

Rostrums

Exhaled breath condensate: An evolving tool for noninvasive evaluation of lung disease

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Exhaled breath condensate (EBC) contains aerosolized airway lining fluid and volatile compounds that provide noninvasive indications of ongoing biochemical and inflammatory activities in the lung. Rapid increase in interest in EBC has resulted from the recognition that in lung disease this easily sampled fluid has measurable characteristics that differ prominently from health. These assays have provided evidence of airway and lung redox deviation, acid-base status, and degree and type of inflammation in acute and chronic asthma, chronic obstructive pulmonary disease, adult respiratory distress syndrome, occupational diseases, and cystic fibrosis. Characterized by uncertain and variable degrees of dilution, EBC does not provide precise assessment of individual solute concentrations within native airway lining fluid. However, it can provide useful information when concentrations differ substantially between health and disease or are based on ratios of solutes found in the sample. Because they can be used to measure the targets of modern therapy, EBC assays are likely to become integral components of future clinical studies, and after further technical work is accomplished, they might be used to diagnose and monitor therapy in individual patients. (*J Allergy Clin Immunol* 2002;110:28-34.)

Key words: *Exhaled breath condensate, exhaled biomarkers, pH, cytokines, hydrogen peroxide, nitrogen oxides, oxidant*

Exhaled breath contains aerosols and vapors that can be collected and analyzed for characteristics capable of noninvasively elucidating physiologic and pathologic processes in the lung. To capture the breath for assay, exhaled air is passed through a condensing apparatus; the result is an accumulation of fluid that is referred to as

Abbreviations used

ALF: Airway lining fluid
BAL: Bronchoalveolar lavage
COPD: Chronic obstructive pulmonary disease
EBC: Exhaled breath condensate
FeNO: Fractional expired nitric oxide concentration
NO: Nitric oxide

exhaled breath condensate (EBC).^{*} Although predominantly derived from water vapor, EBC has dissolved within it nonvolatile compounds, including cytokines,^{1,2} lipids,³ surfactant,⁴ ions,⁵ oxidation products,^{6,7} and adenosine, histamine, acetylcholine, and serotonin.⁸ In addition, EBC traps potentially volatile water-soluble compounds, including ammonia,⁹ hydrogen peroxide,^{10,11} and ethanol, and other volatile organic compounds.¹² EBC has readily measurable pH.^{13,14}

In 1980, investigators in the former Soviet Union began reporting studies of EBC.⁴ Despite the fact that exhaled breath is the major mechanism of insensible water loss, the study of this body fluid was largely ignored in the West until the last several years. The technique is now attracting broad research interest; more than 100 peer-reviewed articles in the international literature (at least 35 in 2000-2001) have described methods and reported its utility in the investigation of lung diseases and occupational illnesses. This interest derives in part from the promise that EBC will provide a substrate by which inflammatory and biochemical components of lung disease can be noninvasively assessed—and that it therefore could substantially improve on spirometry as a noninvasive tool for diagnosis and management.

EBC is critically examined in this article, my aim being to provide investigators with sufficient knowledge to enable them to cautiously apply these techniques in their particular fields of interest.

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^{*}Other commonly used terms include *breath condensate, expired air condensate, breath precipitate, expired air humor, exhaled air humor, expired vapor, airway vapor condensate, condensed exhalate, condensed expirate, and expired condensate*.

THE PHYSICS AND PHYSIOLOGY OF EBC: AEROSOLS AND VAPORS

Two mechanisms have been proposed for aerosolization of airway lining fluid (ALF).^{*} Turbulence, as evidenced by audible airflow, provides sufficient energy to the airway wall to aerosolize particles of ALF.¹⁵ If the energy provided to the airway wall increases—such as with increased ventilation during exercise—a greater yield and initial size of aerosol particles can be expected¹⁶—rather like whitecaps on the sea disturbed by a gale.¹⁵ Anatomic sites of aerosolization can then be expected wherever there is air turbulence. During quiet tidal breathing, this will occur predominantly at the first several generations of carinas, along the bronchial and tracheal walls where the cartilaginous rings alter airflow, and at other sites of airflow direction change, including the glottis and pharynx. Aerosols can be formed during both inspiration and expiration, providing a mechanism of transfer of fluid, and conceivably bioactivity, between different levels of the airway.

