Free Radical Research, September 2006; 40(9): 901–909

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healthcare

Free radicals in exhaled breath condensate in cystic fibrosis and healthy subjects

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Accepted by Professor F. Kelly

(Received 30 November 2005)

Abstract
Many markers of airway inflammation and oxidative stress can be measured non-invasively in exhaled breath condensate (EBC). However, no attempt has been made to directly detect free radicals using electron paramagnetic resonance (EPR) spectroscopy. Condensate was collected in 14 children with cystic fibrosis (CF) and seven healthy subjects. Free radicals were trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide. EPR spectra were recorded using a Bruker EMX® spectrometer. Secondly, to study the source of oxygen centered radical formation, catalase or hydrogen peroxide was added to the condensate. Radicals were detected in 18 out of 21 condensate samples. Analysis of spectra indicated that both oxygen and carbon centered radicals were trapped. Within-subject reproducibility was good in all but one subject. Quantitatively, there was a trend towards higher maximal peak heights of both oxygen and carbon centered radicals in the children with CF. Catalase completely suppressed the signals in condensate. Addition of hydrogen peroxide resulted in increased radical signal intensity. Detection of free radicals in EBC of children with CF and healthy subjects is feasible using EPR spectroscopy.

Keywords: *Cystic fibrosis, electron paramagnetic resonance, electron spin resonance, exhaled breath condensate, free radicals, reactive oxygen species*

Introduction

The collection of exhaled breath condensate (EBC) is a simple and non invasive technique to measure mediators of airway inflammation. EBC consists not only of water vapor, but also contains aerosolized respiratory fluid droplets released from the respiratory epithelial lining fluid. These fluid droplets contain traces of non-volatile solutes, which can be recovered in EBC samples [1]. EBC is collected by guiding and cooling exhaled air of a tidally breathing subject through a condenser system. The collection of EBC does not disturb the airways, in contrast to bronchial biopsy, bronchoalveolar lavage and induced sputum, and thus, can be obtained with minimal risk and inconvenience in both adults and children [2]. Many inflammatory mediators, such as 8-isoprostane, have been demonstrated in EBC of adults and children with chronic inflammatory lung diseases [3–12].

Oxidative stress appears to play an important role in several inflammatory lung diseases, including asthma

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760500522648

and cystic fibrosis (CF). Oxidative stress is defined as an imbalance between oxidants (reactive oxygen species, ROS, and reactive nitrogen species, RNS) and antioxidants (superoxide dismutase, catalase, glutathione, uric acid, lactoferrin, vitamin C, vitamin E) in favor of oxidants [13]. By virtue of their unpaired electrons, many ROS are unstable compounds, capable of initiating widespread oxidative events. Increased production of ROS has been directly linked to protein, DNA and lipid oxidation, which may cause direct lung injury or induce a variety of cellular responses, through the generation of secondary metabolic reactive species [14].

The measurement of F_2-isoprostanes is currently the most reliable approach to assess oxidative stress status *in vivo* [11]. In EBC, 8-isoprostane can be measured in both adults and children, although reproducibility remains controversial [11–12,15–16]. On the other hand, spin trapping is the only direct method for the detection of free radicals [14,17]. ROS and RNS can be detected by electron paramagnetic resonance (EPR) spectroscopy using various spin traps, e.g. 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) [14,18,19].

However, as the increased production of radicals appears to play an important role in inflammatory lung diseases, as far as we know, no attempt has been made to detect free radicals in EBC. The aim of this study was the detection of free radicals in EBC of children with CF using EPR spectroscopy.

Materials and methods

Study subjects

EBC was collected in 14 children with CF, routinely attending the CF centre of the Maastricht University Hospital, and seven healthy controls (including one adult). CF was defined as a positive quantitative sweat test in conjunction with chronic airway obstruction and/or recurrent infections of the airways and/or exocrine pancreatic insufficiency and maldigestion. Children with CF were clinically stable at the time of the assessment. Healthy subjects were asked to complete a standardized ISAAC questionnaire in order to exclude the presence of asthma or allergic disease [20]. The subject characteristics are shown in Table I. The study was approved by the medical ethical committee of the University of Maastricht.

