sub>2</sub> Puls (ml O <sub>2</sub> /Hs)	External Load (Watt)
femal	Rest	8.71 ±1.76	0.306 ±0.071	0.240 ±0.068	0.80 ±0.16	29.73 ±8.80	5.2 ±1.5	81.6 ±7.6	3.80 ±1.01	
	P <sub>Max</sub>	97.74 ±10.69	2.710 ±0.211	3.129 ±0.295	1.15 ±0.05	36.11 ±3.42	44.9 ±3.3	186.3 ±9.5	14.59 ±1.45	200.2 ±23.6
male	Rest	13.64 ±2.63	0.461 ±0.137	0.381 ±0.108	0.83 ±0.11	32.59 ±13.63	6.10 ±0.75	75.7 ±7.6	6.15 ±1.90	
	P <sub>Max</sub>	122.66 ±21.27	3.756 ±0.467	4.300 ±0.506	1.14 ±0.07	32.91 ±5.68	49.6 ±6.6	179.6 ±6.3	20.95 ±2.77	240.7 ±24.9

gas mixture of 5%  $CO_2$  in oxygen are shown. At rest, the pH was  $6.08 \pm 0.23$ , the  $PCO_2$  was  $32.8 \pm 3.5$  mmHg, and the  $HCO_3^-$  was calculated at  $1.03 \pm 0.40$

mmol/l. After maximal exercise, we found pH  $6.18 \pm 0.17$ ,  $PCO_2$   $35.1 \pm 1.8$  mmHg, and  $[HCO_3^-]$   $1.13 \pm 0.40$  mmol/l. The results show that EBC is slightly

buffered by a small amount of  $\text{HCO}_3^-$ , which is almost not altered during exercise; no acids seem to be added to the EBC under this condition. The pH of EBC is found to be near the pK of the  $\text{CO}_2$ -bicarbonate system. After exercise, a small alkaline shift of 0.1 pH is found, indicating, that no acids are added during exercise.

## DISCUSSION

Release of  $\text{H}_2\text{O}_2$  in exhaled breath as a maker of oxidative stress and/or inflammatory processes increased during exhausting external load. We observed exercise induced alterations of blood pH and bicarbonate. pH and bicarbonate of the equilibrated EBC remained almost constant.

### $\text{H}_2\text{O}_2$ CONCENTRATIONS IN EBC

$\text{H}_2\text{O}_2$  in EBC is unstable and has to be analyzed directly after collection or should be frozen for later analysis [6]. In our investigation samples were stored in a cooling unit on ice cubes at  $-20^\circ\text{C}$ . Analysis was performed within 90 min after collection. So, no major loss of  $\text{H}_2\text{O}_2$  should be considered.

A number of studies reported from highly variable concentrations of  $\text{H}_2\text{O}_2$  for healthy adults ranging from  $\leq 50$  nmol/l EBC [7] through 250-300 nmol/l EBC [8, 9], up to 480 nmol/l [10]. One reason could be that the collection time is different from subject to subject. The higher the ventilation the greater is the dilution of the exhaled breath. Thus, it seems necessary to calculate the release of  $\text{H}_2\text{O}_2$  in both exhaled breath and EBC. A other reason could be that there is an intra-individual release of markers like  $\text{H}_2\text{O}_2$  in the EBC [11].

### $\text{H}_2\text{O}_2$ AS AN INFLAMMATORY MARKER OF LUNG DISEASES

In the respiratory tract,  $\text{H}_2\text{O}_2$  is released from neutrophils and eosinophils and from macrophages and epithelial cells [12] in inflammatory processes. Compared with healthy subjects, increased level of  $\text{H}_2\text{O}_2$  concentrations can be found in EBC from smokers [13], patients with bronchial asthma [14-17], COPD [14, 16, 18-21], bronchiectasis [8, 22] cystic fibrosis [23, 24], and ARDS [24, 25]. In induced sputum,  $\text{H}_2\text{O}_2$ -concentrations at moderate asthma correlated to the amount of eosinophiles and airway responsiveness [26].

$\text{H}_2\text{O}_2$  as an inflammatory marker is of special interest for time course of disease and the therapy control of asthma. Under inhaled steroid therapy the altered  $\text{H}_2\text{O}_2$  concentration in EBC reversal [31], exacerbations of COPD cause increased levels of  $\text{H}_2\text{O}_2$  compared to stable phases [18]. First investigations show, that anti-oxidative therapy with N-acetylcystein result in a decrease of  $[\text{H}_2\text{O}_2]$  in EBC [20].

In our investigation at resting conditions a mean value for the  $\text{H}_2\text{O}_2$  concentration of 190 nmol/l was measured in EBC. Individual values were ranging from 80 to 1330 nmol/l. It is unknown whether the highest values derive from a subject with a symptom free fresh infection or from a contamination with saliva. A pro-

cessing fault seems unlikely since the subject had the highest values also during exercise.