In contrast to the turbulence theory is the recent proposal that aerosolization of ALF can occur in the absence of bulk flow, during the popping-open of closed respiratory bronchioles and alveoli (S. Kharitonov, oral communication, June 2001). In this regard, studies have reported the presence of surfactant in EBC,⁴ though the site of aerosolization of the surfactant could certainly be more proximal in the airway tree. The individual contributions to EBC of these 2 processes for generating aerosols, and of surfactant in enhancing aerosolization by decreasing surface tension, have not yet been investigated.

Regardless of the precise anatomic source, aerosols are generated within the milieu of air that is almost saturated with gas-phase water vapor.¹⁷ On cooling, aerosol particles serve as seeds for condensation of the water vapor, increasing in size and inertia while the air dehydrates. This is what occurs when we see our breath in cold weather; the particles become large enough to be visible. If we were meteorologists, we would call the collected fluid “breath precipitate.”

Water condensation can serve to trap volatile substances with a high water partition coefficient—substances that can emanate from any site in the lung, including the alveoli. Therefore, though it is uncertain whether distal airways and alveoli contribute to either the water volume or the nonvolatile solute content of EBC during quiet breathing, they might well significantly contribute to levels of volatile compounds.

COLLECTION OF EBC: GENERAL PRINCIPLES

EBC collection is a noninvasive process that is both simple and safe. As exhaled air cools below the dew point by transfer of heat to a chilled condenser surface, condensa-

tion occurs on available aerosolized ALF particles, and the thus-enlarged droplets of EBC impact and collect on the condenser wall. The systems reported in the literature generally collect 1 mL of EBC in 5 to 15 minutes. The volume collected depends on total expired air volume, condenser material and temperature, and turbulence characteristics.

Contamination by salivary fluid is of concern if the collection is made orally. Investigators repeatedly have not found salivary amylase in collected breath samples, which is reassuring. But to accomplish this, some form of saliva trap or filter is used to prevent gross contamination by liquid saliva. It needs to be noted that the amylase assays used might not be sufficiently sensitive to identify small contributions of saliva to EBC.

COLLECTION DEVICES

Most researchers have used custom devices, fashioned in individual laboratories, consisting of jacketed cooling pipes, tubes in buckets of ice, or glass chambers in ice with inhalation and exhalation ports. The least expensive and simplest technique involves exhaling through a tube that is suspended through a bucket of ice (Fig 1). This can require a collection time of 15 minutes or longer, because the sample is held by surface tension to the condenser wall until enough fluid is collected that gravity and/or airflow can propel the larger drops distally.

There are 2 commercially available devices of which I am aware. The ECoScreen, manufactured by Eric Jaeger, GmbH (Hoechberg, Germany), is an electric refrigerated system modified from a cold air challenge device. Designed for the pulmonary function laboratory, it has an extendable arm that allows the subject to sit upright on a chair and exhale into a cooled chamber. It is noted to be efficient at condensing fluid, inasmuch as the condenser maintains a cold temperature (−10°C) throughout the 5- to 15-minute collection period. The size of a desktop personal computer, it is not easily portable. It uses a salivary trap consisting of a hole near the mouthpiece that reportedly keeps the collected fluid amylase-free. Pooling of the condensate requires either sufficient sampling time to overcome surface tension issues or centrifuging of the collection chamber.

Another commercially available device is the RTube, from Respiratory Research, Inc (Charlottesville, Va; Fig 2). This is a portable device that uses an exhalation valve that also serves as a syringe-style plunger to pool fluid off the condenser walls, allowing for collection times of as little as 1 minute. Cooling is achieved by placing an aluminum cooling sleeve over the disposable polypropylene condensation chamber, which can also serve as the sample storage container. The temperature of the cooling sleeve can be chosen by the investigator. The device inherently prevents salivary contamination, and the company reports no evidence of salivary amylase in the condensed fluid. An attachable microbial filter (0.3 μm) is provided to prevent transmission of infectious particles. There is a separate device in this family, called the *pHTube*, that

^{*}As used here, the term *airway lining fluid* refers to the fluid lining the entire respiratory tract, not excluding the mouth and nose.

