Collection of exhaled breath and exhaled breath condensate in veterinary medicine. A review

Zollinger, E; Clauss, M; Steinmetz, H W; Hatt, J M
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Abstract

Collection of exhaled breath (EB) and exhaled breath condensate (EBC) is a noninvasive method for obtaining samples from the lower airways. While this technique has been well established for the diagnosis of lower respiratory tract diseases in human medicine, only a few studies have been performed in veterinary medicine. This article critically reviews the collection methods and parameter values measured in various animal species published to date and points out directions for further research.
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E. Zollinger, M. Clauss, H.W. Steinmetz and J.-M. Hatt*

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SUMMARY

Collection of exhaled breath (EB) and exhaled breath condensate (EBC) is a noninvasive method for obtaining samples from the lower airways. While this technique has been well established for the diagnosis of lower respiratory tract diseases in human medicine, only a few studies have been performed in veterinary medicine. This article critically reviews the collection methods and parameter values measured in various animal species published to date and points out directions for further research.

Keywords: Exhaled breath; Exhaled breath condensate; Hydrogen peroxide; Leukotriene B₄; Nitric oxide; Respiratory tract diseases; Review.

Abbreviations

EB Exhaled breath
EBC Exhaled breath condensate
COPD Chronic obstructive pulmonary disease
H₂O₂ Hydrogen peroxide
LTB₄ Leukotriene B₄
NO Nitric oxide

Introduction

In human medicine, analysis of exhaled breath (EB) and exhaled breath condensate (EBC) plays an important role in the diagnosis of many lung diseases including asthma, chronic obstructive pulmonary disease (COPD) and bronchiectasis (41). In contrast to the traditional methods of sampling secretions from the lower airways (e.g. sputum collection, sputum induction and bronchoscopy with bronchoalveolar lavage), EB and EBC analysis is noninvasive, simple to perform and may be repeated frequently, even in children. EB consists of a gaseous phase, which contains volatile compounds such as nitric oxide (NO) and carbon monoxide, and a water-vapor-saturated phase containing aerosol particles that can be condensed by breathing through a cooling system (59). EBC contains many non-volatile substances such as hydrogen peroxide (H₂O₂), eicosanoids, cytokines and electrolytes, and reflects the composition of the airway-lining fluid (64).

In veterinary medicine, analysis of EB and EBC as a noninvasive diagnostic tool to diagnose lung diseases is not yet established. Only few studies have been performed. While in EB exhaled NO is of particularly interest, leukotriene B₄ (LTB₄) and hydrogen peroxide (H₂O₂) were the parameters detected in EBC of different species like cats, dogs, calves and horses. In 2004, Wyse et al. (91) published a review about breath analysis in veterinary medicine. That article described the range of parameters that can be used to exploit EB and EBC for studying digestive and respiratory functions, but did not compare measurements with respect to the diagnostic value of EB and EBC analysis for diagnosing diseases of the lower respiratory tract. Therefore, the aim of this review is to critically compare the different collecting methods in animals and to show the discrepancies in the data presented in the literature to date.

Parameters in exhaled breath

Nitric oxide (NO)

The presence of endogenous NO in exhaled breath of animals and humans was first described in 1991 (25). In human medicine, most studies have focused on exhaled nitric oxid (NO), and abnormalities in exhaled NO have been documented in many lung diseases (40), particularly asthma (24,38). NO is derived from the semi-essential amino-acid L-arginin by the action of the enzyme nitric oxide synthase (NOS). NO in exhaled breath may originate from endogenous sources such as alveoli (epithelium or endothelium), airway epithelium, macrophages and other inflammatory cell types (7). External sources like ambient air, bacteria, NO produced in the stomach, nitrate, and nitrite may interfere with NO output from the lung (8). Different authors suggested that nasal and nasopa-
ryngeal NO concentrations in exhaled air were higher than those in the oral exhaled air. They concluded that in healthy subjects almost all NO in exhaled air originates from the upper airways, including paranasal sinuses with the highest levels reported (43,54), with only minor contribution from lower airways (23,55). Thus, it must be ensured that nasal NO does not contaminate the exhaled breath. Nose clips are used to prevent nasal exhalation (42,67) in humans, or the patients are trained to voluntarily close the soft palate while breathing through the mouth to isolate the nose and nasopharynx from the rest of respiratory tract (43). The same effect is achieved when subjects exhale against a resistance (39).

