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# Evaluation of $H_2O_2$ and pH in exhaled breath condensate samples: methodical and physiological aspects

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**Abstract**

This veterinary study is aimed at further standardization of $H_2O_2$ and pH measurements in exhaled breath condensate (EBC). Data obtained in the study provide valuable information for many mammalian species including humans, and may help to avoid general pitfalls in interpretation of EBC data. EBC was sampled via the 'ECoScreen' in healthy calves (body weight 63–98 kg). Serum samples and condensates of ambient (indoor) air were collected in parallel. In the study on $H_2O_2$, concentrations of $H_2O_2$ in EBC, blood and ambient air were determined with the biosensor system 'ECoCheck'. In EBC, the concentration of $H_2O_2$ was found to be dependent on food intake and increased significantly in the course of the day. Physiologically, lowest $H_2O_2$ concentrations at 06:00 varied within the range 138–624 nmol $l^{-1}$ EBC or 0.10–0.94 nmol per 100 l exhaled breath and individual concentrations were significantly different indicating a remarkable intersubject variability. Highly reproducible results were seen within each subject (three different days within 4 weeks). No correlation existed between $H_2O_2$ concentrations in EBC and blood, and EBC–$H_2O_2$ was not influenced by variables of spontaneous breathing. Further results confirmed that standardization of $H_2O_2$ measurements in EBC requires (1) the re-calculation of the concentration exhaled per 100 l exhaled breath (because the analyzed concentration in the liquid condensate underlies multiple methodological sources of variability given by the collection process), and (2) subtracting the concentration of inspired indoor $H_2O_2$. In the study on pH use of the ISFET electrode (Sentron, the Netherlands) and a blood gas analyzer ABL 550 (Radiometer, Denmark) led to comparable results for EBC–pH ($r = 0.89$, $R^2 = 79.3\%$, $p \leq 0.001$). Physiological pH data in non-degassed EBC samples varied between 5.3 and 6.5, and were not significantly different between subjects, but were significantly higher in the evening compared with the morning. EBC–pH was not dependent on variables of spontaneous breathing pattern or ambient conditions, and no significant correlation was found between serum and EBC for pH.

**Keywords:** *Exhaled breath condensate (EBC), hydrogen peroxide ($H_2O_2$), pH, respiratory physiology, calves*

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## Introduction

Early and non-invasive detection of inflammatory processes within the respiratory system of mammals is an important but also challenging diagnostic tool in both veterinary and human medicine for successful intervention and treatment of respiratory diseases. The exhaled breath condensate (EBC) is a medium of increasing interest for detecting or monitoring inflammatory processes within the respiratory system, and a variety of mediators measurable in EBC has been described as being influenced by the degree of several diseases of airways or lung parenchyma as well as by treatment modalities in humans and in animals (Scheideler et al. 1993, Kharitonov & Barnes 2002, 2006, Horváth et al. 2005, Reinhold et al. 2005). The use of exhaled breath condensate (EBC) analysis in animals has direct applications in animal studies and veterinary medicine, especially large animals models. It can also serve as a surrogate for humans because it offers the opportunity to use the same techniques as applied to humans in study designs that may not be appropriate, or are difficult to perform, or are less accepted in human subjects.

In EBC samples of human beings, increased concentrations of hydrogen peroxide ($H_2O_2$) have been reported to be markers of oxidative stress with respect to airway inflammation (asthma and chronic obstructive pulmonary disease (COPD)), bronchiectasis, lower and upper respiratory tract infections, community acquired pneumonia, idiopathic pulmonary fibrosis, in human tuberculosis, or in conditions of lung tissue toxicity (Antczak et al. 1997, Horvath et al. 1998, De Benedetto et al. 2000, Jobsis et al. 2001, Loukides et al. 2002, Svensson et al. 2004, Gerritsen et al. 2005, Caglieri et al. 2006, Psathakis et al. 2006, Kwiatkowska et al. 2007), and treatment protocols led successfully to decreasing $H_2O_2$ concentrations in EBC in patients with community acquired pneumonia (Majewska et al. 2004, Stolarek et al. 2006) or COPD (De Benedetto et al. 2005). In animal studies related to airway inflammation or airway sensitization, elevated $H_2O_2$ in EBC was found to be correlated with inflammatory cells obtained in the bronchoalveolar lavage fluid in horses and cats (Deaton et al. 2004, Kirschvink et al. 2005). In rats, sustained hypoxia as well as reoxygenation increased $H_2O_2$ formation in the lung (Hitka et al. 2003). Furthermore, the exhaled breath hydrogen peroxide concentration has been measured successfully in human and canine models of reperfusion lung injury (Wilson et al. 1993).

Furthermore, endogenous airway acidification, as assessed by the pH of exhaled breath condensates (EBC–pH) has been proposed to be used as a diagnostic tool for respiratory disorders as well (Vaughan et al. 2003, Walsh et al. 2006). In human patients, a reduced EBC–pH has been reported with respect to allergic upper and lower airway inflammation, chronic airway diseases, pneumonia, cystic fibrosis – especially when combined with infective exacerbations – acute lung injury, or mechanical stress during ventilation (Hunt et al. 2000, Kostikas et al. 2002, Tate et al. 2002, Gessner et al. 2003, Carpagnano et al. 2004, Niimi et al. 2004, Carraro et al. 2005, Ojoo et al. 2005, Brunetti et al. 2006, Prince et al. 2006, Profita et al. 2006). In calves undergoing an experimental respiratory infection with *Mycoplasma*, a decrease in the pH of EBC was seen after infection and has been interpreted as an early marker of subclinical pulmonary inflammation (Schroeder, 2006).

Comparing results of numerous studies related to measurements of either $H_2O_2$ or pH in EBC, a wide range of absolute data becomes evident, and often no correlations were found with other indicators of lung injury (Wilson et al. 1993, van Beurden et al.

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2003) indicating that standardization of both EBC collection and analytical tests applied to EBC samples are actually the biggest problems concerning incoherent results and their interpretations (Rahman & Biswas 2004, Horváth et al. 2005). The apparent simplicity of EBC collection must not be overstated, as numerous fundamental problems and pitfalls associated with analysis of samples of EBC have yet to be overcome (Effros et al. 2004, 2005, Rosias et al. 2004, Harrison & Andersen 2005, Montuschi 2005).

Consequently, the purpose of this study was to examine physiological and methodological influences on both the concentration of $H_2O_2$ and pH data in EBC. The veterinary study was performed in calves, but animals of this size may also serve as models for EBC collection in humans because sampling devices that are used in human medicine are directly applicable due to comparable body weights and volumes of ventilation (Reinhold et al. 2006). To clarify physiological aspects on results obtained for $H_2O_2$ and pH in EBC samples, attention was focused on (1) intrasubject reproducibility within 1 day and within 4 weeks, (2) intersubject variability of physiological data, (3) influence of food intake, and (4) influence of variables of ventilation. Possible correlations between EBC and the peripheral blood were examined. In addition to EBC and serum, $H_2O_2$ and pH were determined in ambient air to clarify methodological influences of the ambient background. Furthermore, two devices for pH measurement were compared for methodological reasons. Data obtained in this study are important for further standardization of $H_2O_2$ and pH measurements in EBC.

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## Animals, material and methods

### *Animals*

The individuals that had been used in the study were six conventionally reared calves (breed 'Holstein') aged 2–3 months. The body weight was $70 \pm 5$ kg at the beginning and $93 \pm 6$ kg at the end of the study (means $\pm$ SD). The calves were housed according to the guidelines for animal welfare in the European Union and were fed with commercially available milk substitutes and coarse meal. Water and hay were supplied *ad libitum*. Daily clinical control (food intake, rectal temperature respiratory rate, behaviour) confirmed that all calves were symptom-free (no fever, diarrhoea, or respiratory symptoms such as cough, nasal discharge or ocular secretions) and could be regarded as clinically healthy subjects over the complete period of the study. None of the calves was sedated, anaesthetized or euthanized.

All phases of the study were performed in a specialized veterinary institute (Federal Research Institute for Animal Health, Germany) under supervision of a veterinarian, and had ethical approval from the Institutional Commission for the Protection of Animals.

### *Collection of exhaled breath condensates*

For the collection of the EBC samples the commercially available system ECoScreen (Viasys Healthcare, Hoechberg, Germany) was used as described and validated for calves (Reinhold et al. 2006). First, the individual respired into a fitted facemask (Heiland, Germany) that was connected to a cooling trap. The trap inlay consisted mainly of a Teflon-coated condenser that cooled the expired air in a counter-flow

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principle. The gained condensate was collected in a fitted plastic vessel. On the inspiratory side, a PALL filter (PRO TEC; Pall Europe Ltd. Portsmouth, UK) had been attached to avoid any contamination such as bacteria and particulate matters. On the expiratory side, an electronic spirometer ('ECoVent', Viasys Healthcare) recorded the following ventilatory characteristics of spontaneous breathing during each EBC collection:

- time spent on EBC collection (min)
- respiratory rate (RR) ($min^{-1}$)
- maximal expiratory flow ($V'_{Emax}$) ($l\ s^{-1}$)
- expiratory tidal volume ($V_{tex}$) (l)
- expiratory minute ventilation ($V_E = V_{tex} \star RR$) (l)
- total exhaled volume ($V_{total} = V_E \star$ sampling duration in min) (l)

Expiratory tidal volume per kg body weight ($V_{tex}\ kg^{-1}$) and expiratory minute ventilation per kg body weight ($V_E\ kg^{-1}$) were calculated using the body weight measured before each EBC collection. EBC collection was finished after 400 l of exhaled air. In a few cases, when the animal was getting tired or worried, the collection was terminated earlier. Data describing the physiological conditions for EBC collection in this study are given in Table 1.