Collection of exhaled breath condensate

Each subject was asked to exhale tidally, while wearing a nose clip, through a mouthpiece connected to a two-way non-rebreathing valve and a cooled (at 0°C) double-jacketed 50 cm long borosilicate glass tube as shown in Figure 1 and described previously [3,10]. In order to prevent salivary contamination of EBC samples, the two-way valve and tubing were used as a saliva gravity trap. Condensate was collected at the open end of the glass condenser, as shown in Figure 1, directly into vials containing spin trap (DMPO) (Table II). Thus, no resistance was created during exhalation. Each vial was wrapped in aluminum paper to protect DMPO against day light. The condensate collection continued until the minimal required volume of EBC was obtained necessary to perform one test (Table II). Samples were analyzed immediately. Four different test conditions were applied, as shown in Table II. Test 1–4 were performed to study DMPO-condensate samples under different volumes and concentrations of EBC and DMPO (Table II).

Table I. Subject characteristics.

	Cystic fibrosis	Healthy subjects
Number of subjects	14	7
Age (years)	14(6–17)*	13(9–25)*
Male/female ratio	9/5	4/3
Weight (kg)	38(18–60)*	44(37–85)*
Height (cm)	153(114–176)*	164(144–179)*
FEV_1 (%predicted)	66(31–108)*	–
Use of corticosteroids	4	0
Use of antibiotics	4	0

* Median and (range).
FEV_1 forced expiratory volume in 1 s.

Electron paramagnetic resonance spectroscopy

EPR spectra of the DMPO trapped radicals were recorded at room temperature using a Bruker

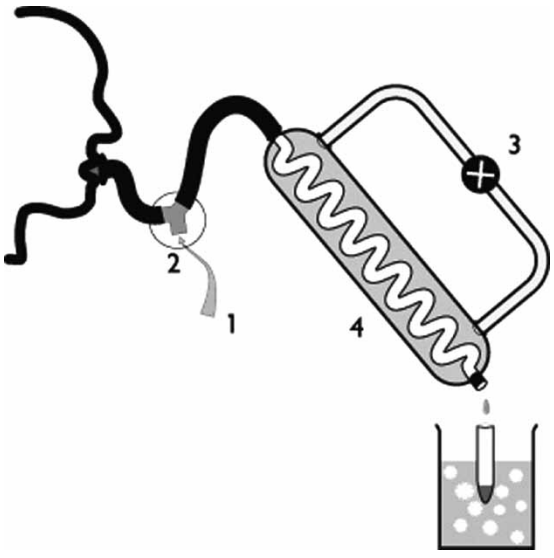

Figure 1. Schematic representation of the EBC collection device. The subject breaths tidally and inhales room air (1) through a two-way non-rebreathing valve (2). Exhaled air is guided by the tubing through a double-wall glass condenser (4), which is cooled by a counter-current circulating ice-water pump (3). The two-way non-rebreathing valve (2) and tubing also serve as a saliva trap.

Table II. Specific test conditions.

Test	CF	Healthy	EBC (ml)	DMPO (ml)
1	2	1	1	1 (50 mM)
2	4	–	0.5	0.5 (100 mM)
3	5	6	0.5	0.25 (100 mM)
4	3	–	0.3	0.25 (100 mM)

CF—number of CF patients; Healthy—number of healthy subjects; EBC—volume of collected EBC; DMPO—volume and (concentration) of spin trap (5,5-dimethyl-1-pyrroline *N*-oxide).

EMX® EPR spectrometer (Bruker Biospin GmbH, Karlsruhe, Germany). The experimental conditions were as follows: Klystron frequency 9.85 GHz, microwave power 30 mW, field modulation amplitude 2G at 100 kHz frequency, time constant 40.96 ms and 1.0×10^6 gain, and a sweep width of 60 G. Each sample was scanned 50 times. The signal of DMPO in H_2O was subtracted from the DMPO-condensate signal to obtain condensate-specific spectra (Figure 2).

Within-subject reproducibility of the EPR signals was assessed in five subjects by measuring EPR spectra in EBC collected with a 1 h interval. The results were expressed as coefficients of variation (CV = [SD/mean] × 100%).

In order to quantify the method, we assigned to each EPR signal an arbitrary unit derived from it's maximal peak height, as shown in Figure 3.