Two different methods were developed to measure exhaled NO: on-line and off-line measurement. In 1999 the American Thoracic Society (ATS) (1) and in 2005 the ATS together with European Respiratory Society (ERS) (2) published recommendations for standardized procedures for online and offline measurement of exhaled NO. It must be considered that different expiratory flow rates significantly alter the mean concentrations of NO obtained. Byrnes et al. (14) showed that an increase in expiratory flow rate results in a decreased concentration of NO.

NO concentrations in many lung diseases (40) and particularly in human asthma (26,27,31,36,60,67), COPD (56,61) and bronchiectasis (42) were determined. However, it is difficult to compare the results from different studies.

**Parameters in exhaled breath condensate**

**Hydrogen peroxide \( (H_2O_2) \)**

\( H_2O_2 \) is produced after converting the superoxide anions \( O_2^- \) to \( H_2O_2 \) by superoxide dismutase in several cell types (15,85). In the respiratory tract, \( H_2O_2 \) may be released from activated inflammatory cells such as neutrophils, macrophages and eosinophiles during a respiratory burst (4) as well as from epithelial cells (32). \( H_2O_2 \) in EBC is unstable. Therefore, EBC should be rapidly frozen after collection and kept at \( 70^\circ C \) until determination of its peroxide concentration (32). \( H_2O_2 \) was detected in EBC of healthy human subjects (31) and increased levels in patients with various lung diseases such as asthma (3,19,31,37), bronchiectasis (50,52) or COPD (18,65,84,47). The concentration of \( H_2O_2 \) varies from study to study. It depends on the expiratory flow rate (77) as well on the methods of measurement such as spectrofluorimetric assay (75), colorimetric assay (22) or fluorimetric assay (35).

**Leukotriene \( B_4 \)**

Leukotrienes are eicosanoids that are derived from phospholipids of the cell membrane.

\( LTB_4 \) may be released from macrophages, epithelial cells and activated neutrophils (11,33). Eosinophiles, which are absent from the airways of healthy subjects, migrate into the airways of asthmatic patients in large numbers and may synthesize \( LTB_4 \) too (60). \( LTB_4 \) is a classic chemottractant that activates neutrophiles (13,21,26,60,78) and can also cause eosinophilic chemotaxis (66). \( LTB_4 \) was found in EBC of healthy subjects and was increased in patients with asthma (26,45,60) or COPD (10,45,62).

**pH**

Airway homeostasis is maintained by a balance of different buffer systems and the production and release of acids and bases in the airways (32). The acidity (pH) of EBC can be easily measured using pH electrodes and indicator dyes (34,86). In healthy subjects, the pH of EBC tends to be unstable immediately after the end of collection. To enhance the stability of the readings, the EBC is de-aerated with a \( CO_2 \)-free gas such as argon. During this de-aeration, the pH gradually converges to a point at which a stable reading can be obtained (at this point, no further \( CO_2 \) can be removed by de-aeration). The mean pH after de-aeration of orally sampled EBC averaged 7.7 in healthy subjects, with a range of 7.4-8.8 (32).

Lowering of airway pH has been shown to cause bronchoconstriction (74), to impair the ciliary motility (30,53), to increase the airways mucus viscosity (29) and to damage airway epithelial cells (30). These mechanisms are important for the development of respiratory inflammation which plays a central role in development and progression of many lung diseases (46).

It has been reported that the pH in EBC decreased over two log orders in patients with asthma (34, 46), COPD (12,46) or bronchiectasis (46).

**Collection and analysis of exhaled breath and exhaled breath condensate in veterinary medicine**

Up to now, invasive methods such as bronchoalve-
olar lavage or lung biopsy were routinely used to analyse immune parameters or inflammatory mediators in the lung. In human medicine, the advantages of a noninvasive diagnostic investigation like collecting exhaled breath and exhaled breath condensate were demonstrated. Currently in veterinary medicine, various explorative studies have been carried out to find an noninvasive method to diagnose lung diseases.

NO in exhaled breath of different animal species

Primates

Rhesus monkeys
Schedin et al. (76) examined the NO concentrations in airway gas of three healthy, anaesthetised, spontaneously breathing Rhesus monkeys (Macaca mulatta). Gas was aspirated from one nostril, with a sampling flow rate of 0.02 L min⁻¹, to exclude room air on that side. NO concentrations were measured by chemiluminescence. In the three monkeys high and stable NO concentrations were recorded from the nose, which averaged 237±10 ppb, when sampling with a constant flow rate.