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*Collection of condensates of ambient air*

For ambient air condensate collection, a commercially available air pump was adapted to the ECoScreen. The pump on the vacuum side provided the ambient air to the cooling trap. A total ambient air volume of $1166 \pm 170$ l within a $60.7 \pm 1.2$ min time span was pulled through. The average flow was 19.2 l $min^{-1}$ and the volume of condensate obtained was $2.82 \pm 0.98$ ml (all means $\pm$ SD).

*Collection of blood and serum preparation*

Venous blood samples were obtained via catheterized vena jugularis dextra using serum 'MONOVETTE' (Sarstedt, Nuernbrecht, Germany). After collection, the blood sample was centrifuged (Labofuge 400R; Heraeus, Germany) for 15 min at 15°C and 3939 *g*. Rectal temperature of the animal was measured with a commercially available thermometer before each blood collection.

*Methodology of $H_2O_2$ measurement*

For each sample, $H_2O_2$ concentration was analyzed immediately after collection by means of the glucose-based biosensor system ('ECoCheck', FILT Inc., Berlin, Germany). This device consists of a disposable one-way biosensor based on a silver/silver chloride system. The key reaction is the reduction of hydrogen peroxide to water. The resulting current which is directly proportional to the existing $H_2O_2$ concentration in the sample is detected and amplified for analysis.

For the determination of the $H_2O_2$ concentration in condensates of exhaled breath and ambient air, a three-tube cassette was used. An equilibration solution, a $H_2O_2$ generating glucose/glucose oxidase standard solution and the sample to be analyzed were filled into the cassette. Afterwards, the measurement was started and high

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Table 1. Body weight, rectal temperature, and variables of ventilation during EBC collection in six healthy calves ($n = 18$)[a].

| | Median | Min | Max |
|---|---|---|---|
| Body weight (kg) | 81.1 | 62.6 | 98.2 |
| Rectal temperature (°C) | 39.0 | 38.2 | 39.8 |
| Respiratory rate (breaths $min^{-1}$) | 28.1 | 18.1 | 53.3 |
| Expiratory tidal volume ($V_{tex}$) (l) | 0.7 | 0.5 | 1.1 |
| $V_{tex}$ per kg body weight ($V_{tex}$/kg) (ml $kg^{-1}$) | 8.8 | 5.3 | 12.2 |
| Expiratory volume per minute ($V_E$) (l) | 20.0 | 9.4 | 34.2 |
| Maximal airflow during expiration ($V'_{Emax}$) (l/s) | 0.8 | 0.3 | 1.4 |
| Collection time for one EBC sample (min) | 20.0 | 12.0 | 46.0 |
| Volume of collected EBC (total) (ml) | 5.9 | 1.1 | 10.8 |
| Volume of EBC obtained per minute (ml $min^{-1}$) | 0.3 | 0.1 | 0.4 |
| Volume of EBC per 100 l exhaled breath (ml per 100 l) | 1.5 | 0.3 | 2.8 |

Min, minimum; max, maximum.
[a]Six calves, each examined three times (mean interval between two time points per animal $12 \pm 1$ days).

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accuracy pumps pumped the liquids from the three tubes to the sensor where horseradish peroxidase reduced $H_2O_2$ to water. Based on the measured current, the computer calculated the $H_2O_2$ concentration in the sample tube referring the current to analyzed glucose/glucose oxidase standard solution. Accuracy was $\pm 20$ nmol $l^{-1}$ in both exhaled breath condensate and ambient air condensate.

For $H_2O_2$ analyses in blood serum, the given method was modified. Distilled water (Milli-Q quality) was used for dilution (1:18) since serum pH is about 7.4 and ionic strength of serum samples had to be reduced to obtain conductivity of buffered breath samples. Analyses were performed in duplicate.

For methodological evaluation, the specificity of the $H_2O_2$ measurement in serum samples was investigated adding artificial $H_2O_2$ and catalyze (both Sigma Aldrich) to serum samples which led to an increase and decrease in the measured signal. Methods were further verified by comparing breath/air condensate samples and serum. A difference between both methods was investigated and eliminated by adding the mean difference ($129.5 \pm 38.4$ nmol $l^{-1}$; mean $\pm$ SD) to the values obtained from serum analysis.

For data analyses, datasets obtained in liquid condensates were normalized to 100 l air (exhaled or ambient) by re-calculation for two reasons. First, only this approach allowed a direct comparison between exhaled breath and ambient air concentrations. A direct comparison of $H_2O_2$ concentrations in liquid condensate would be inappropriate because 1.5 ml EBC derived from approximately 100 l exhaled air (Table 1) while approximately 1.5 ml of ambient air condensate derived from about 600 l ambient air (see Collection of condensates of ambient air). Second, the volume of EBC collected per volume exhaled breath cannot be regarded as constant in spontaneously breathing subjects resulting in different volumes of EBC condensed from 100 l exhaled volume (Reinhold et al. 2006).

### *Methodology of pH measurement*

In condensates of exhaled breath and ambient air, pH was measured immediately after finishing the collection process (without de-aeration) with two different commercially

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available devices in parallel: (1) ISFET electrode (Sentron, the Netherlands) and (2) blood gas analyzer (ABL 550, Radiometer, Copenhagen, Denmark).

The ISFET electrode, an ion sensitive field effect transistor, consists of two n-doped contacts, 'DRAIN' and 'SOURCE'. In addition, a third electrode, the 'GATE', is established. The GATE electrode is made sensitive to $H_3O^+$ ions by immobilizing an appropriate coating. The potential between the GATE and the reference electrode is influenced by the concentration of $H_3O^+$ ions, and is used for the pH measurement.

The ABL550 blood gas analyzer works with a standard glass electrode ($H_3O^+$ electrode G707) and a calomel reference electrode ($Hg_2Cl_2$ electrode K606). In contrast to the ISFET electrode, the pH is measured by relating the potential difference (between the outer membrane and the inner buffer solution) of the measuring electrode to the reference electrode with a constant potential.

Both devices were calibrated at the pH = 4.0 or pH = 7.0, respectively. Accuracies for both devices were pH $\pm$ 0.01. All pH data measured with ABL 550 (working at 37°C) were temperature corrected for both, (1) the actual body temperature of the individual animal measured immediately before each EBC collection and (2) the actual temperature of the condensate sample as measured by the ISFET electrode (in parallel to pH measurement). The latter was used for further analysis.

Blood pH was measured in heparinized venous blood samples using the blood gas analyser ABL725 (Radiometer), which is based on the glass electrode (E777; accuracy of pH $\pm$ 0.02). All pH data measured in blood samples were corrected for the actual body temperature as measured before blood collection.

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*Protocol to evaluate physiological concentrations, reproducibility and biological variability of $H_2O_2$ and pH in EBC and blood serum*

Each individual underwent three identical tests, each lasting from 06:00 to 20:00 per day and calf. The time difference between consecutive tests per subject was 12 $\pm$ 1 days (mean $\pm$ SD).

The following experimental protocol was used per day and animal. The calf to be examined was checked for its health and weighed. In the EBC collection room ('Room $_{EBC}$'; an empty animal room of the same size as the group's room) eight EBC collections were performed across the day at 2-h intervals starting at 06:00. Each EBC collection was followed by rectal temperature measurement and venous blood sampling. Between sample collections, the calf was bedded on straw in another empty animal room. Feeding times for milk substitutes were 07:00, 15:00 and 21:00. After finishing the daily study profile, the calf went back to the animals' group.

*Protocols to evaluate the concentration of $H_2O_2$ and pH data in ambient air*

While the ambient conditions in the animal house were kept constant during the study (ambient air temperature 23.0°C $\pm$ 0.3; ambient air humidity 70.0 $\pm$ 5.2%), different room conditions were examined with respect to the concentration of $H_2O_2$ and the pH of ambient air condensates using the following protocols.

First, ambient air condensates were sampled in an 'empty' room (no calf experiment inside) on four different days. To test both, the influence of different filters and the influence of the time of the day (morning, midday, and afternoon), nine condensates of room air were taken per day. For every part of the day there were three ambient air condensate collections, one collection per type of filter in a randomized order: (1)

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without any filter, (2) PALL filter (PRO TEC, Pall Europe Ltd. Portsmouth, UK), and (3) NO filter (MSA Auer, Combination filter 89 ABEK CO, NO, Hg/St).

Second, condensates of ambient air of the same room used for EBC collections of calves were obtained in between collections (10:45, 16:45 and 18:45) on four different days. In order to allow comparability to EBC samples, the same type of filter (PALL) was used for ambient air condensate collection.

### *Statistical analyses*

Box and whisker plots are used to present multiple data. The central box represents the 25% and 75% percentiles (i.e. 50% of data). Within the box, the line represents the median value while the cross symbolizes the average value (mean). Outliers are symbolized as separated squares. For statistical analysis of multiple data with normal distribution, the multifactor analysis of variance (ANOVA) was used. Statistically differences were identified by the multiple range test based on the least significant difference (LSD). The linear regression model ($y = a + bx$) was used to quantify linear correlations between different parameters. In addition, Spearman's rank correlation analysis was applied to data in order to identify possibly existing relationships based on ranked data instead of absolute data. For statistical significance, probability levels are given with the results. The probability of error ($p$) was defined as 5% which indicates a significant limit with $\leq 0.05$.