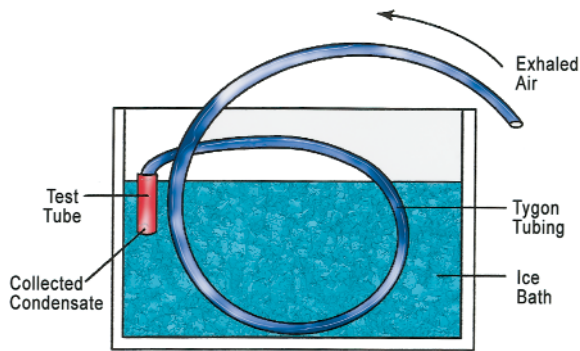


FIG 1. Representation of a custom-built prototypical EBC collection device of the type used for many of the early studies. Air is exhaled through a tube, and droplets of condensate are collected in a container. This is inexpensive, but collection times can be long and saliva contamination is a concern.



FIG 2. A commercially available collection device. The RTube collection system is portable and disposable, and it can collect a sample in as little as 1 minute.

allows deaeration for removal of carbon dioxide—a process helpful for pH measurement.

It is important to note that the collection of sample is simple, regardless of the device used. Children 4 years old and even younger cooperate and perform the procedure without difficulty or pain. Samples can be collected orally, nasally, or from endotracheal or tracheostomy tubes.

VARIABILITY IN EBC ASSAYS

Although multiple reports suggest that assays for compounds of interest in EBC have sufficient intrasubject reproducibility, published reports of formal investigation into this issue are as yet scant. The one report to raise concerns about reproducibility unfortunately relied on homemade collection systems and difficult assays.⁵ The investigators' findings of marked variability in certain EBC substances raise questions about the collection and

assay systems used in this particular study and should not be generalized to more standardized systems or to assays used by other investigators. My experience is that some condensate solute concentrations indeed differ in back-to-back assays by as much as 50%. However, this degree of variability might not diminish the utility of EBC assays for differentiating group means and thus elucidating disease mechanisms. More strict intrasubject reproducibility might be required if certain EBC assays will be used to diagnose, treat, or monitor a given individual. In most of the papers published to date, investigators have used EBC assays in mechanistic studies, comparing group means. They have provided insights into mechanisms of asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) by revealing disease-associated alterations in inflammatory mediators and reaction products of oxygen and nitrogen redox species.

The effect size—the size of the difference in the EBC concentration of a given compound between health and disease—also determines how much intrasubject variability is acceptable, particularly when the assays are to be used to follow an individual patient. If certain compounds are present in the EBC of patients with asthma at 10- or 100-fold higher concentrations than in the EBC of controls, then even a 2- to 3-fold variation in back-to-back sample collections might not be a matter for concern. If, however, the effect size is small, then the ability to distinguish health from disease might easily be overwhelmed by the errors in the collection and assay process.

There are several factors that lead to variability. Assay sensitivity is an issue. Although many compounds are in concentrations fully in the range of available assays (leukotriene B₄, isoprostane, ammonia, hydrogen ion, nitrate), some compounds are identified in concentrations close to their lower limits of detection by commercially available assay techniques. In EBC, levels of nitrite (0.5–2 $\mu\text{mol/L}$) are very close to the lower limit of detection for colorimetric assays. However, chemiluminescent assays for nitrite (after reduction to nitric oxide [NO]) are sufficiently sensitive to reproducibly quantify low nanomolar (50 nmol/L) concentrations of nitrite. As new assays are developed—optimized for the low levels of solutes in EBC—we can expect to see an increasing array of inflammatory, redox active, and smooth muscle-affecting molecules being investigated as useful noninvasive indicators of disease activity.

Issues regarding sample collection and storage that need to be investigated include the effects of total collection time, rate of exhalation, and device temperature, turbulence, and saliva-trapping characteristics. It seems likely that each compound of interest will have its own optimum conditions for collection and storage and that these conditions will need to be individually determined.

Of interest is the possibility, suggested by Effros et al,⁵ that an increase in an EBC solute concentration either is caused by an increase in the ALF concentration of the solute or simply reflects the fact that more aerosolized particles are captured within the same volume of condensate. Given that most airway disorders studied with

EBC have noisy and turbulent airflow—and presumably more particle formation—this issue is in need of careful investigation, and dilution markers need to be identified before EBC can be used to confidently predict ALF concentrations of individual solutes.