Baboons
High exhaled or nasal NO levels were found in humans, elephants and rhesus monkeys - mammals with paranasal sinuses. In a study with 17 healthy baboons (Papio hamadryas), which are the only mammals known to lack paranasal sinuses, Lewandowski et al. (49) measured the exhaled and nasal NO to determine whether significant nasal NO concentrations could be found in the absence of paranasal sinuses. For sampling EB, the baboons were breathing spontaneously synthetic air through a face mask. Gas was continuously sampled for 60 s via a thin plastic tube with a constant flow rate of 1.35 L min⁻¹ and analyzed online for NO concentration by chemiluminescence. To measure NO in the upper airways, a thin plastic tube was connected to a nasal olive that was gently introduced into the vestibulum of one nostril. Gas sampling and analysis were performed as described previously. The concentration of the exhaled NO was significantly lower than the nasal NO (1.00±0.59 ppb vs. 4.79±2.08 ppb). Compared to humans and other animals, the NO concentration in nasal sampling are very low and hardly detectable in exhaled gas. But when comparing the NO concentrations found in baboons with the NO levels found in the lower airways of tracheotomi-zed or intubated mammals with paranasal sinuses, including humans, values were similar. Hence, the authors conclude that the NO concentrations found in baboons derive mainly from the lower airways.

Rodents and rabbits
Endogenous NO was first demonstrated by chemiluminescence and mass spectrometry in 1991 in exhaled air of humans, rabbits (Oryctolagus cuniculus) and guinea pigs (Cavia aperea porcellus) (25). Rabbits and guinea pigs were anaesthetised, tracheotomized and ventilated by a ventilator with synthetic air. The expired air was sampled from an open-end reservoir tube immediately after the ventilator exhaust valve and the NO measured as described above. In rabbits, the NO concentration in the expired gas was 15±0.8 ppb. NO was measurable in guinea pigs too, but the results cannot be converted in the conventional unit (ppb).

In an other study (9), rabbits, guinea pigs and rats (Rattus norvegicus domesticus) were anaesthetised, tracheotomized and artificially ventilated with synthetic air. The exhaled NO was measured with a chemiluminescence analyser sampling at a flow rate of 0.1 L min⁻¹. In all animals tested, stable levels of exhaled NO were detected. Rabbits exhibited the highest concentrations (12.9±1.0 ppb), followed by guinea pigs (6.2 ± 0.7) and rats (0.9±0.01). In this study, the detected NO was derived from the lower airways due to the tracheotomy. There are striking differences among the species studied in NO-levels.

Weicker et al. (87) succeeded to measure exhaled NO from a single, spontaneously breathing mouse (Mus musculus) placed inside a Plexiglas chamber (volume: 85 ml). The chamber was perfused with synthetic air with different animal chamber flow rate. It was demonstrated that the optimal animal chamber flow rate was 0.06 L min⁻¹ to yield the greatest signal while the mouse remained comfortable. Thus, the exhaled NO was highly dependent on the animal chamber flow rate. The outflow from the chamber passed through a Nafion dryer ensuring that all samples had the same final humidity. The sample was then intermittently collected in a NO-inert Mylar balloon over 120 s and subsequently measured using a chemiluminescence-based NO analyzer.

The time of day had no effects on exhaled NO levels. Mean exhaled NO concentrations for 25
mice was $10.1 \pm 1.1$ ppb. When measured daily for 7 days, the exhaled NO concentration of six mice were in the same range.

Further, exhaled NO from six mice was measured before and 30 min after an intraperitoneal injection of ketamin/xylazine. Anesthesia significantly decreased exhaled NO concentration levels by $42 \pm 7\%$.

At last, a 21-gauge silastic catheter was inserted into the lower trachea of anaesthetised mice to determine the contribution of the upper airways to the exhaled NO signal. In sharp contrast to the high concentration of NO in the upper airways of humans, which may contribute significantly to exhaled NO, the upper airways contributed minimally to exhaled NO in the spontaneously breathing mouse.

In the literature, a number of studies tested the effects of specific drugs on NO production in different animal species like mouse, rat, guinea pig and rabbits (5,57,69,70,81,82)

**Carnivores**

**Weddell seals**

Stanek et al. (80) performed a study with five subadult male Weddell seals (*Leptonychotes weddellii*) to measure exhaled NO by chemiluminescence. The seals were brought into a ice hole and allowed to dive voluntarily. An acrylic plastic chamber was placed over the ice hole to capture exhaled gases from the seal surfacing between dives. They did not observe any increase of exhaled NO levels above ambient concentrations (1-2 ppb) during the recovery period between dives.

**Artiodactyls**

**Minipigs**

The NO concentration of the upper respiratory system of two anaesthetised, intubated and ventilated minipigs (*Sus scrofa domestica*) were investigated (76). NO concentrations in airway gas were measured by chemiluminescence. Sampling flow rate was 0.02 L min$^{-1}$. Gas was aspirated from one nostril, excluding room air on that side. In the two pigs nasal NO concentration were found to average $18.1 \pm 0.5$ ppb.