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## Results

### *Physiological concentration of $H_2O_2$ in EBC*

Taking the detection limit of $\geq 20$ nmol $l^{-1}$ for $H_2O_2$ measurements into account, $H_2O_2$ was measurable in 142 out of 144 exhaled breath condensates (i.e. 24 EBC samples per subject; six subjects) at concentrations varying between 24 and 2270 nmol $l^{-1}$ EBC. After calculating the concentration of $H_2O_2$ per 100 l exhaled breath, data ranged between 0.04 and 3.24 nmol per 100 l and were less variable. Intersubject variability was significant for both $H_2O_2$ per l EBC (data not shown) and $H_2O_2$ per 100 l exhaled breath (Figure 1A). The lowest individual concentration was $354 \pm 196$ nmol $l^{-1}$ EBC or $0.46 \pm 0.28$ nmol per 100 l exhaled breath (subject no. 3; mean $\pm$ SD), and the highest one was $960 \pm 453$ nmol $l^{-1}$ EBC or $1.43 \pm 0.69$ nmol per 100 l exhaled breath (subject no. 1).

Intrasubject variability was found to be significant in the course of the day (Figure 2A). Minimal concentrations were seen in non-fed individuals at 06:00 ($391 \pm 147$ nmol $l^{-1}$ EBC or $0.50 \pm 0.24$ nmol per 100 l exhaled breath; mean $\pm$ SD) while maxima of the day were measured at 20:00 ($846 \pm 508$ nmol $l^{-1}$ EBC or $1.26 \pm 0.81$ nmol per 100 l exhaled breath). A significant increase was regularly observed after morning feeding (08:00) indicating a significant influence of food intake. Expressed in percentages, the mean concentration of $H_2O_2$ in 100 l exhaled breath increased during the day as follows (baselines at 06:00 were set 100%): 208% at 08:00 and 10:00, 219% at 12:00, 225% at 14:00, 265% at 16:00, 292% at 18:00, 348% at 20:00.

No significant differences were seen between results of the three different days per calf indicating a good intrasubject reproducibility over at least 4 weeks (data not shown). No significant correlations were seen between the concentration of $H_2O_2$ in EBC and any variable of ventilation (breathing pattern).

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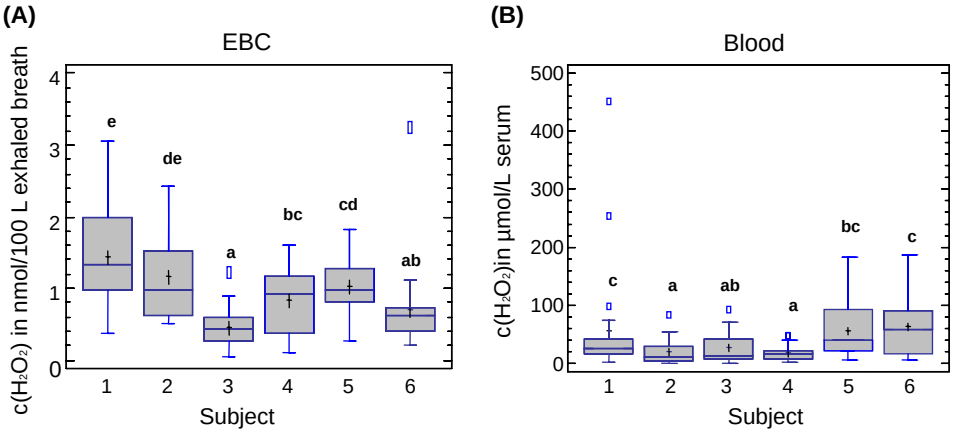


Figure 1. Interindividual variation of the concentration of $H_2O_2$ in 100 l exhaled breath as recalculated from the concentration in EBC samples (A) and in blood sera (B) obtained from six clinically healthy calves. Different letters indicate significant differences between subjects (ANOVA; LSD; $p \leq 0.05$); $n = 24$ measurements per subject (3 days per calf, eight time points per day).

### *Physiological concentration of $H_2O_2$ in blood*

Significantly different concentrations of $H_2O_2$ were measured in sera of different calves (Figure 1B). The lowest individual blood concentration was $17 \pm 13$ µmol $l^{-1}$ (subject no. 4; mean $\pm$ SD), and the highest individual concentration was $63 \pm 51$ µmol $l^{-1}$ (subject no. 6). Neither the time of the day nor the day of experiment had any significant influence on $H_2O_2$ in blood (data not shown). Across 18 different measurement days (each single day in each individual including always eight pairs of data), a significant linear correlation between the concentration of $H_2O_2$ in serum and in EBC was only seen on one single day in one calf ($r = 77$, $R^2 = 58.55\%$, $p \leq 0.05$).

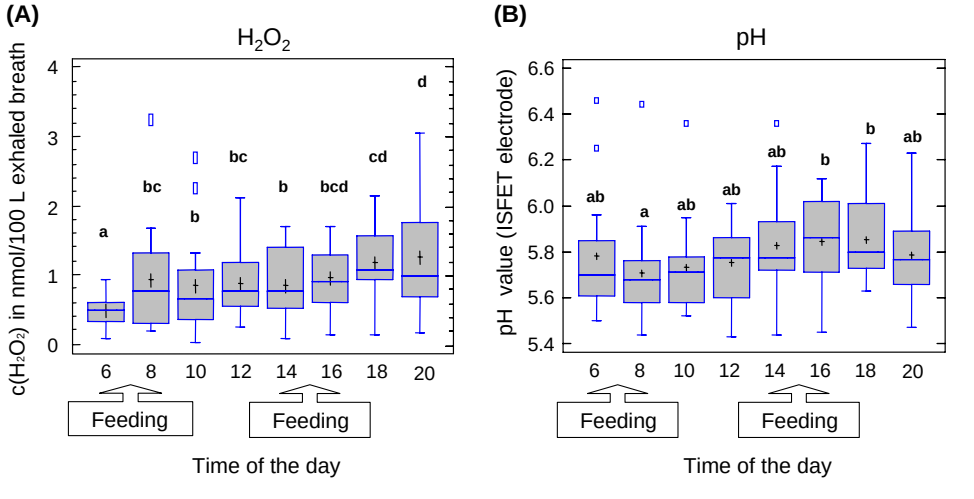


Figure 2. Concentration of $H_2O_2$ in 100 l exhaled breath as recalculated from the concentration in EBC samples (A) and pH data of EBC (B) in the course of the day as measured in clinically healthy calves. Different letters indicate significant differences between time points (ANOVA; LSD; $p \leq 0.05$); $n = 18$ measurements per time point (six calves, 3 days per calf).

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*Concentration of $H_2O_2$ in ambient air*

While highest ambient $H_2O_2$ concentrations were seen in unfiltered air condensates, significantly lower levels were detected depending on the different types of filters (Figure 3). Comparing four different days without using the room to be examined for calf experiments (Figure 3A), reproducible and not significantly different concentrations of $H_2O_2$ were detected when ambient air condensate was sampled without any filter or through the PALL filter. In contrast, $H_2O_2$ concentrations of air condensate rose significantly when the room air was sampled through the NO filter (from $0.025 \pm 0.025$ to $0.094 \pm 0.021$ nmol per 100 l ambient air; mean $\pm$ SD, $p \leq 0.05$).

A significant increase in the concentration of $H_2O_2$ was observed in the course of the day (Figure 3B). Depending on filtration type, remarkably higher $H_2O_2$ concentrations were measured in the afternoon compared with morning data as follows: unfiltered air – sixfold increase; PALL filter – 2.4-fold increase; NO filter – 2.0-fold increase ($p \leq 0.001$).

Figure 4 compares EBC–$H_2O_2$ concentrations with ambient $H_2O_2$ concentrations measured either in the room where EBC samples were collected from calves (Room $_{EBC}$) or in an empty room, and using the same type of filter *in vivo* and *in vitro*. Compared with EBC, the condensates of ambient air contained less $H_2O_2$. In the room where the animal experiments took place, 2.1 times higher $H_2O_2$ concentrations were measured in comparison to the 'empty' room at all time points.

*Methodological aspects of pH measurement in EBC*

Since EBC–pH was measured using two different means in parallel, first a methodological comparison was made between the results given by the ISFET electrode ($pH_1$) and those obtained by the ABL550 blood gas analyser ($pH_2$).

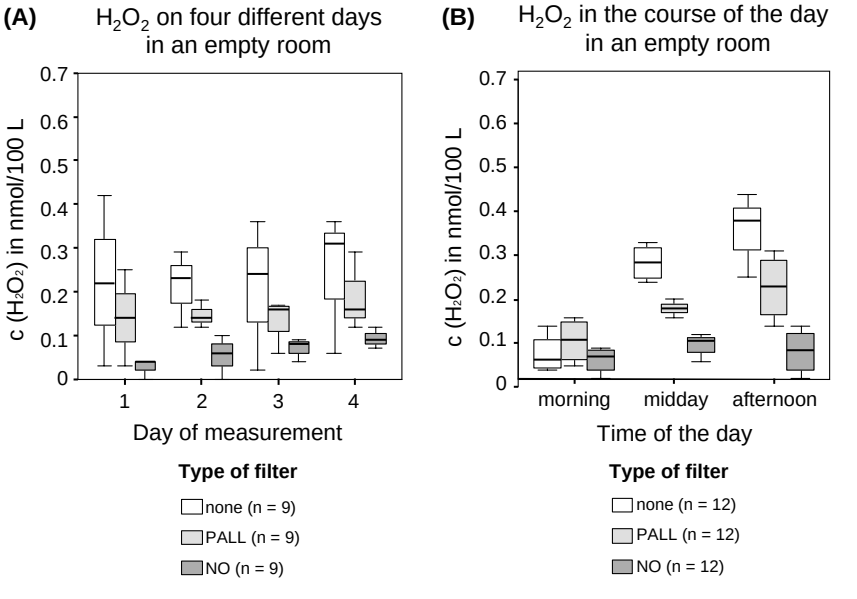


Figure 3. Concentrations of $H_2O_2$ in ambient air on four different days (A) and in the course of the day (B) as measured in an empty room.