Additionally, test A and B were designed to study the source of oxygen centered radical formation. In test A, catalase (50 μl of a 5000 U/ml catalase solution) was added to the DMPO vials of test three. Catalase converts hydrogen peroxide in water and oxygen. Therefore, catalase was expected to reduce free radical signals derived from hydrogen peroxide. In test B, hydrogen peroxide (2 μl of a 9.7 M hydrogen peroxide solution) was added to the DMPO vials of test four. When hydrogen peroxide is considered to be the main source of free radical generation, hydrogen peroxide should amplify free radical signals.

Finally, saliva and sputum were investigated on the presence of free radical signals, in order to exclude free radical contamination by these biofluids.

Results

The median duration of EBC collection was 16.5 min (range 13–18 min). As shown in Table III, optimal capture of free radical adducts was obtained using 0.25–0.50 ml DMPO (100 mM) and 0.5 ml condensate. Free radical adduct signals were detected in 18

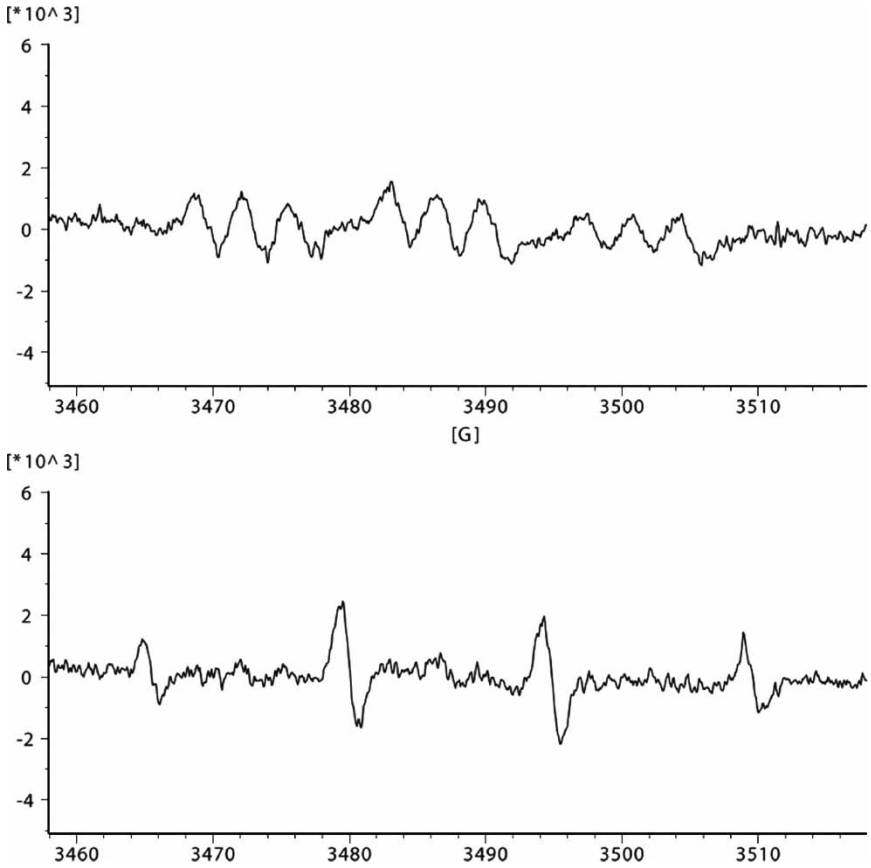

Figure 2. The DMPO-background signal and the condensate-specific radical adduct signal. *X*-axis: magnetic field strength (Gauss). *Y*-axis: intensity of the EPR signal. Upper part: signal of DMPO in water, or DMPO-background signal. Lower part: the condensate-specific radical adduct signal after subtraction of the DMPO-background signal.

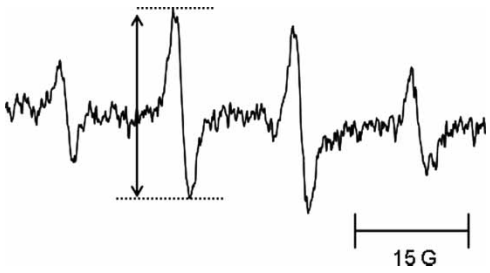

Figure 3. Quantification of EPR spectra in EBC by assessing maximal peak height in arbitrary units. *X*-axis: magnetic field strength (Gauss). *Y*-axis: intensity of the EPR signal.

out of 21 DMPO-condensate samples of children with CF and healthy controls (Table III).