**Perissodactyls**

**Horses**

Mills et al. (58) examined the effects of exercise on the production rate of NO in exhaled air. Four horses (*Equus caballus*) were exercised on the treadmill at 3 speeds: walk, trot, canter. A vacuum pump was used to collect exhaled air through a polypropylene tube attached to the mask on the horse. The suction was regulated by a valve which had been calibrated for two flow rates. The exhaled air was collected into evacuated polypropylene bags and the NO concentration was measured within 10 min using a chemiluminescence analyser.

Walk, trot and canter were alternated. Exhaled air was collected for 1 min by suction. Each collection consisted of consecutive 30 s collections at two flow rates: 20 or 80 L min$^{-1}$ The exhaled NO concentration during trot was almost 50% lower than during canter (1.23 ± 0.14 ppb vs. 2.25 ± 0.32 ppb). At the higher flow rate at 80 L min$^{-1}$ the NO concentration of the exhaled air was significantly lower during both trot (1.23 ± 0.14 ppb vs. 0.78 ± 0.15 ppb) and canter (2.25 ± 0.32 ppb vs. 1.69 ± 0.31 ppb). This agrees with the observations in humans (14).

**Proboscids**

**Elephants**

Lewandowski et al. (48) analysed the NO concentrations in EB of four awake captive Asian elephants (*Elephas maximus*). Two different measurements techniques were used: 1) the syringe technique; and 2) the continuous registration technique.

The first method could be applied to all elephants. The tip of the 1 L calibration syringe with a two-way stopcock valve was cautiously introduced 5 cm into the elephants trunk und 1 L samples were drawn when the animal exhaled through its trunk. The samples were immediately transferred into a 15 L non-diffusing metallic gas collection bag, which was also closed with a two-way stopcock valve. After 3 L were collected in the bag in less then 3 min, the gas collected in the bag was immediately analysed for NO using chemiluminescence analyser.

Two elephants were co-operative for the continuous recording of exhaled NO. A thin plastic tube was cautiously introduced 20 cm into the elephants trunk. Respiratory gas was continuously aspirated through the tube with a sample flow rate of 1.35 L min$^{-1}$ and on-line analysed for NO concentration by chemiluminescence.

NO in exhaled gas of four asian elephants sampled with syringe technique was 4.99 - 26.45 ppb, but the samples seemed to have been diluted by
ambient air. The NO concentration at the continuous on-line measurement of NO concentrations varied with in- and expiration of the elephants: lower concentrations were found during inspiration phases. During expiration the NO concentration was in the range of the syringe technique.

The published data on NO in veterinary medicine are summarized in Table 1.

**Evaluation of NO**

When comparing detected NO concentrations derived from airways, it is important to know the source of measured NO and to distinguish between nasal, tracheal and exhaled NO. Nasal NO represents the NO concentration in the upper, tracheal NO that in the lower airways and exhaled NO, sampled by face mask, reflects the NO concentration of entire respiratory system and thus, it is not valid to compare the different methods. Lewandowski et al. (49) showed in their study that the nasal and exhaled NO concentration in baboons were lower than in mammals with paranasal sinus. This suggests, that the paranasal sinus (anatomic dead space) might be an anatomic requirement for production of relevant nasal NO concentrations, i.e. the nasal NO level is normally higher than tracheal NO level in the same species. Considering this example, it is interesting, whether the NO level in exhaled breath of birds is significantly higher than in mammals, because of the anatomic differences. Birds have only one paranal sinus, but the respiratory system is composed of comparatively small lungs and several airsacs, that act as bellows but do not participate in gas exchange. These airsacs are anatomic dead spaces too, and may therefore increase the NO production in airways.

Concerning NO levels, there are striking differences among the species even when comparing the NO concentration of the same sampling methods as shown by Bernareggi et al. (9). Different measurement conditions such as expiratory flow rate could also affect measured NO levels. The higher the flow rate the lower the concentrations of NO obtained. This phenomenon was showed by Byrnes et al. (14) in human medicine, by Mills et al. (58) in their study with horses and by Weicker et al. in their studies with mice in a plexiglas chamber (87). Besides, exercise increased the production rate of NO in exhaled air of horses (58) and anaesthesia decreased the NO level measured in exhaled air of mice (87); increased and decreased tidal volume could be an explanation. Therefore, collection and analysis of exhaled, nasal and tracheal NO may easily be done. However, it is critical to establish a standardized collection protocol for animals as in human medicine (2).