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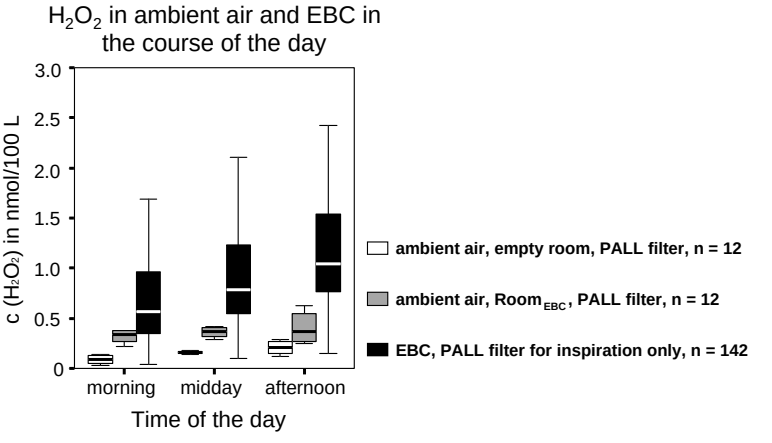


Figure 4. $H_2O_2$ concentration in ambient air in comparison to the calves' exhaled breath condensate (EBC) in the course of the day.

Including all EBC samples ($n = 144$) and correcting both pH data for the same temperature as measured in the EBC sample immediately after collection, the comparison of the two applied pH measurement methods revealed a significant linear correlation as shown in Figure 5.

*Physiological data of the pH in blood and EBC samples*

Blood–pH was found to be nearly constant ($7.37 \pm 0.03$; mean $\pm$ SD) in clinically healthy calves without being influenced by any factor taken into account (subject, day,

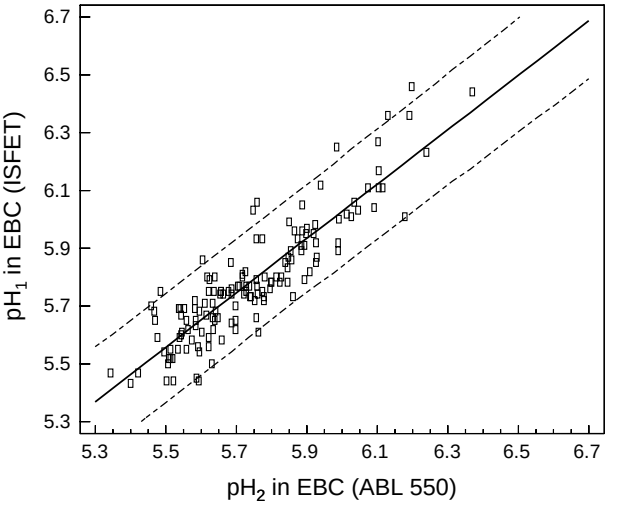


Figure 5. Linear correlation between the pH measured by means of the ISFET electrode ($pH_1$) and the pH measured with the blood gas analyzer ($pH_2$) in exhaled breath condensate samples, and both corrected for the temperature of the sample. ———, line of linear regression; ----------, 95% prediction limits for new observations. Equations of linear regression ($y = a + bx$): $pH_1 = 0.89 + 0.84\, pH_2$. Coefficient of linear correlation: $r = 0.89$. Coefficient of determination: $R^2 = 79.25\%$. Sample size: $n = 142$. Probability level: $p \leq 0.001$.

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time of the day). EBC–pH in non-degassed EBC samples was acid ranging either 5.43–6.46 (mean ± SD: 5.79 ± 0.21) as measured with the ISFET electrode, or 5.34–6.37 (mean ± SD: 5.74 ± 0.20) as measured with the blood gas analyzer. No significant differences were detected between subjects, and no significant correlation was found between EBC–pH and blood–pH.

EBC–pH was not dependent on any variables of ventilation or spontaneous breathing pattern, but was significantly higher at 16:00 and 18:00 compared with 08:00 (Figure 2B). Although there was no obvious tendency for increasing pH data in EBC samples within the 4-week period, data measured on the third day per calf provided significantly higher pH data in EBC ($p \leq 0.001$) compared with the two previous measurements (Figure 6).

### *Data of the pH in ambient air condensates*

Data of pH in ambient air condensates varied between 5.74 and 7.03 (median: 6.23, $n = 47$). No influence of either the day of measurement or the time of day was detected in both the 'empty' room and the 'Room $_{EBC}$' (data not shown). Ambient pH was not dependent on the usage of any filter.

### *Correlations between $H_2O_2$ concentrations and pH*

Analyzing all *in vivo* data using both linear regression model and Spearman's rank correlation analysis, no significant correlation was found between pH and the concentration of $H_2O_2$ in either blood or EBC samples. However, a positive linear relationship was found between pH in ambient air condensate and the concentration of $H_2O_2$ per 100 l ambient air ($r = 0.41$, $R^2 = 17.05$ %, $n = 47$, $p \leq 0.01$), whilst no

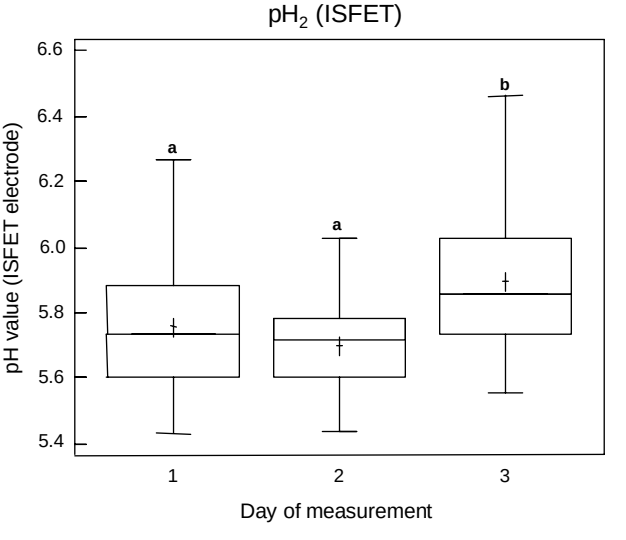


Figure 6. pH data in exhaled breath condensate samples (EBC) of clinically healthy calves on three different days. pH was measured in non-degassed EBC samples by the ISFET electrode, and immediately after finishing the collecting process. Time difference between days was 12 ± 1 day (mean ± SD). Different letters indicate significant differences between days (ANOVA; LSD; $p \leq 0.05$); $n = 48$ measurements per day (six calves, eight measurements per calf at 2-h intervals between 06:00 and 20:00).

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significant correlation was detectable between pH and the $H_2O_2$ concentration per l ambient air condensate.

## Discussion

Because a number of substances, including $H_2O_2$ and pH, have been measured in EBC without adequate published details of validation and optimization (Montuschi 2005, Brooks et al. 2006b) this study focused on the complexity of methodological and biological influences affecting an accurate assessment and interpretation of both EBC–$H_2O_2$ and EBC–pH. Calves were studied with direct application to veterinary medicine, but serving additionally as models because body weights and physiological ventilation data were comparable with adult humans. Since this animal study was controlled for uniformity in housing, diet, and other aspects of management, many issues related to experimental protocol for human subjects not housed under controlled conditions for weeks were less likely to confound the present study. In addition, a greater degree of homogeneity of the experimental group (same breed, age and gender) could be achieved than is possible for human studies removing potential confounding variables. Results of the study are relevant for different mammalian species including human patients demonstrating multiple methodological and biological interactions in EBC analyses as well as potential pitfalls with respect to both interpretation and further standardization of $H_2O_2$ and pH measurements in exhaled breath.

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### *Measurements of hydrogen peroxide*

In mammalian organisms, $H_2O_2$ is known for two contrasting effects (Goth 2006). Generated by physiological processes such as glycation, phagocytosis (as a result of unspecific immune response), or cell metabolism, low concentrations of $H_2O_2$ have physiological functions in degradation of some proteins, as a messenger in cell signalling, could contribute to apoptosis or may be involved in signal transduction processes (Weissmann et al. 2004, Goth 2006). Increased $H_2O_2$ production results from pathological changes or side-effects of some drugs, and higher concentrations are known to be toxic for mammalian organs. The enzyme catalase is the main regulator of $H_2O_2$ metabolism destroying toxic concentrations effectively without changing its low, physiological concentration. Other enzymes as glutathione peroxidase and haemoglobin play a limited role in these processes (Goth 2006).

Hydrogen peroxide found in exhaled breath condensate is thought to be produced physiologically by airway epithelia (Forteza et al. 2005). Enhanced exhalation of $H_2O_2$ may reflect activated phagocytes influx and free radical generation in the airways (Nowak et al. 2001). In this context, various cells including polymorphonuclear leukocytes, alveolar macrophages and type-II pneumocytes may be a source of exhaled $H_2O_2$, and increased concentrations in exhaled breath may either reflect elevated unspecific immune response (for example permanently occurring phagocytosis) or even pathological processes in the airway system. However, before applying $H_2O_2$ as a biomarker it is necessary to understand factors influencing $H_2O_2$ exhalation in healthy subjects (Nowak et al. 2001).

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*Concentration of hydrogen peroxide in exhaled breath condensate samples.* The commercially available equipment used in this study for $H_2O_2$ analysis is based on a biosensor technique. The detection limit of $\geq 20$ nmol $l^{-1}$ is comparable with detection limits of spectrofluorimetric or chemiluminescent methodologies (Zappacosta et al. 2001, Brooks et al. 2006b) and is much lower than those of spectrometric or colorimetric methods (showing a lack of sensitivity when applied to EBC samples; Van Hoydonck et al. 2004). A previous validation of the device 'ECoCheck' led to the result that the linearity of the biosensor went up to 4000 nmol $l^{-1}$ which is high enough to obtain results in clinical ranges (Gerritsen et al. 2005).