Analysis of EPR spectra indicated that both oxygen and carbon centered radicals were trapped by DMPO, as shown in Figure 4A and B, respectively.

Within-subject reproducibility of EPR spectra in EBC was good in four subjects (coefficients of variation ranged from five to twelve percent) (Figure 5). However, in one subject the coefficient of variation was 87%.

Quantitative results expressed as maximal peak height (in arbitrary units) of both oxygen and carbon centered radicals in children with CF and healthy controls had a normal distribution and are shown in Figure 6. The maximal peak heights of oxygen as well as carbon centered radical adduct signals seemed higher in the CF group compared with the healthy controls. However, the difference in oxygen and carbon centered radicals failed to reach statistical significance ($p = 0.828$ and 0.253, respectively, student's *t*-test).

Catalase completely suppressed most oxygen centered radical adduct signals in EBC of children with CF (four out of five) and healthy controls (three out of six) (Figure 7A). On the other hand, addition of hydrogen peroxide resulted in a substantial increase of oxygen centered radical adduct signals in EBC of children with CF (two out of three) (Figure 7B).

Table III. Detection of oxygen (O) and carbon (C) centered radical adduct signals in EBC of children with CF and healthy controls.

	Cystic fibrosis			Healthy controls		
Test	O	C	O or C	O	C	O or C
1	1/2	0/2	1/2	0/1	0/1	0/1
2	1/4	3/4	4/4	–	–	–
3	5/5	3/5	5/5	6/6	4/6	6/6
4	2/3	0/3	2/3	–	–	–
Total			12/14			6/7

O—number of oxygen centered radical adduct signals to number of subjects; C—number of carbon centered radical adduct signals to number of subjects; O or C—number of free radical adduct signals to number of subjects; Total—total number of free radical adduct signals to total number of subjects.

Saliva contained little or no free radicals (three out of four samples were negative). It was not possible to detect free radicals in sputum ($n = 1$) due to its viscosity.

Discussion

The presence of oxidative stress in children with CF was assessed by direct measurement of free radicals in EBC using EPR spectroscopy. Oxygen as well as carbon centered radicals were trapped by DMPO in EBC in children with CF and healthy controls. Quantification demonstrated a difference that failed to reach statistical significance. Short-term reproducibility was good, except in one subject. Furthermore, most oxygen centered radical adduct signals were completely suppressed by catalase, while signal amplification by hydrogen peroxide was observed, indicating that hydrogen peroxide is a main source for the generation of free radicals in EBC.

Although within-subject reproducibility was good in four subjects, reproducibility was questionable in one subject, maybe as a consequence of physical exertion between the two tests.

Our quantitative approach using maximal peak height of an EPR signal in EBC did not show a statistical significant difference in oxygen or carbon centered radicals, between the CF children and healthy controls. However, there was a trend towards higher maximal peak heights of both oxygen and carbon centered radicals in the CF group, that may reach statistical significance in a larger study population.

Various indirect biomarkers of oxidative stress in condensate of subjects with chronic respiratory inflammation are known, as recently reviewed [3,14]. Currently, comparative reports on the direct measurement of free radicals in EBC are, to our knowledge, not available.

Oxidative stress appears to play an important role in several inflammatory lung diseases. It is defined as an imbalance between oxidants (ROS, RNS) and antioxidants (e.g. catalase) in favor of oxidants [13]. Chronic inflammatory lung diseases, such as asthma and CF, are characterized by the activation of epithelial cells and resident macrophages, and the recruitment and activation of neutrophils, eosinophils, monocytes and lymphocytes [14]. The activation of macrophages, neutrophils and eosinophils results in the generation of ROS [14]. Lung epithelial cells also release ROS, and when this occurs these substances stimulate inflammatory cells directly, thereby amplifying lung inflammatory and oxidant events [14,21].