Exhaled gas phase NO concentration is known to be flow-dependent because of steady-state transfer of NO out of the airway wall.¹⁸ Lower flows lead to higher concentrations. Flow might affect breath condensate solute concentration in a different and opposite manner: as flow rate increases, turbulence will increase and occur further down the airway, generating more particles and perhaps allowing sampling of more distal lung. Studies of potential flow dependence of breath condensates are more complicated than those pertaining to NO, because slow or fast flows cannot easily be maintained for the several minutes usually needed for breath condensate collection. The results of investigations into flow effects are eagerly anticipated and might help clarify questions of anatomic source and volatility.

Temperature of the collected fluid might be relevant beyond the effects on efficiency. Certain compounds of interest are unstable, and collection as frozen fluid seems beneficial. On the other hand, volatile gases that are water-soluble might be better captured if the EBC is in liquid form, near 0°C. Each laboratory should be aware of the volatility and stability characteristics of the compounds of interest.

Contamination from nonrespiratory sources must be considered where appropriate. This is particularly relevant for assays for inorganic substances at low concentrations, such as nitrate and nitrite. Both of these nitrogen oxides are found abundantly in the laboratory environment, in the water, and on fingers.

ABSENCE OF A GOLD STANDARD

As of this writing, there have been only limited head-to-head comparisons of EBC assays with other methods of assessing airway biochemistry and inflammation, such as exhaled NO, bronchoalveolar lavage (BAL), induced sputum, and biopsy. In general, there does appear to be correlation among EBC assays and other assays of lung inflammation. It can be expected that like these other modalities, EBC will not precisely represent the ALF. Indeed, it is clear that ALF will be diluted in EBC, perhaps a great deal more than it is in BAL. It must be kept in mind that we do not know the ionic composition of the human ALF in health, to say nothing of disease, so there is as yet no available gold standard by which to make comparisons. However, inasmuch as direct microcapillary sampling techniques of ALF are beginning to show promise, we might soon learn the true ALF concentrations in health and disease; we would then have a gold standard.

CURRENT APPLICATIONS

As a research tool, EBC has several commendable features. Through use of appropriate equipment, samples

have been collected at worksites, schools, hospitals, and clinics as well as in homes. After undergoing brief instruction, subjects can perform the sample collections multiple times in a day, completely unsupervised. Patients tend to find the experience interesting, and its negligible risk profile assures almost 100% successful recruitment into a study protocol. Frequent collections allow for the kind of intense longitudinal data collection^{13,19} that is to all intents and purposes impossible to accomplish with invasive techniques. Furthermore, whereas induction of sputum can both cause bronchoconstriction in some patients²⁰ and affect the cell counts of subsequent sampling, there is no theoretic or empiric suggestion that EBC collection should or does alter the airway environment.

The list of disease states for which EBC assays have been used for research purposes is steadily growing; it includes asthma, cystic fibrosis, chronic obstructive pulmonary disease, viral respiratory infections, and idiopathic pulmonary fibrosis. Similarly, the number of recognized compounds of interest is increasing rapidly. Assays for nitrogen oxides, oxidants, surfactant, and leukotrienes dominate the literature, but many additional compounds have been recently added to the list, including ammonia, volatile organic compounds, and nitrotyrosine. There has been recent interest in using EBC as a sample for proteomic evaluation. Several cytokines are found in EBC solidly within the sensitivity range of enzyme-linked immunoassays. In each report, abnormalities in EBC levels of compounds are consistent with and/or add to our current understanding of the studied disease.

Hydrogen peroxide

Assays for hydrogen peroxide (H₂O₂) captured in EBC reveal increased production of this potent volatile oxidant during symptomatic colds,²¹ as has also been noted by multiple investigators during acute and stable asthma,²²⁻²⁴ COPD,²⁵ cystic fibrosis,²⁶ bronchiectasis,²⁷ and acute respiratory distress syndrome.¹¹ Hydrogen peroxide is formed during the cellular respiratory burst. The capability of stimulated neutrophils from asthmatic subjects to generate superoxide correlates well with the patients' EBC hydrogen peroxide levels,²⁸ and the diseases in which EBC hydrogen peroxide has been found to be elevated have a prominent neutrophilic component.²⁹ Hydrogen peroxide is the product of superoxide (O₂⁻) dismutation, the presence of which is suggested in part by high levels of oxidative and nitrative stress evident in the airways of subjects with asthma.³⁰ This is also revealed in the elevated EBC levels of the prostaglandin peroxidation product 8-isoprostane in adult respiratory distress syndrome,⁶ COPD,³¹ asthma,⁷ and cystic fibrosis.³² Oxidative injury is now suspected of contributing much to the pathology of these lower airway diseases.