**Parameters in exhaled breath condensate in different animals**

**Carnivores**

**Cats**

In 2004, the first description of EBC collection in cats with a noninvasive system, and successful measurement of H₂O₂ was published (79). A collection system was designed based on an acrylic plastic chamber (volume: 25 L). An U-shaped welded stainless steel condensing tube was immersed in an ice-water bath at ~4°C so that the exhaled breath condensed. The air flow through the chamber was set to 900 ml/kg/min. To collect a condensate volume of approximately 1 ml the cats had to be in the chamber for 20-30 min. The cats showed no signs of distress. Within 20 min the EBC sample collected was analysed for H₂O₂ concentrations using a spectrophotometric assay. In the six healthy cats, EBC H₂O₂ concentrations ranged from <0.1 to 2.1 μmol/l. It was demonstrated that, using this system, EBC can be successfully collected in a safe, noninvasive and well tolerated way.

In a further study the aim was to describe a standardised and non-invasive method to collect EBC in cats and to test whether determination of H₂O₂ in EBC might be used as a marker of lower airway inflammation (44). Eighteen conscious cats were placed in a hole body plethysmograph, which was combined with a system to condense the effluent air and to collect the EBC. The EBC was kept on ice until analysis that was performed within 10 min of collection using a spectrophotometric assay. The H₂O₂ concentration in EBC was compared with bronchoalveolar lavage (BAL) cytology. The mean H₂O₂ concentration was 0.685 ± 0.286 μmol/L, thus in agreement with healthy humans (31,51,83) and healthy horses (17).