In the present study, concentrations of $H_2O_2$ in EBC in clinically healthy bovine subjects as obtained at 06:00 in the morning (i.e. lowest individual concentrations during the day) varied between 138 and 624 nmol $l^{-1}$. Concentrations below 1.0 μmol $l^{-1}$ EBC are comparable to data reported in healthy children (Griese et al. 2001), healthy cats (Kirschvink et al. 2005), healthy horses (Deaton et al. 2004), or spontaneously breathing rabbits (Weissmann et al. 2004). In contrast, higher individual mean concentrations of $H_2O_2$ in EBC (above 1.0 μmol $l^{-1}$) were found in healthy dogs (Wyse et al. 2004). In a number of studies performed in human beings, highly variable concentrations of $H_2O_2$ were reported for healthy adults ranging from $\leq 50.0$ nmol $l^{-1}$ EBC (Zappacosta et al. 2001, Gerritsen et al. 2005) via means of approximately 250–300 nmol $l^{-1}$ EBC (Nowak et al. 2001, Loukides et al. 2002, Szkudlarek et al. 2003) or 480 nmol $l^{-1}$ (Svensson et al. 2004), to concentrations above 1.0 μmol $l^{-1}$ EBC (Griese et al. 2001, Latzin et al. 2003).

*Biological and methodological influences on the variability of $H_2O_2$ in EBC.* Beside methodological aspects in $H_2O_2$ analyses, species-specific differences could be one reason for differences in data reported in different mammalian species; however, there is no report in literature to verify this hypothesis. Another potential source of differing data comparing animal studies and human studies may be contamination by nasal contribution. While EBC collection in animals does necessarily include nasal airflow, this is not the case in the majority of studies in humans. In a comparative study performed in humans, significantly higher concentrations of $H_2O_2$ were seen in orally collected EBC in comparison to nasally collected EBC (Latzin et al. 2003).

Mean concentrations of $H_2O_2$ in EBC were significantly different between individuals. Even minimal individual concentration averaged at 06:00 varied between 283 and 533 nmol $l^{-1}$ EBC (i.e. intersubject variability of 188%). Notable intersubject variability must be accepted as one factor contributing to biological variability among healthy subjects because it cannot be explained by a different health status or ambient conditions (all animals came from the same farm, were housed at the same time under identical conditions with respect to environment, ambient conditions and nutrition, and were free of any respiratory symptoms). Upper airway tract infections acting as confounders (Jobsis et al. 2001) or systemic diseases that have also been reported to increase the exhaled $H_2O_2$ concentration in humans (Luczynska et al. 2003) could be excluded because each animal was checked clinically on a daily basis. In agreement, a high intersubject variation of more than 300% has also been reported in healthy dogs (Wyse et al. 2004).

In the course of the day, significantly increasing concentrations of $H_2O_2$ were observed in exhaled breath condensate indicating the presence of a remarkable circadian variability. Exhaled $H_2O_2$ concentrations at night (20:00) were found to be

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approximately 350% higher compared with those in the early morning (06:00). This observation is in line with data reported in humans demonstrating a significantly increasing exhaled $H_2O_2$ concentration during the day (09:00, 12:00, and 15:00) in both healthy subjects and COPD patients (van Beurden et al. 2002). In another study including healthy humans, the $H_2O_2$ exhalation also revealed diurnal variation; however, the described pattern was different with two peaks (each $>400$ nmol $l^{-1}$) at 12:00 and 24:00, and lowest concentrations ($\leq 250$ nmol $l^{-1}$) at 08:00 and 20:00 (Nowak et al. 2001).

In addition to the general upward trend during the day, food intake was identified to have a significant influence leading to striking higher concentration of exhaled $H_2O_2$ as measured approximately 1 h after feeding (08:00 and 16:00). This effect has not been pointed out before in the literature, but might explain conflicting results of different within-day studies or even the non-significance of changes in exhaled $H_2O_2$ at different time points during the day as reported for healthy children (Griese et al. 2001) or healthy cats (Kirschvink et al. 2005).

Evaluating the day-to-day variability, three identical full-day studies were performed in each calf (each lasting 14 h per day) with a time difference between consecutive tests of $12 \pm 1$ days. The day of measurement had no significant influence indicating high within-subject reproducibility and no significant growth related changes within the 4-week period. Acceptable within-subject reproducibility has also been reported for humans within studies lasting 2–3 weeks (Jobsis et al. 2001, van Beurden et al. 2002) and for cats assessing the day-to-day variability (Kirschvink et al. 2005). In contrast, the $H_2O_2$ concentration in EBC samples of healthy 'never smoked' subjects decreased significantly within a 2-week observation, but was found to be stable in smokers (Nowak et al. 2001).

Whether the concentration of $H_2O_2$ is age-dependent is a point of controversial discussion in the literature. While $H_2O_2$ in EBC was found to be not age dependent in healthy children aged 4 weeks to 18 years (Griese et al. 2001), a positive correlation to age has been reported in 'never smoked' adult subjects (Nowak et al. 2001). In rapidly growing calves with increases in body weight of about 1 kg per day, no influence of growth or increasing body weight was seen in the short 4-week period taken into account in this study.

A study performed in humans breathing at different flow rates clearly demonstrated decreasing concentrations of $H_2O_2$ in EBC with increasing expiratory flow rates (Schleiss et al. 2000). Flow dependence suggests that the exhaled hydrogen peroxide mainly originates from conductive airways. Following this, one has to consider that a different breathing pattern during EBC collection might lead to different $H_2O_2$ concentrations in EBC for two reasons: (1) the flow dependence of $H_2O_2$, and (2) the dilution of EBC. The latter has been pointed out in a previous study demonstrating that deeper breaths (i.e. low dead space ventilation in relation to alveolar ventilation) led to relatively smaller EBC volumes whilst a higher dead space ventilation is correlated to higher EBC volumes, and consequently to a higher dilution factor of EBC (Reinhold et al. 2006). Although no significant correlation was seen between the concentration of $H_2O_2$ in EBC and spirometric data recorded while EBC was collected in the present study, the known influence of ventilatory characteristics should not be ignored. Consequently, the concentration of EBC–$H_2O_2$ was calculated per 100 l exhaled breath in addition to the concentration of $H_2O_2$ as analyzed per l EBC in order to eliminate breathing pattern dependence. This step of standardization

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has been introduced previously by our group for other components of EBC (Reinhold et al. 2006). As EBC collection cannot be strictly controlled in spontaneously breathing subjects, this procedure is recommended for further studies before comparing data.

*In vitro* data reporting that the quantitation of $H_2O_2$ in EBC is pH-dependent (Brooks et al. 2006b) and the positive correlation between $H_2O_2$ and pH we found in ambient air raise the questions (1) whether or not the concentration of $H_2O_2$ in EBC might be influenced by the pH of EBC *in vivo*, and (2) whether or not the quantitation of $H_2O_2$ in native EBC samples requires standardized conditions with respect to pH. Data obtained in this study do not imply a need for action because there was no significant correlation between $H_2O_2$ and pH in either EBC or blood within the biologically relevant ranges.

*Relationships between the concentrations of $H_2O_2$ in EBC and blood.* Despite exhaled $H_2O_2$ being considered as an indicator of lung inflammatory and oxidative stress in most of the literature, it is not fully elucidated to what extent $H_2O_2$ is being released from the intravascular compartment, and whether systemic $H_2O_2$ generation contributes to exhaled $H_2O_2$. In rabbits (exhaled air was collected via tracheal tube) the $H_2O_2$ concentration in EBC represented pulmonary $H_2O_2$ generation with a negligible contribution of the remaining body (Weissmann et al. 2004). In contrast, elevated exhaled $H_2O_2$ has been reported in patients with different systemic diseases (systemic sclerosis (Luczynska et al. 2003) or uraemia (Rysz et al. 2004)) suggesting involvement of reactive oxygen species in systemic disease processes. Furthermore, $H_2O_2$ exhalation in healthy 'never smoked' subjects was found to be dependent on the ability of blood phagocytes to generate reactive oxygen species (Szkudlarek et al. 2003).

In our study including eight pairs of data per day on 18 different measurement days (six animals; 3 days per animal), a significant linear correlation between exhaled concentration of $H_2O_2$ per 100 l and the concentration of $H_2O_2$ in the peripheral blood was only seen once for one individual. Consequently, the $H_2O_2$ concentration in EBC might be influenced by the $H_2O_2$ concentration in blood even in physiological conditions; however, more significant influences have to be taken into account that might obscure this relationship.

*$H_2O_2$ in ambient air.* To understand the impact of ambient conditions on the $H_2O_2$ level in EBC, the concentration of $H_2O_2$ in ambient air was examined. Comparing first the data as measured in the liquid ambient air condensate, the $H_2O_2$ concentration ranged from 92 to 1976 nmol $l^{-1}$, and this range showed a large overlapping with the range found for $H_2O_2$ concentrations in exhaled breath condensates (24–2270 nmol $l^{-1}$). Because different collection conditions (exhaled breath contains more water and is warmer compared with ambient air, different airflow rates exist in a living subject compared with a pump) might cause significant differences in the dilution factor of the condensate, both concentrations of $H_2O_2$ in exhaled breath condensates and those in ambient air condensates were recalculated in relation to 100 l exhaled or ambient air, respectively. Comparing these standardized concentrations, ambient $H_2O_2$ was found to be much lower (0.02–0.63 nmol per 100 l ambient air) than exhaled $H_2O_2$ (0.04–3.24 nmol per 100 l exhaled breath) which indicates that concentrations in the liquid condensate are clearly confounded by the

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collection process. This new insight may help to understand huge differences between data reported in literature for ambient air condensates collected with different methods (Latzin & Griese 2002, Kirschvink et al. 2005).

Although ambient air contained only concentrations below 1.0 nmol per 100 l (which corresponds on average to approximately 30% of the concentration in exhaled breath), an overlap still existed with respect to low concentrations in EBC leading to the conclusion that direct comparisons under identical conditions were absolutely essential in order to understand possible interactions between ambient air and exhaled breath.