By virtue of their unpaired electrons, many ROS are reactive compounds, that cause widespread oxidative events [14]. One such species, the superoxide anion, is rapidly reduced to hydrogen peroxide

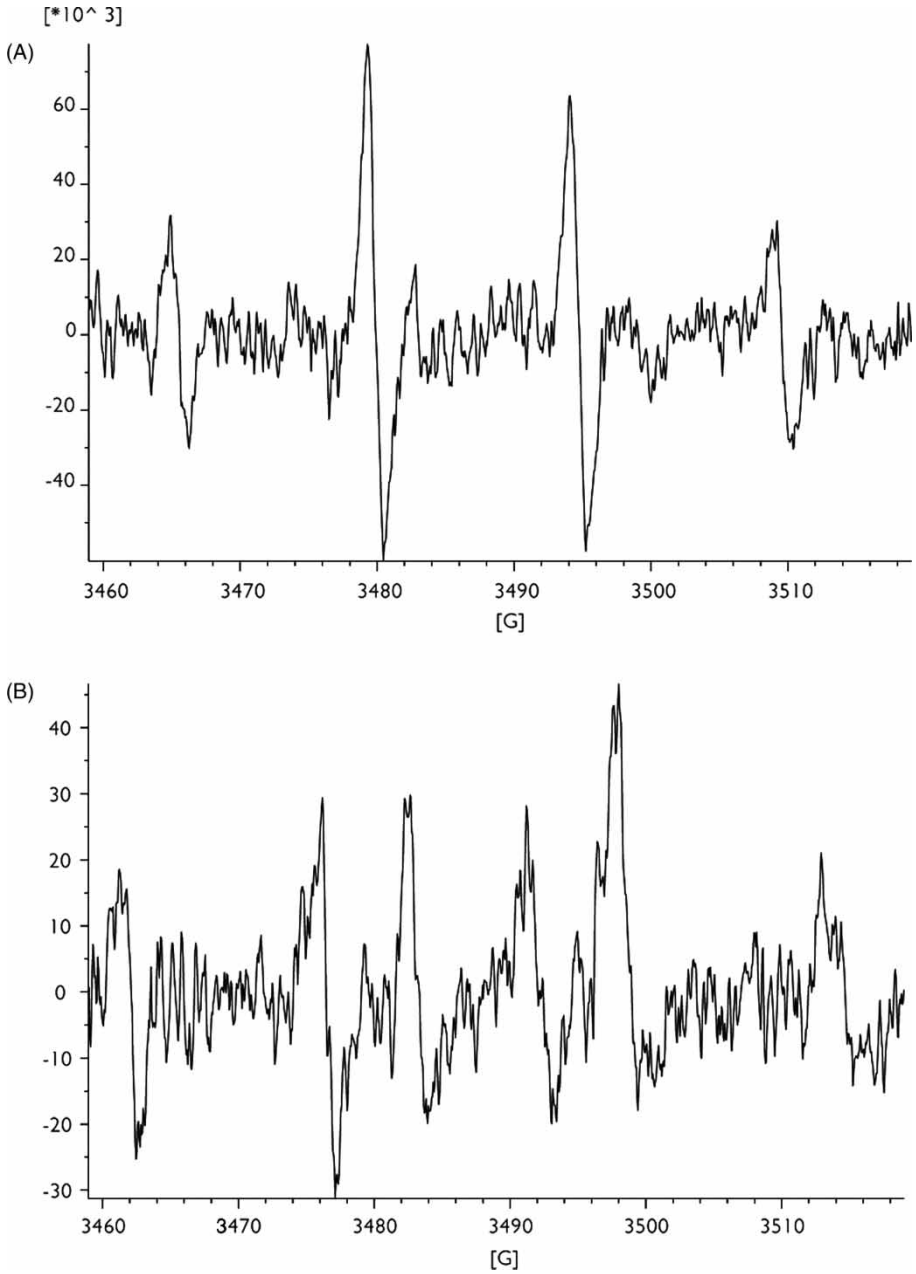

Figure 4. (A) Oxygen (1:2:2:1) centered radical adduct signal in the mixture of condensate and spin trap (DMPO). (B) Carbon (1:1:1:1:1:1) centered radical adduct signal in the mixture of condensate and spin trap (DMPO). *X*-axis: magnetic field strength (Gauss). *Y*-axis: intensity of the EPR signal.