**Dogs**

Currently, two parameters have been measured in
<table>
<thead>
<tr>
<th>SPECIES</th>
<th>PARAMETER</th>
<th>STATUS</th>
<th>METHOD</th>
<th>FLOW RATE</th>
<th>VALUES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhesus monkey</td>
<td>Nasal NO</td>
<td>Healthy, anesthetized, spontaneously</td>
<td>Continuously</td>
<td>0.02 L/min</td>
<td>237 ± 10 ppb (SEM)</td>
<td>(76)</td>
</tr>
<tr>
<td></td>
<td>Exhaled NO</td>
<td>breathing</td>
<td>sampling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baboon</td>
<td>Exhaled NO</td>
<td>Healthy, anesthetized, spontaneously</td>
<td>Face mask</td>
<td>1.35 L/min</td>
<td>1.0 ± 0.6 ppb (SD)</td>
<td>(49)</td>
</tr>
<tr>
<td></td>
<td>Nasal NO</td>
<td>breathing</td>
<td>Continuously</td>
<td>1.35 L/min</td>
<td>4.8 ± 2.1 ppb (SD)</td>
<td>(49)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Tracheal NO</td>
<td>Healthy, anesthetized, ventilated</td>
<td>Tracheotomized</td>
<td>No specification</td>
<td>15.0 ± 0.8 ppb (SEM)</td>
<td>(25)</td>
</tr>
<tr>
<td></td>
<td>Tracheal NO</td>
<td>Healthy, anesthetized, ventilated</td>
<td>Tracheotomized</td>
<td>0.05 L/min</td>
<td>17.4 ± 2.7 ppb (SEM)</td>
<td>(70)</td>
</tr>
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<td></td>
<td>Tracheal NO</td>
<td>Healthy, anesthetized, ventilated</td>
<td>Tracheotomized</td>
<td>0.15 L/min</td>
<td>15.3 ± 2.0 ppb (SEM)</td>
<td>(68)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Tracheal NO</td>
<td>Healthy, anesthetized, ventilated</td>
<td>Tracheotomized</td>
<td>No specification</td>
<td>12.9 ± 1.0 ppb (SEM)</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>Tracheal NO</td>
<td>Healthy, anesthetized, ventilated</td>
<td>Tracheotomized</td>
<td>0.1 L/min</td>
<td>12.9 ± 1.0 ppb (SEM)</td>
<td>(9)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Exhaled NO</td>
<td>Healthy, conscious</td>
<td>Plexiglas chamber</td>
<td>0.06 L/min</td>
<td>10.1 ± 1.1 ppb (SEM)</td>
<td>(87)</td>
</tr>
<tr>
<td></td>
<td>Exhaled NO</td>
<td>Healthy, anesthetized, spontaneously</td>
<td>Plexiglas chamber</td>
<td>0.06 L/min</td>
<td>4.0 ± 0.8 ppb (SEM)</td>
<td>(87)</td>
</tr>
<tr>
<td>Rat</td>
<td>Tracheal NO</td>
<td>Healthy, anesthetized, ventilated</td>
<td>Tracheotomized</td>
<td>0.05 L/min</td>
<td>4.2 ± 1.7 ppb (SEM)</td>
<td>(70)</td>
</tr>
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<td>Tracheal NO</td>
<td>Healthy, anesthetized, ventilated</td>
<td>Tracheotomized</td>
<td>No specification</td>
<td>5-10 ppb</td>
<td>(81)</td>
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<td>Tracheal NO</td>
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<td>Tracheotomized</td>
<td>0.1 L/min</td>
<td>0.9 ± 0.01 ppb (SEM)</td>
<td>(9)</td>
</tr>
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<td>Seal</td>
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<td>Plexiglas chamber</td>
<td>No specification</td>
<td>No concentration found</td>
<td>(80)</td>
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<tr>
<td>Mini-pig</td>
<td>Nasal NO</td>
<td>Healthy, anesthetized, intubated, ventilated</td>
<td>Continuous</td>
<td>0.02 L/min</td>
<td>18.1 ± 0.5 ppb (SEM)</td>
<td>(76)</td>
</tr>
<tr>
<td>Horse</td>
<td>Exhaled NO</td>
<td>Healthy, conscious, trot</td>
<td>Face mask</td>
<td>20 L/min</td>
<td>1.2 ± 0.1 ppb (SEM)</td>
<td>(58)</td>
</tr>
<tr>
<td></td>
<td>Exhaled NO</td>
<td>Healthy, conscious, canter</td>
<td>Face mask</td>
<td>20 L/min</td>
<td>2.3 ± 0.3 ppb (SEM)</td>
<td>(58)</td>
</tr>
<tr>
<td></td>
<td>Exhaled NO</td>
<td>Healthy, conscious, trot</td>
<td>Face mask</td>
<td>80 L/min</td>
<td>0.8 ± 0.2 ppb (SEM)</td>
<td>(58)</td>
</tr>
<tr>
<td>Elephant</td>
<td>Nasal NO</td>
<td>Healthy, conscious</td>
<td>Syringe technique</td>
<td>1.35 L/min</td>
<td>5.0 ± 0.1 – 26.5 ± 0.4 ppb (SEM)</td>
<td>(48)</td>
</tr>
<tr>
<td></td>
<td>Nasal NO</td>
<td>Healthy, conscious</td>
<td>Continuous sampling</td>
<td>1.35 L/min</td>
<td>23.1 – 26.4 ppb</td>
<td>(48)</td>
</tr>
</tbody>
</table>
the EBC of dogs, LTB$_4$ (71) and H$_2$O$_2$ (90).
In the first study (71), the EBC was collected under general anaesthesia using a manufactured instrument formed by a glass cylinder containing a coil connected to a refrigerator. The eleven dogs were intubated using an orotracheal tube, which was connected to the glass cylinder on one side and to a tube cooled in an ice-bath on the other. The best conditions for EBC collection was using a 56 ml glass cylinder, a temperature of 0°C and a collection time of 30 min. Thus, an EBC volume, varying between 1 and 3 ml depending on body size, was collected from each dog. These samples were immediately frozen at -82°C and then measured using an enzyme immunoassay (EIA). The concentration of LTB$_4$ varied from 15 to 94 pg/ml. This was lower than reported in the literature for humans and bovines. But the range of LTB$_4$ concentrations measured in dogs were wider than reported in humans (the concentrations in calves span an even wider range). This method prevents the contamination of the lower airways, but the dogs had to be anaesthetised.

In the second study (90), the EBC was collected from ten healthy conscious dogs, lying in lateral or sternal recumbency, using a face mask connected to a condensing chamber filled with an ethanol/liquid nitrogen slurry. The sampling collection was well tolerated by all dogs. After 10 to 15 min breathing through the collection apparatus, at least 0.1 ml EBC was collected from each dog. H$_2$O$_2$ was measured immediately using the spectrophotometric method. H$_2$O$_2$ was detected in all EBC samples, but there was considerable variation between and within subjects (1.85 ± 0.84 µmol/l). The H$_2$O$_2$-concentrations were higher then those reported in healthy humans, but in the range of healthy horses.

**Artiodactyls**

**Calves**

Reinhold et al. (72,73) evaluated the measurement of LTB$_4$ concentrations as a noninvasive method for assessing mediators of inflammation in the lungs of calves. LTB$_4$ was determined in 35 EBC samples. The 35 conscious calves were spontaneously breathing through a special face mask with two valves to separate inspired and expired air. Exhaled air was directed via a nonrebreathing valve into a cooled (-15 to -20°C) collection tube. Sample collection of each calf was 30 min. The amount of condensate gained in the 35 healthy calves ranged either from 0.1-9.1 ml (73) or 0.1-2.8 ml (72). After collecting, the tube was immediately sealed at both ends and stored at -80°C until analyzed. The authors state that LTB$_4$ was measured using a commercially available EIA-Kit-System (73) in one publication and a commercially available ELISA (72) in the other. The concentration ranged in both studies from 31.8 to 225.4 pg/ml. However, the fact that the data given are identical, suggests that only one method was used.