From morning to afternoon, the concentration of ambient $H_2O_2$ increased significantly. This observation fits in with the literature reporting that the concentration of hydrogen peroxide in the atmospheric gas phase is strongly correlated with UV-radiation (and is therefore higher in summer than in winter) with a typical diurnal variation increasing from minima in the early morning to maxima in the late afternoon (Jacob et al. 1990, Dommen et al. 1995, Moeller et al. 2003). Comparing $H_2O_2$ concentrations in two different rooms with identical artificial ventilation rate (one was completely empty for weeks before collecting ambient air condensates; the other one was used day by day for EBC collection in one animal per day at 2-h intervals), the concentration of $H_2O_2$ was significantly higher in the room frequently having one animal and one or two persons inside. This finding indicates that, even at constant ambient temperature and humidity, the concentration of $H_2O_2$ in inspired air can be expected to be higher in intensively used rooms. Within each room, however, reproducible concentrations were found on different days showing the diurnal increase as described above. Following this, it can be assumed that the increase in the concentration of indoor $H_2O_2$ is a result of enrichment processes by both the environment and the individuals present in the room. Although it cannot be concluded from this study to what extent the present indoor $H_2O_2$ contributes to exhaled $H_2O_2$, the conclusion may be drawn that precise measures of exhaled $H_2O_2$ would required subtracting the concentration of inspired indoor $H_2O_2$ for each subject.

Another point of discussion is to what extent filters (that are used frequently in respiratory medicine) modify the concentration of $H_2O_2$ measured in exhaled breath condensate. In this study, the highest concentration of $H_2O_2$ was found in unfiltered ambient air while different types of filter were able to remove $H_2O_2$ with a different efficiency. Collecting ambient air through a PALL filter, the concentration of $H_2O_2$ in ambient air condensate was significantly reduced. Since this filter type has been routinely attached to the inspiratory valve of the EBC collection system used in calves (in order to avoid contamination of the respiratory tract by bacteria or particles present in the stable), one can retrospectively assume that the impact of ambient $H_2O_2$ on the exhaled $H_2O_2$ was reduced, too. The second filter tested in this study was related to nitric oxide (NO) because Latzin & Griese (2002) described a significant influence of environmental NO on $H_2O_2$ measurements in EBC of healthy children ($r = -0.50$; $p \leq 0.0001$). Collecting ambient air, the NO filter reduced the concentration of $H_2O_2$ in ambient air condensate most efficiently, but lost its power over the four measuring days (Figure 5A). For *in vivo* conditions, the NO filter used in this study appears unsuitable due to size and the compact inlet for NO retention that would increase breathing resistance.

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*Conclusions for $H_2O_2$.* Taking all information regarding methodological and biological influences on the concentration of $H_2O_2$ in EBC into account, the following conclusions arise:

- In order to avoid confounding effects of varying breathing pattern during EBC collection or EBC dilution, the concentration of exhaled $H_2O_2$ should be standardized per 100 l exhaled breath (i.e. re-calculation from EBC concentration per l condensate to 100 l exhaled breath).
- Within-subject comparisons (for example, pre-post comparisons or individual follow-up studies) might be much more sensitive compared with intersubject comparisons that might lack sensitivity due to the significant biologically caused intersubject variability.
- In addition, influences of food intake, and the time of the day should be carefully taken into account.

With respect to ambient conditions, the following conclusions have been drawn:

- Ambient concentration of $H_2O_2$ may result – at least in part – from subjects expiring in the room. Variable concentrations of $H_2O_2$ in the inspired ambient air may contribute to exhaled $H_2O_2$. Consequently, ambient $H_2O_2$ should be controlled.
- Doing this, the concentration in a given volume of liquid (i.e. condensate) cannot be regarded as an appropriate approach. It is recommended to evaluate the concentration of $H_2O_2$ in a volume of gas (exhaled breath, ambient air) and subtract the inhaled (i.e. ambient) concentration from the exhaled concentration.
- Inspiring through a filter system that does not add significant resistive loads to breathing but reduces $H_2O_2$ may help to increase the preciseness of exhaled $H_2O_2$ measurements.

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*Measurements of pH*

EBC–pH is a biomarker of ongoing discussion. It has been reported to be a robust and reproducible assay of airway acidity (Vaughan et al. 2003), and the phenomenon 'acidopnea' has been attributed mainly to airway inflammation but also to other respiratory disorders (Brunetti et al. 2006, Leung et al. 2006, Prince et al. 2006, Profita et al. 2006, Ratnawati et al. 2006, Walsh et al. 2006). Inhalation of aerosolized fine particulate matter contained in metal fumes was also associated with an acute increase in EBC–pH (Boyce et al. 2006). In contrast to both, there is a third category of studies unable to identify any significant difference between the EBC–pH in patients with inflammatory airway diseases compared to controls (MacGregor et al. 2005). According to Montuschi (2005), the lack of standardization of the EBC analysis is the primary limitation of this technique making comparisons of data obtained in different laboratories difficult.

*Methodological aspects of pH measurements.* Since different EBC–pH data may result from different measurement techniques, two methods of pH measurement were compared in this study. A blood gas analyzer (based on a glass electrode) and an ion sensitive field effect transistor (ISFET) electrode were successfully applied to EBC samples, leading to comparable and highly reproducible results, and can therefore this

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can be recommended. Blood gas analyzers are widely available in clinics and have the advantage of very small sample volumes required (95 µl in this study). An IFSET electrode, however, is transportable and robust and can therefore be easily used in field studies. EBC–pH measurements may also be dependent on the devices used for EBC collection (Leung et al. 2006), but this aspect was not taken into account in this study (all EBC samples were collected with one device).

Another methodological point of discussion is the dependence of EBC–pH on the concentration of $CO_2$ within the condensate, leading to the question of whether or not EBC samples should be de-aerated before pH measurements. Bubbling EBC samples with argon gas aims to standardize for carbon dioxide, and EBC–pH (originally acidic) has been reported to become alkaline and less variable after de-aeration (Vaughan et al. 2003, Borrill et al. 2005) with a normative interquartile range (25–75%) of pH 7.8–8.1 in healthy humans (Paget-Brown et al. 2006). A similar EBC–pH of 7.6–7.8 has been reported in clinically healthy dogs after argon de-aeration (Hirt et al. 2003). A recent study suggests measuring pH in EBC samples first de-aerated and afterwards $CO_2$ loaded to standardize $CO_2$ at 5.33 kPa (which should correspond to the physiological alveolar $CO_2$ partial pressure) in order to obtain most reproducible EBC–pH data (Kullmann et al. 2007). In the present study, however, EBC–pH was measured immediately after EBC collection in non-deaerated samples for the following reasons. Although there is no doubt that a decreasing concentration of $CO_2$ in the EBC sample increases its pH (especially during storage processes), $CO_2$ is not the only factor determining pH in a fresh EBC sample. This assumption is underlined by results of a previous interspecies comparison demonstrating that significant differences in partial pressure of EBC–$CO_2$ (which is significantly negatively dependent on the duration of collection period) were not associated with significant differences in EBC–pH (Reinhold et al. 2006). Furthermore, no significant correlation was found between EBC–pH and the peak alveolar $CO_2$ concentration (Tate et al. 2002). These findings suggest that acidic pH of fresh native EBC must be influenced by multiple factors beside $CO_2$. Indeed, several acid base equivalents including sodium, ammonia, potassium, lactate, acetate, bicarbonate, chloride, or different $H^+$-transport mechanism may contribute to EBC–pH (Hunt et al. 2002, Gessner et al. 2003, Inglis et al. 2003, Niimi et al. 2004, Carraro et al. 2005, Svensson et al. 2005, Effros et al. 2005, 2006), and the exact site of origin of many different substances influencing EBC–pH has yet to be defined. The possibility of salivary acidification influencing EBC–pH (Effros et al. 2006) could be excluded from this study because EBC collection in large animals depends on nose breathing and, therefore, does not include oral contamination.

Following this, non-deaerated samples reflect most likely the 'biological' pH of the airway surface, and pH measurements should be carefully undertaken immediately after collection of EBC in order to avoid any falsification of pH (either by an escape of volatile components or by ongoing biochemical processes). According to Leung et al. (2006), pH data measured immediately in non-deaerated EBC samples are recommended to obtain reproducible results with biological background.

*Biological aspects of pH in EBC.* EBC–pH was found to be completely independent of blood pH. While the individual mean pH in venous blood was found to be within the small physiological range of 7.33–7.38, EBC was acidic without any exception, and no correlation was found.

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EBC–pH data obtained in this study (range 5.3–6.5) are in agreement with EBC–pH data reported for healthy calves and pigs when native EBC samples were analyzed immediately after finishing the collection process (Reinhold et al. 2006). These findings also agree with means or medians reported for human EBC–pH being 6.1 to 6.2 in healthy adults (Ojoo et al. 2005, Bloemen et al. 2006, Prince et al. 2006). No significant intersubject variability was observed for EBC–pH in healthy animals either in the study presented here or in previous studies (Reinhold et al. 2006, Schroeder 2006). Furthermore, EBC–pH does not appear to be affected by age in either humans (Brooks et al. 2006a, Paget-Brown et al. 2006) or bovines (Schroeder 2006). Consequently, no systematic or significant change in EBC–pH was expected during this 4-week study despite the calves growing up rapidly. Nevertheless, the third day provided the highest average pH for unknown reasons, and this result was confirmed by both methods.