by superoxide dismutase [14]. The combination of hydrogen peroxide with transition metal ions yields the hydroxyl radical [14]. ROS and RNS can also be generated intracellular from several sources, including mitochondrial respiration, the NADPH oxidase system and xanthine/xanthine oxidase, leading to cell necrosis or apoptosis [14]. When generated close to cell membranes, the ROS oxidize membrane phospholipids (lipid peroxidation) [14]. This initiates a chain reaction, which in turn leads to the generation and accumulation of lipid peroxidation products such as malondialdehyde, F_2-isoprostanes, 4-hydroxy-2-nonenal and acrolein [14]. The peroxidative breakdown of polyunsaturated fatty acids within the membrane impairs function, inactivates membrane-bound receptors and enzymes and increases tissue permeability and cell fragility [14]. In addition to their cytotoxic properties, lipid peroxides are increasingly recognized as being important in redox-sensitive signal transduction pathways, especially those activated during an inflammatory response [14,22,23].

To study the source of the formation of oxygen centered radicals, two additional tests (test A and B) were performed. Test A shows the absence of a radical adduct signal in the mixture condensate, catalase and

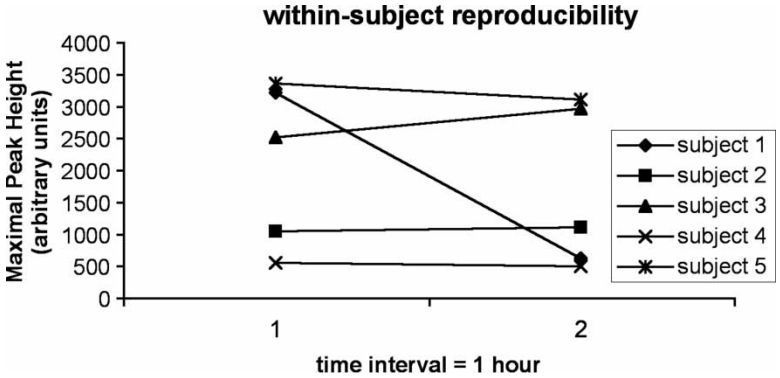

Figure 5. Within-subject reproducibility of EPR spectra in EBC. Within-subject reproducibility was assessed in five subjects by measuring EPR spectra in EBC that was collected twice with 1 h time interval. The coefficients of variation (%) were 87, 5, 12, 8 and 5%, respectively.

spin trap (in seven out of eleven samples), which suggests that hydrogen peroxide may play an important role in the generation of oxygen centered radicals measured in EBC after spin trapping. Increased concentrations of hydrogen peroxide in EBC have been demonstrated in patients with asthma and CF [3]. In sixteen children with CF, Jöbsis et al. reported increased hydrogen peroxide levels in EBC, and a significant decrease of these levels during intravenous antibiotic treatment [24]. Test B demonstrates an increased formation of most oxygen centered radicals (in two out of three samples), after addition of hydrogen peroxide. These amplified EPR signals suggest that EBC may contain compounds that enable the formation of radicals from hydrogen peroxide, such as transition metal ions and/or compounds that reduce these ions to their Fenton active states. Griese and co-workers found higher concentrations of zinc in EBC in CF patients, compared to healthy controls [25]. Zinc is known as a redox-active metal that may generate both hydrogen peroxide and hydroxyl radicals by Fenton reaction [26].