Furthermore, Reinhold et al. (72) showed, that in a group of 12 healthy calves, which were examined on two consecutive days, no differences were detected between results of day 1 and 2 (neither the obtained volume of EBC nor LTB$_4$ concentrations differed significantly between sample collection days). In order to evaluate the long-term variability of LTB$_4$ concentrations, EBC (8 samples/calve) were obtained throughout a 53-day period from three healthy calves. The LTB$_4$ concentration was highly reproducible and there was low variability in concentrations in EBC.

In the same study, seven healthy calves with basic LTB$_4$ value ranging within the values of the other 35 healthy calves, were inoculated intratracheally with *Pasteurella multocida* serovar D. In all calves, EBC was collected 3 days before and 3 days after the infection and LTB$_4$ concentrations were measured. Five days after inoculation, visible lesions of the lung surface were observed at necropsy. The LTB$_4$ concentrations in EBC significantly increased after bacterial infection, but the increased LTB$_4$ concentrations were still within the reference range for healthy calves.

At last, four calves were inoculated with bovine respiratory syncytial virus. A significant increase of LTB$_4$ concentration could only be detected in two of the four calves.

**Perissodactyls**

**Horse**

EBC from 22 horses with different degrees of recurrent airway obstruction (RAO) were collected and analysed for LTB$_4$ (20). Standing horses were intubated with an endotachecial tube, which was connected with a refrigerated (-20°C) teflon tube to condensate exhaled air. A valve ensured that just expired air passed the collection system. The horses breathed for 10 minutes through the teflon
tube, which then was removed, and the frozen breath condensate was allowed to thaw. The fluid EBC was transferred into liquid nitrogen (−80°C) and analysed by an enzyme immuno assay (EIA). LTB₄ was detected in all samples in a range of 52−330 pg/ml.

In an other study (17), eight healthy and ten RAO horses, subdivided into two groups, were studied. The first group of RAO horses had significant airway inflammation, and the second RAO group was investigated in the absence of airway inflammation and after a period of at least two month at pasture. EBC was collected from conscious unsedated horses fitted with a face mask with a non-rebreathing valve. A flexible pipe connected the valve to the 5 L “U”-shaped stainless steel condensing tube surrounded by ice and water (0°C). After ten minutes sampling time, 2-4 ml breath condensate was collected. The H₂O₂ concentration in EBC collected was analysed using a spectrophotometric assay.

RAO horses without inflammation had a significantly lower concentration of H₂O₂ than those with inflammation.

The published data on H₂O₂ and LTB₄ in animals are collected in Tables 2 and 3.

**Evaluation of the parameters determined in exhaled breath condensate**

Collection of EBC is a noninvasive diagnostic tool and can be performed in conscious as well as in anaesthetised animals. In anaesthetised animals an orotracheal tube can be used to prevent inflammatory contaminations of the upper airways, and sampling conditions can be standardized, but each general anaesthesia involves some risk, and frequent repetition in small intervals, to monitor respiratory diseases as made in humane medicine, is not practical.

The sample collection procedure using face mask or plexiglass chamber was well tolerated by all animals. In healthy animals, a wide range of parameters measured was detected. Because animals breathe predominantly through the nose, substances originating from the nasal mucosa may be included in the condensate and may influence the concentrations measured in EBC. In the study with calves, a differentiation of healthy and sick animals was not possible by means of LTB₄, whereas in studies with cats and dogs only clinical-

ly healthy animals were used. Therefore, in these species, no statement can be made concerning variation of parameters in disease. Deaton et al. (17) succeeded in differentiating between healthy and RAO (recurrent airway obstruction) affected horses in their study using H₂O₂ concentration in EBC as diagnostic parameter.

In summary, EBC can be collected successfully and inflammatory parameters can be determined, but the interpretation of results is difficult due to the wide range of the concentration of inflammatory markers measured in healthy animals. Furthermore, the sample collection procedure has to be standardized with regard to the sampling technique, the surface of the sample collection tube, the temperature of fluid around the condensing chamber, the ambient humidity, the respiratory rate and the storage of EBC. When a plexiglass chamber technique is used, the flow rate plays an important role too, because an effect on H₂O₂ concentration can not be excluded (77); decrease in flow rate increases H₂O₂ levels.