Interestingly, a significant circadian variation of EBC–pH was detected leading to increasing pH data in the course of the day – a phenomenon that has not been described in the literature before. At a first glance, this observation seems to be conflicting with observations published by Bloemen et al. (2006) who did not identify any significant difference between sampling times on the same day or on different days for EBC–pH in human subjects. However, because the increase in EBC–pH during the day was observed repeatedly for 3 days per animal, this effect must be considered as reproducible. The reason, however, could not be identified by this study, because no significant influence of feeding was seen on EBC–pH. Furthermore, EBC–pH was not found to be dependent on variables of ventilation determining the pattern of breathing which is in accordance with the literature (McCafferty et al. 2004).

*pH in ambient air.* Since the pH in ambient air condensate was determined as nearly constant, neither the time of day nor the day of measurement had a statistical impact. Furthermore, none of the applied filters changed the pH at any time. This suggests that there are no impact factors in the ambient air condensate that may change the pH. Consequently, changes in the pH of EBC are most likely not influenced by the pH of ambient air.

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## References

Antczak A, Nowak D, Shariati B, Krol M, Piasecka G, Kurmanowska Z. 1997. Increased hydrogen peroxide and thiobarbituric acid-reactive products in expired breath condensate of asthmatic patients. European Respiratory Journal 10:1235–1241.

Bloemen K, Lissens G, Desager K, Schoeters G. 2006. Determinants of variability of protein content, volume and pH of exhaled breath condensate. Respiratory Medicine 101:1331–1337.

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Borrill Z, Starkey C, Vestbo J, Singh D. 2005. Reproducibility of exhaled breath condensate pH in chronic obstructive pulmonary disease. European Respiratory Journal 25:269–274.

Boyce PD, Kim JY, Weissman DN, Hunt J, Christiani DC. 2006. pH increase observed in exhaled breath condensate from welding fume exposure. Journal of Occupational & Environmental Medicine 48:353–356.

Brooks SM, Haight RR, Gordon RL. 2006a. Age does not affect airway pH and ammonia as determined by exhaled breath measurements. Lung 184:195–200.

Brooks WM, Lash H, Kettle AJ, Epton MJ. 2006b. Optimising hydrogen peroxide measurement in exhaled breath condensate. Redox Report 11:78–84.

Brunetti L, Francavilla R, Tesse R, Strippoli A, Polimeno L, Loforese A, Miniello VL, Armenio L. 2006. Exhaled breath condensate pH measurement in children with asthma, allergic rhinitis and atopic dermatitis. Pediatric Allergy & Immunology 17:422–427.

Caglieri A, Goldoni M, Acampa O, Andreoli R, Vettori MV, Corradi M, Apostoli P, Mutti A. 2006. The effect of inhaled chromium on different exhaled breath condensate biomarkers among chrome-plating workers. Environmental Health Perspectives 114:542–546.

Carpagnano GE, Barnes PJ, Francis J, Wilson N, Bush A, Kharitonov SA. 2004. Breath condensate pH in children with cystic fibrosis and asthma: a new noninvasive marker of airway inflammation? Chest 125:2005–2010.

Carraro S, Folesani G, Corradi M, Zanconato S, Gaston B, Baraldi E. 2005. Acid-base equilibrium in exhaled breath condensate of allergic asthmatic children. Allergy 60:476–481.

Deaton CM, Marlin DJ, Smith NC, Smith KC, Newton RJ, Gower SM, Cade SM, Roberts CA, Harris PA, Schroter RC, Kelly FJ. 2004. Breath condensate hydrogen peroxide correlates with both airway cytology and epithelial lining fluid ascorbic acid concentration in the horse. Free Radical Research 38:201–208.

De Benedetto F, Aceto A, Dragani B, Spacone A, Formisano S, Cocco R, Sanguinetti CM. 2000. Validation of a new technique to assess exhaled hydrogen peroxide: results from normals and COPD patients. Monaldi Archives of Chest Disease 55:185–188.

De Benedetto F, Aceto A, Dragani B, Spacone A, Formisano S, Pela R, Donner CF, Sanguinetti CM. 2005. Long-term oral n-acetylcysteine reduces exhaled hydrogen peroxide in stable COPD. Pulmonary Pharmacology & Therapeutics 18:41–47.

Dommen J, Neftel A, Sigg A, Jacob DJ. 1995. Ozone and hydrogen peroxide during summer smog episodes over the Swiss Plateau: measurement and model simulations. Journal of Geophysical Research 100:8953–8966.

Effros RM, Dunning MB, Biller J, Shaker R. 2004. The promise and perils of exhaled breath condensates. American Journal of Physiology. Lung Cellular & Molecular Physiology 287:L1073–L1080.

Effros RM, Su J, Casaburi R, Shaker R, Biller J, Dunning M. 2005. Utility of exhaled breath condensates in chronic obstructive pulmonary disease: a critical review. Current Opinion in Pulmonary Medicine 11:135–139.

Effros RM, Casaburi R, Su J, Dunning M, Torday J, Biller J, Shaker R. 2006. The effects of volatile salivary acids and bases on exhaled breath condensate pH. American Journal of Respiratory & Critical Care Medicine 173: 386–392. [Comment in: American Journal of Respiratory & Critical Care Medicine 2006; 173:1047–1048].

Forteza R, Salathe M, Miot F, Forteza R, Conner GE. 2005. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. American Journal of Respiratory Cell & Molecular Biology 32:462–469.

Gerritsen WB, Zanen P, Bauwens AA, van den Bosch JM, Haas FJ. 2005. Validation of a new method to measure hydrogen peroxide in exhaled breath condensate. Respiratory Medicine 99:1132–1137.

Gessner C, Hammerschmidt S, Kuhn H, Seyfarth HA, Sack U, Engelmann L, Schauer J, Wirtz H. 2003. Exhaled breath condensate acidification in acute lung injury. Respiratory Medicine 97:1188–1194.

Goth L. 2006. The hydrogen peroxide paradox [Article in Hungarian]. Orvosi Hetilap 147:887–893.

Griese M, Latzin P, Beck J. 2001. A noninvasive method to collect nasally exhaled air condensate in humans of all ages. European Journal of Clinical Investigation 31:915–920.

Harrison CM, Andersen CC. 2005. Exhaled breath measures of inflammation: are they useful in neonatal chronic lung disease? Archives of Disease in Childhood. Fetal & Neonatal Edition 90:F6–F10.

Hirt RA, Gütl A, Delvaux F, Gustin P, Marlin D, Kirschvink N. 2003. Hydrogen peroxide and pH analysis in exhaled breath condensate of healthy dogs [abstract]. Journal of Veterinary Internal Medicine 17:746.

Hitka P, Vizek M, Wilhelm J. 2003. Hypoxia and reoxygenation increase H2O2 production in rats. Experimental Lung Research 29:585–592.

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RIGHTSLINK

Horvath I, Donnely LE, Kiss A, Kharitonov SA, Lim S, Chung KF, Barnes PJ. 1998. Combined use of exhaled hydrogen peroxide and nitric oxide in monitoring asthma. American Journal of Respiratory & Critical Care Medicine 158:1042–1046.

Horváth I, Hunt J, Barnes PJ., Alving K, Antczak A, Baraldi E, Becher G, van Beurden WJ, Corradi M, Dekhuijzen R, Dweik RA, Dwyer T, Effros R, Erzurum S, Gaston B, Gessner C, Greening A, Ho LP, Hohlfeld J, Jobsis Q, Laskowski D, Loukides S, Marlin D, Montuschi P, Olin AC, Redington AE, Reinhold P, van Rensen EL, Rubinstein I, Silkoff P, Toren K, Vass G, Vogelberg C, Wirtz H. ATS/ERS Task Force on Exhaled Breath Condensate. 2005. Exhaled breath condensate: methodological recommendations and unresolved questions. European Respiratory Journal 26:523–548.

Hunt J, Erwin E, Palmer L, Vaughan J, Malhotra N, Platts-Mills TAE, Gaston B. 2002. Expression and activity of pH-regulatory glutaminase in the human airway epithelium. American Journal of Respiratory & Critical Care Medicine 165:101–107.

Hunt JF, Fang K, Malik R, Snyder A, Malhotra N, Platts-Mills TAE, Gaston B. 2000. Endogenous airway acidification: implications for asthma pathophysiology. American Journal of Respiratory & Critical Care Medicine 161:694–699.

Inglis SK, Wilson SM, Olver RE. 2003. Secretion of acid and base equivalents by intact distal airways. American Journal of Physiology. Lung Cellular & Molecular Physiology 284:L855–L862.

Jacob P, Tavares TM, Rocha VC, Klockow D. 1990. Atmospheric $H_2O_2$ field measurements in a tropical environment: Bahia, Brazil. Atmospheric Environment 24A:377–382.

Jobsis RQ, Schellekens SL, Fakkel-Kroesbergen A, Raatgeep RH, de Jongste JC. 2001. Hydrogen peroxide in breath condensate during a common cold. Mediators of Inflammation 10:351–354.

Kharitonov SA, Barnes PJ. 2002. Biomarkers of some pulmonary diseases in exhaled breath. Biomarkers 7:1–32.

Kharitonov SA, Barnes PJ. 2006. Exhaled biomarkers. Chest 130:1541–1546.

Kirschvink N, Delvaux F, Leemans J, Clercx C, Marlin D, Sparkes A, Gustin P. 2005. Collection of exhaled breath condensate and analysis of hydrogen peroxide as potential marker of lower airway inflammation in cats. Veterinary Journal 169:385–396.

Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P, Loukides S. 2002. pH in exspired breath condensate of patients with inflammatory airway diseases. American Journal of Respiratory Cell & Molecular Biology 165:1364–1370.

Kullmann T, Barta I, Lazar Z, Szili B, Barat E, Valyon M, Kollai M, Horvath I. 2007. Exhaled breath condensate pH standardised for CO2 partial pressure. European Respiratory Journal 29:496–501.