## OXIDATIVE STRESS

Physical exercise is characterised by an increase in reactive oxygen species (ROS) production [27]. The main sources of ROS during exercise are the mitochondrial respiratory chain, xanthine oxidase-catalyzed reaction, and neutrophils' activation. ROS are known to cause oxidative modifications of lipids, proteins and nucleic acids leading to cell and tissue damage [28].  $\text{H}_2\text{O}_2$  considered a ROS because of its capacity to cause ROS formation. Earlier studies have shown increased levels of oxidative stress associated with physical work [29], because  $\text{H}_2\text{O}_2$  correlates well with the oxygen consumption at resting conditions, as well as under moderate and exhausting exercise [30]. The higher the oxygen consumption the greater is the  $\text{H}_2\text{O}_2$ -release in EBC, because  $\text{H}_2\text{O}_2$  is produced after converting superoxide anions  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$ .

### NORMAL VALUES OF HEALTHY SUBJECTS

In young and healthy non-smokers, Nowak et al. [9] reported  $\text{H}_2\text{O}_2$  concentrations from 0.0 to 0.9  $\mu\text{mol/l}$ . Compared with young non smokers, the values were significantly increased in older healthy subjects and smokers [9]. The authors found a circadian rhythm in  $\text{H}_2\text{O}_2$  concentration with the highest values at 12:00 and 24:00 o'clock. We found  $\text{H}_2\text{O}_2$  concentrations of  $190 \pm 169$  nmol/l, which are in accordance with the literature (250-300 nmol/l) [8, 9]. At rest,  $\text{H}_2\text{O}_2$  release was  $31 \pm 18.3$  pmol/min and increased to  $84.4 \pm 39.9$  pmol/min at exhausting exercise. The results show a tendency of increased  $\text{H}_2\text{O}_2$  concentration (2.5 times) during exercise, but a significant increase in  $\text{H}_2\text{O}_2$  release during exercise ( $P < 0.05$ ), as a marker of oxidative stress in young and healthy subjects.

### pH OF EXHALED BREATH CONDENSATE

Acidification of the airways results in airway smooth muscle constriction [31], impaired ciliary motility [32], increases airway mucus viscosity [33], and damage of the airway epithelium [34]. These mechanisms are known to be involved in the development of airway inflammation. In patients with exacerbated asthma, COPD and bronchiectasis, the pH of EBC was essentially lower compared to normal conditions, and returned towards normal values after anti-inflammatory therapy and/or remission of exacerbations [35].

In healthy control subjects, Vaughan et al. [36] found a pH of  $7.7 \pm 0.49$  in argon deaerated EBC samples with intraweek and intraday coefficients of variation of 4.5% and 3.5%. The authors conclude that pH is mainly controlled by the lower airway source fluid. Kostikas et al. [35] reported a mean value of 7.56 for EBC pH of healthy subjects and lower values for patients with COPD (7.16) and bronchiectasis (7.11) moderate asthma (7.25). In steroid sensitive patients pH values normalized during therapy. In our investigation, we equilibrated the EBC solutions with 5%  $\text{CO}_2$ , conditions close to body fluids or epithelial



lining fluid. In CO<sub>2</sub> controlled EBC, mean pH at rest was 6.08, or 1.4 units lower compared with deaerated samples. After exhausting exercise, the mean pH was found at 6.18, which was not significantly different from resting values. In uncontrolled CO<sub>2</sub> conditions, pH of EBC, measured within 15 min after collection was found at 6.99 under resting conditions and at 6.90 after exercise.

#### EBC pH IN EXERCISE

In an earlier study Riediker et al. [37] had shown an increase of EBC pH after 60 min post exercise. The exercise change on the treadmill was moderate and corresponded to fast walking. The pH increased by 0.07, which was about one half to one fourth of the positive effect observed for inhaled corticosteroid therapy in asthmatics.

The pH of EBC is largely influenced by acids and bases which are released to maintain airway pH homeostasis of the airway extra cellular fluid. This fluid seems to be the source of the EBC the pH of which is in particular influenced by the CO<sub>2</sub> of the lung compartment and by other acids and bases swept away by the airflow [38]. In healthy subjects we found an EBC pH of about 6.1 (near the pK of the CO<sub>2</sub>-bicarbonate buffer system, which is equilibrated with a gas mixture containing 5% CO<sub>2</sub>, thus simulating the lung compartment in relation to CO<sub>2</sub>. However the bicarbonate concentration of EBC was found to be 1.1 mmol/l. The pH of EBC is largely influenced by acids and bases which are released to maintain airway pH homeostasis of the airway extracellular fluid. This fluid seems to be the source of the EBC the pH of which is in particular influenced by the CO<sub>2</sub> of the lung compartment and by other acids and bases swept away by the airflow [38].

Kullman et al. [5] could show that reproducibility of pH measurements in 5.3% CO<sub>2</sub> equilibrated EBC was 6 times higher compared to de aerated samples. pH in deaerated samples may be stable after 10 min of argon equilibration [6]; however these samples are not completely free from CO<sub>2</sub> and its influence on pH. From the physiological point of view, pH and acid base measurements should be performed under conditions as close to the normal conditions as possible, e.g., equilibrated with 5.3% CO<sub>2</sub>. In future, measurements of H<sub>2</sub>O<sub>2</sub> and acid-base parameters in EBC may become an effective tool for estimation of oxidative stress in sports and occupational medicine.

*Conflicts of interest:* No conflicts of interest were reported in relation to this article

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