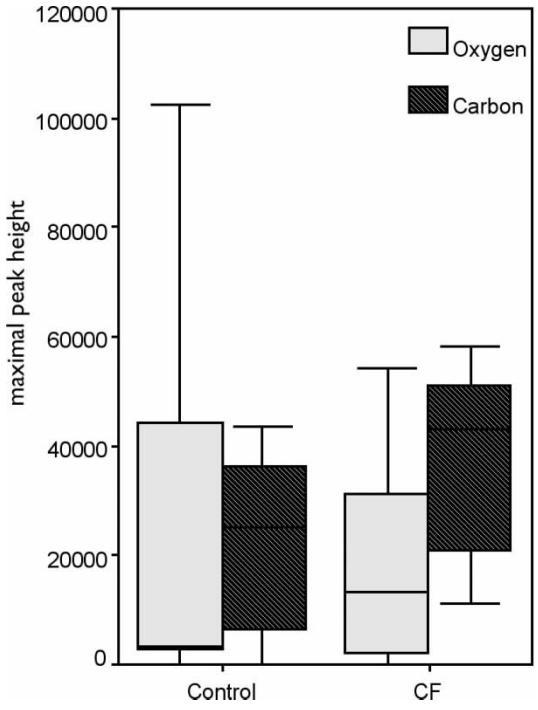

Figure 6. Quantitative results expressed as maximal peak height (in arbitrary units) of both oxygen and carbon centered radical adduct signals in EBC in children with CF and healthy controls. The maximal peak height of oxygen and carbon centered radical adduct signals did not differ significantly between the CF group and healthy controls ($p = 0.828$ and 0.253, respectively, *t*-test). Control: healthy control group. CF: children with CF. Oxygen: oxygen centered radical adduct signal. Carbon: carbon centered radical adduct signal.

Carbon centered radicals may originate from various sources, such as fatty acids, alkane, ethanol or carbohydrates. However, we are not able to exclude with certainty whether free radicals are formed *in situ* in the collection vial. A possible explanation for the presence of carbon centered radicals, in the absence of oxygen centered radicals, may be that carbon containing molecules react with hydroxyl radicals. By consequence, carbon containing molecules become carbon centered radicals and the former hydroxyl radical is no longer an oxygen centered radical. Similarly, hydroxyl radicals may be formed *in situ* from hydrogen peroxide in the presence of certain compounds such as transition metals. In order to reduce the risk of *in situ* free radical formation, one may develop a collecting device that allows direct contact between exhaled air and a DMPO containing filter. Spin trapping remains highly dependent on the spin trap used and the antioxidant status of the condensate [27]. Furthermore, the outcome of EPR results is not only influenced by the amount of free radicals trapped by constituent compounds of the EBC and the presence of some electrolyte ions, such as sodium and potassium, but also influenced by the spin trap-free radical interaction (each free radical has a specific affinity for a given spin trap) [27].

Finally, as most saliva samples were negative, free radicals in EBC do not likely result from contamination