Kwiatkowska S, Szkudlarek U, Luczynska M, Nowak D, Zieba M. 2007. Elevated exhalation of hydrogen peroxide and circulating IL-18 in patients with pulmonary tuberculosis. Respiratory Medicine 101:574–580.

Latzin P, Griese M. 2002. Exhaled hydrogen peroxide, nitrite and nitric oxide in healthy children: decrease of hydrogen peroxide by atmospheric nitric oxide. European Journal of Medical Research 7:353–358.

Latzin P, Beck J, Bartenstein A, Griese M. 2003. Comparison of exhaled breath condensate from nasal and oral collection. European Journal of Medical Research 8:505–510.

Leung TF, Li CY, Yung E, Liu EK, Lam CW, Wong GW. 2006. Clinical and technical factors affecting pH and other biomarkers in exhaled breath condensate. Pediatric Pulmonology 41:87–94.

Loukides S, Bouros D, Papatheodorou G, Lachanis S, Panagou P, Siafakas NM. 2002. Exhaled $H_2O_2$ in steady-state bronchiectasis: relationship with cellular composition in induced sputum, spirometry, and extent and severity of disease. Chest 121:81–87.

Luczynska M, Szkudlarek U, Dziankowska-Bartkowiak B, Waszczykowska E, Kasielski M, Sysa-Jedrzejowska A, Nowak D. 2003. Elevated exhalation of hydrogen peroxide in patients with systemic sclerosis. European Journal of Clinical Investigations 33:274–279.

Majewska E, Kasielski M, Luczynski R, Bartosz G, Bialasiewicz P, Nowak D. 2004. Elevated exhalation of hydrogen peroxide and thiobarbituric acid reactive substances in patients with community acquired pneumonia. Respiratory Medicine 98:669–676.

McCafferty JB, Bradshaw TA, Tate S, Greening AP, Innes JA. 2004. Effects of breathing pattern and inspired air conditions on breath condensate volume, pH, nitrite, and protein concentrations. Thorax 59:694–698.

MacGregor G, Ellis S, Andrews J, Imrie M, Innes A, Greening AP, Cunningham S. 2005. Breath condensate ammonium is lower in children with chronic asthma. European Respiratory Journal 26:271–276.

Moeller D, Biermann UM, Wieprecht W, Acker K, Kalass D, Hofmeister W, Tian-Kunze X. 2003. New insights into atmospheric hydrogen peroxide under polluted and remote influence. 14th International

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RIGHTSLINK

Conference on Air Quality – Assessment and Policy at Local, Regional and Global Scale, Dubrovnik, Croatia, 6–10 October 2003.

Montuschi P. 2005. Exhaled breath condensate analysis in patients with COPD. Clinica Chimica Acta 356:22–34.

Nowak D, Kalucka S, Bialasiewicz P, Krol M. 2001. Exhalation of H2O2 and thiobarbituric acid reactive substances (TBARs) by healthy subjects. Free Radical Biology & Medicine 30:178–186.

Niimi A, Nguyen LT, Usmani O, Mann B, Chung KF. 2004. Reduced pH and chloride levels in exhaled breath condensate of patients with chronic cough. Thorax 59:608–612.

Ojoo JC, Mulrennan SA, Kastelik JA, Morice AH, Redington AE. 2005. Exhaled breath condensate pH and exhaled nitric oxide in allergic asthma and in cystic fibrosis. Thorax 60:22–26.

Paget-Brown AO, Ngamtrakulpanit L, Smith A, Bunyan D, Hom S, Nguyen A, Hunt JF. 2006. Normative data for pH of exhaled breath condensate. Chest 129:426–430.

Psathakis K, Mermigkis D, Papatheodorou G, Loukides S, Panagou P, Polychronopoulos V, Siafakas NM, Bouros D. 2006. Exhaled markers of oxidative stress in idiopathic pulmonary fibrosis. European Journal of Clinical Investigations 36:362–367.

Prince P, Boulay ME, Boulet LP. 2006. A fast, simple, and inexpensive method to collect exhaled breath condensate for pH determination. Annals of Allergy & Asthma Immunology 97:622–627.

Profita M, La Grutta S, Carpagnano E, Riccobono L, Di Giorgi R, Bonanno A, Pace E, Bonsignore G, Bousquet J, Vignola AM, Gjomarkaj M. 2006. Noninvasive methods for the detection of upper and lower airway inflammation in atopic children. Journal of Allergy & Clinical Immunology 118:1068–1074.

Rahman I, Biswas SK. 2004. Non-invasive biomarkers of oxidative stress: reproducibility and methodological issues. Redox Reports 9:125–143.

Ratnawati R, Morton J, Henry RL, Thomas PS. 2006. Exhaled breath condensate nitrite/nitrate and pH in relation to pediatric asthma control and exhaled nitric oxide. Pediatric Pulmonology 41:929–936.

Reinhold P, Deaton C, Marlin D. 2005. Potential for and limitations of exhaled breath analysis in animal models. In: Amann A, Smith D, editors. Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring. Singapore: World Scientific Publishing Co Pte Ltd. p. 503–514.

Reinhold P, Jaeger J, Schroeder C. 2006. Evaluation of methodological and biological influences on the collection and composition of exhaled breath condensate. Biomarkers 11:118–142.

Rosias PP, Dompeling E, Hendriks HJ, Heijnens JW, Donckerwolcke RA, Jöbsis Q. 2004. Exhaled breath condensate in children: pearls and pitfalls. Pediatric Allergy & Immunology 15:4–19.

Rysz J, Kasielski M, Apanasiewicz J, Krol M, Woznicki A, Luciak M, Nowak D. 2004. Increased hydrogen peroxide in the exhaled breath of uraemic patients unaffected by haemodialysis. Nephrology Dialysis Transplantation 19:158–163.

Scheideler L, Manke H-G, Schwulera U, Inacker O, Hammerle H. 1993. Detection of nonvolatile macromolecules in breath. A possible diagnostical tool? American Review of Respiratory Disease 148:778–784.

Schleiss MB, Holz O, Behnke M, Richter K, Magnussen H, Jorres RA. 2000. The concentration of hydrogen peroxide in exhaled air depends on expiratory flow rate. European Respiratory Journal 16:1115–1118.

Schroeder C. 2006. Untersuchung zu Inhaltsstoffen des Atemkondensates beim Kalb. Dissertation. Freie Universität Berlin, Fachbereich Veterinärmedizin.

Stolarek RA, Kasielski M, Rysz J, Bialasiewicz P, Nowak D. 2006. Differential effect of cigarette smoking on hydrogen peroxide and thiobarbituric acid reactive substances exhaled. Monaldi Archives for Chest Disease 65:19–25. [Comment in: Monaldi Archives for Chest Disease 2006 65:3–4].

Svensson S, Isacsson AC, Ljungkvist G, Torén K, Olin AC. 2005. Optimization and validation of an ion chromatographic method for the simultaneous determination of sodium, ammonium and potassium in exhaled breath condensate. Journal of Chromatography B 814:173–177.

Svensson S, Olin AC, Larstad M, Ljungkvist G, Toren K. 2004. Determination of hydrogen peroxide in exhaled breath condensate by flow injection analysis with fluorescence detection. Journal of Chromatography B 809:199–203.

Szkudlarek U, Maria L, Kasielski M, Kaucka S, Nowak D. 2003. Exhaled hydrogen peroxide correlates with the release of reactive oxygen species by blood phagocytes in healthy subjects. Respiratory Medicine 97:718–725.

Tate S, MacGregor G, Davis M, Innes JA, Greening AP. 2002. Airways in cystic fibrosis are acidified: detection by exhaled breath. Thorax 57:926–929.

van Beurden WJC, Dekhuijzen PNR, Harff GA, Smeenk FWJM. 2002. Variability of exhaled hydrogen peroxide in stable COPD patients and matched healthy controls. Respiration 69:211–216.

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RIGHTSLINK

van Beurden WJ, Smeenk FW, Harff GA, Dekhuijzen PN. 2003. Markers of inflammation and oxidative stress during lower respiratory tract infections in COPD patients. Monaldi Archives for Chest Disease 59:273–280.

Van Hoydonck PG, Wuyts WA, Vanaudenaerde BM, Schouten EG, Dupont LJ, Temme EH. 2004. Quantitative analysis of 8-isoprostane and hydrogen peroxide in exhaled breath condensate. European Respiratory Journal 23:189–192. [Comment in: European Respiratory Journal 2004 23:183–184].

Vaughan J, Ngamtrakulpanit L, Pajewski TN, Turner R, Nguyen T, Smith A, Urban P, Hom S, Gaston B, Hunt J. 2003. Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. European Respiratory Journal 22:889–894.

Walsh BK, Mackey DJ, Pajewski T, Yu Y, Gaston BM, Hunt JF. 2006. Exhaled-breath condensate pH can be safely and continuously monitored in mechanically ventilated patients. Respiratory Care 51:1125–1131.

Weissmann N, Vogels H, Schermuly RT, Ghofrani HA, Hanze J, Fink L, Rose F, Seeger W, Grimminger F. 2004. Measurement of exhaled hydrogen peroxide from rabbit lungs. Biological Chemistry 385:259–264.

Wilson WC, Laborde PR, Benumof JL, Taylor R, Swetland JF. 1993. Reperfusion injury and exhaled hydrogen peroxide. Anesthesia & Analgesia 77:963–970.

Wyse CA, Hammond J, Arteaga A, Cumming D, Cooper JM, McEwan JD, Yam PS. 2004. Collection and analysis of exhaled breath condensate hydrogen peroxide in conscious healthy dogs. Veterinary Record 155:744–746.

Zappacosta B, Persichilli S, Mormile F, Minucci A, Russo A, Giardina B, De Sole P. 2001. A fast chemiluminescent method for H2O2 measurement in exhaled breath condensate. Clinica Chimica Acta 310:187–191.

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