In 2005, Horvath et al. (32) published, on behalf of the American Thoracic Society and the European Respiratory Society Task Force on Exhaled Breath Condensate an article about methodological recommendations on EBC collection and measurements, in which they advised using an EIA for measuring LTB₄ concentration in EBC. The specificity of the EIA for determination of LTB₄ concentration in EBC was confirmed by using reverse-phase high performance liquid chromatography (RP-HPLC) (63).

Further work is necessary to determine normal ranges for values of inflammatory markers in larger numbers of different animals and to investigate changes associated with lung diseases.

**Conclusion**

Sampling of exhaled breath and exhaled breath condensate is a noninvasive diagnostic tool and simple to perform. But to date, the analysis of parameters for clinical use would be premature, because of the lack of reliable baseline values in each animal species, generated by standardized collection methods. Further investigations are necessary to evaluate the effect of collection variables such as sampling rate, sampling method and storage temperature, and to propose a standardized collection protocol for each species.
Table 2: $\text{H}_2\text{O}_2$ in exhaled breath condensate of different animal species

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>PARAMETER</th>
<th>STATUS</th>
<th>METHOD</th>
<th>VALUES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy, conscious</td>
<td>Plexiglas chamber</td>
<td>$2.5 \pm 0.3$ pmol/h</td>
<td>(88)</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy</td>
<td>Intubated</td>
<td>$2.4 \pm 0.4$ pmol/h</td>
<td>(88)</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy conscious</td>
<td>Plexiglas chamber</td>
<td>$3.1$ pmol/h</td>
<td>(89)</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy, anesthetized</td>
<td>Intubated</td>
<td>$20 \pm 10$ pmol/h</td>
<td>13 $\pm$ 5 pmol/h</td>
</tr>
<tr>
<td>Cat</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy</td>
<td>Plexiglas chamber</td>
<td>$0.7 \pm 0.3$ μmol/L</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy</td>
<td>Plexiglas chamber</td>
<td>$&lt; 0.1 - 2.1$ μmol/L</td>
<td>(79)</td>
</tr>
<tr>
<td>Dog</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy, conscious</td>
<td>Face mask</td>
<td>$1.8 \pm 1.5$ μmol/L</td>
<td>(90)</td>
</tr>
<tr>
<td>Horse</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy</td>
<td>Face mask</td>
<td>$0.4 \pm 0.2$ μmol/L</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2$</td>
<td>RAO$^1$ - horses without inflammation</td>
<td>Face mask</td>
<td>$0.9 \pm 0.2$ μmol/L</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2$</td>
<td>RAO$^1$ - horses with inflammation</td>
<td>Face mask</td>
<td>$2.0 \pm 0.5$ μmol/L</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Healthy</td>
<td>Face mask</td>
<td>$0.7 - 1.3$ μmol/L</td>
<td>(16)</td>
</tr>
</tbody>
</table>

Table 3: $\text{LTB}_4$ in exhaled breath condensate of different animal species

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>PARAMETER</th>
<th>STATUS</th>
<th>METHOD</th>
<th>VALUES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>$\text{LTB}_4$</td>
<td>Healthy, anesthetized</td>
<td>Intubated</td>
<td>$15 - 94$ pg/ml</td>
<td>(71)</td>
</tr>
<tr>
<td>Calf</td>
<td>$\text{LTB}_4$</td>
<td>Healthy (35 calves)</td>
<td>Face mask</td>
<td>$116 - 55$ pg/ml</td>
<td>(73, 72)</td>
</tr>
<tr>
<td></td>
<td>$\text{LTB}_4$</td>
<td>Healthy (7 calves)</td>
<td>Face mask</td>
<td>$59.6 - 17.2$ pg/ml</td>
<td>(72)</td>
</tr>
<tr>
<td></td>
<td>$\text{LTB}_4$</td>
<td>3 days after infection with P. multocida</td>
<td>Face mask</td>
<td>$95.7 - 22.1$ pg/ml</td>
<td>(73, 72)</td>
</tr>
<tr>
<td>Horse</td>
<td>$\text{LTB}_4$</td>
<td>RAO$^1$ - horses</td>
<td>Intubated</td>
<td>$163.8 - 62.0$ pg/ml (52-330 pg/ml)</td>
<td>(20)</td>
</tr>
</tbody>
</table>

$^1$RAO = recurrent airway obstruction
References


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60. Montuschi P and Barnes PJ. Exhaled leukotrienes and prostaglandins in asthma. The Journal of Allergy and Clinical Immunology 2002; 109: 615-620.


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