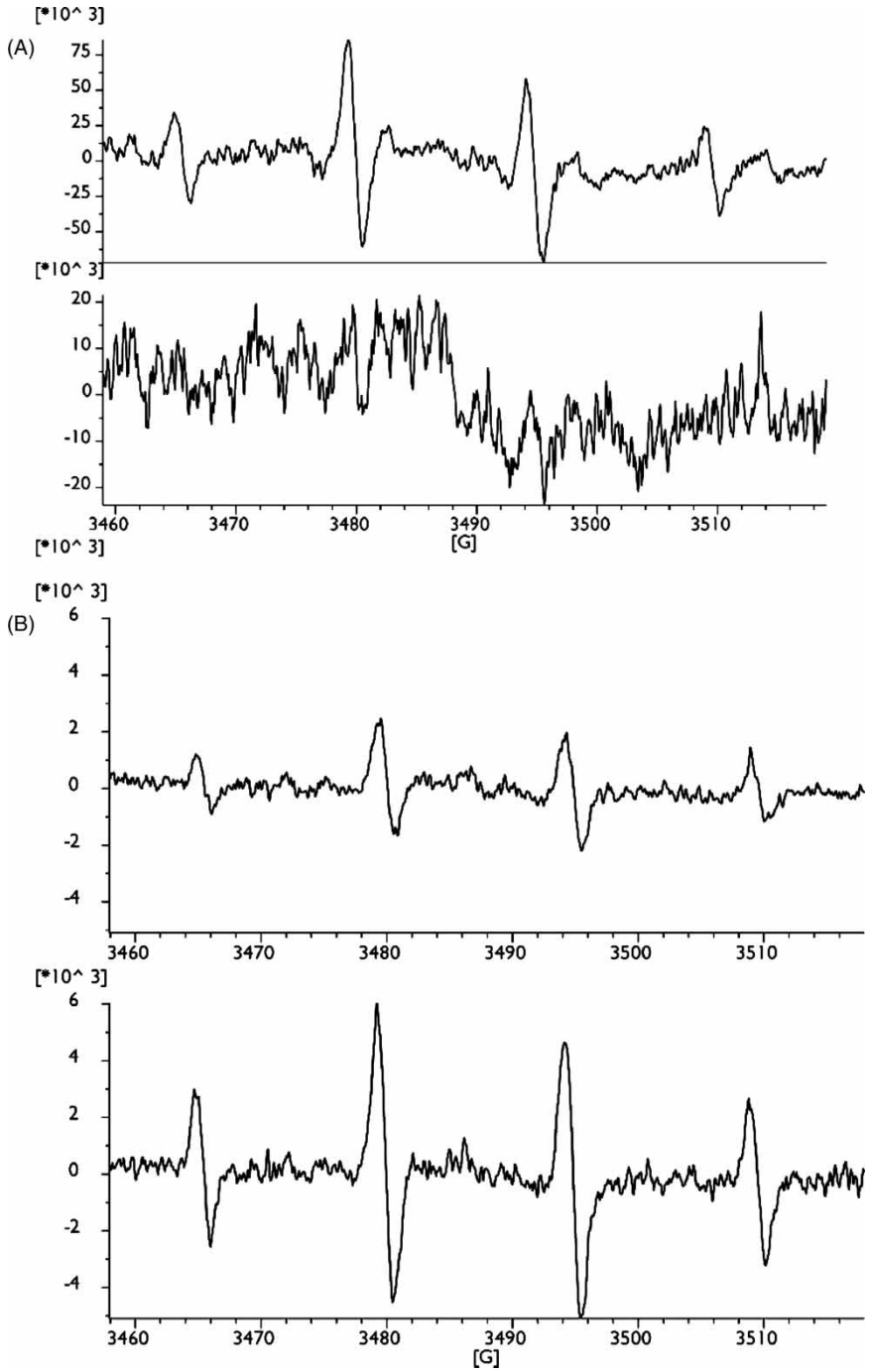

Figure 7. (A) Suppression of oxygen centered radical adduct signal in the mixture of condensate, spin trap (DMPO) and catalase. Upper part: oxygen centered radical adduct signal in the mixture of condensate and spin trap (DMPO). Lower part: addition of catalase results in suppression of the oxygen centered radical adduct signal. (B) Amplification of oxygen centered radical adduct signal in the mixture of condensate, spin trap (DMPO) and hydrogen peroxide. *X*-axis: magnetic field strength (Gauss). *Y*-axis: intensity of the EPR signal. Upper part: oxygen centered radical adduct signal in the mixture of condensate and spin trap (DMPO). Lower part: addition of hydrogen peroxide results in amplification of the oxygen centered radical adduct signal.

with this biofluid. This suggests that free radicals in EBC may originate from the lower respiratory tract, providing further support that EBC reflects the epithelial lining fluid.

Free radicals were also present in condensate of healthy subjects. This may not be surprising, considering that the lung normally exists in an oxygen rich environment which makes it susceptible to oxidative stress, and considering that EPR spectroscopy allows direct but semi-quantitative measurement of free radicals [14,28]. We did not find free radicals in three out of 21 condensate samples,

including one healthy control, one child with CF on longterm prednison treatment and one child with CF on antibiotics. Corticosteroids may have an antioxidant effect by decreasing the numbers as well as the oxidative and chemotactic responses of neutrophils, and antibiotics may have a certain role in reducing oxidative stress by reducing infection and thereby lung inflammation [29].

We aimed at assessing the potential use of EPR spectroscopy in the detection of free radicals in EBC in CF children and healthy subjects. Future studies should include a larger number of subjects and a wider range of disease severity, with repeated measurements of conventional markers and free radicals in EBC.

Other disturbing factors may include counteracting effects of exercise, dietary components, diurnal rhythm, duration of EBC collection and analysis, partial exposition of DMPO to daylight, environmental temperature and inhaled air particles of diverse origin [27,30,31].

As we report on a more direct and non-invasive assessment of oxidative stress, one may speculate on clinical implications. However, the future challenge is to define the source of free radical formation and to extend our understanding of the discriminative power of EPR spectroscopy in the detection of (exacerbations of) chronic lung disease, monitoring of disease severity, and disease progression.

We conclude that detection of free radicals in EBC of children with CF and healthy subjects is feasible using EPR spectroscopy. Furthermore, it is likely that hydrogen peroxide is crucial for the generation of oxygen centered radicals detected in EBC. Moreover, EBC may also contain compounds that enable radical formation.

Acknowledgements

The authors wish to thank the patients and healthy volunteers for their participation in this study.

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