pH

Acidification of EBC is an important characteristic of acute asthma exacerbations¹³ relevant to the underlying pathology of the disease. Of note, EBC collected during

TABLE I. Composition of exhaled breath condensate in lung diseases

Molecule or characteristic	Diseases studied	Effect size	Assay method	Volatile component?	Stability storage	Reference no.	Comments/opinion of the author
pH	Asthma—acute	2-3 log ↓	Electrode	Likely	Stable at RT for years	13	Requires deaeration to remove CO ₂ before assay; pH correlates with severity of disease; largest effect size
	Asthma—stable	0.3 log ↓	Colorimetric			14	
	COPD—stable	0.5 log ↓				39	
	Bronchiectasis	0.5 log ↓					
	Viral URI	2-3 log ↓					
NH ₃ /NH ₄ ⁺	Asthma—acute	1-2 log ↓	Spectrophotometric dipstick	Likely	Stable at RT	9	Oral contamination a concern; simple; best collected at 0°C
	Viral URI	1-2 log ↓					
Leukotrienes	Asthma—acute	2× ↑	ELISA	No	Store in freezer	2	LTB ₄ , C4, D4, E4
	Asthma—stable	<2× ↑				38	
H ₂ O ₂	Asthma—stable	4× ↑	Fluorimetric,	Likely	Stable at -20°C for 1 month; stability can be enhanced by adding reagent after collection	40	Best studied; potent oxidant; potential for volatile component has not been addressed
	Bronchiectasis	4× ↑	spectrophotometric ± HPLC			27	
	COPD—stable	1 log ↑				25	
	COPD—acute	1.5 log ↑				11	
	ARDS	5× ↑	chemiluminescence			41	
	CF—stable	—				26	
	CF—exacerbation	1.5× ↑					
8-isoprostane	Sarcoidosis	2× to 3× ↑	ELISA	No	Stability not completely characterized	42	Correlated with severity of asthma
	ARDS	>1 log ↑				6	
	Asthma	2× to 3× ↑				7	
	CF—stable	3× ↑				32	
	COPD	2× to 4× ↑				31	
Nitrite (NO ₂ ⁻)	Asthma—stable	2× ↑	Chemiluminescence	Possibly	pH-dependent	36	
	Asthma—acute	—				13	
	CF—stable	6× ↑				34	
Nitrate (NO ₃ ⁻)	Asthma—stable	2× ↑	Chemiluminescence	Possibly	Stable at RT	34	
	CF	—					
Nitrotyrosine	Asthma—stable	2× to 4× ↑	ELISA	No	Stable at RT	38	Indicative of nitrative environment (peroxynitrite, acidic conditions w/NO ₂ ⁻)
	CF	4× ↑				37	

Studies have also revealed the presence in EBC of s-nitrosothiols, acetate, adenosine, PGE₂ (which declines in EBC with ibuprofen use),⁴³ TNF- α , IL-1 β , IL-8, IL-10, other cytokines and soluble cytokine receptors, thyroid hormones, histamine, serotonin, cortisol, acetylcholine, catecholamines, calcium, magnesium, surfactant, urea, DNA, cholesterol, and proteins at least as large as 70 kD.

RT, Room temperature; COPD, chronic obstructive pulmonary disease; URI, upper respiratory infection; ARDS, adult respiratory distress syndrome; CF, cystic fibrosis.

acute asthma exacerbations normalizes over several days with corticosteroid therapy.³³ Others have now identified a similar effect in COPD and bronchiectasis.¹⁴ Low pH is associated with neutrophilic and eosinophilic inflammation,¹⁴ though the implications of acid-base disturbance in the airway are more extensive. These EBC pH effects have rigorously been shown not to result from artifacts of ventilatory pattern, orally derived ammonia, or levels of carbon dioxide. Given the ease of the assay, after additional ongoing research, EBC pH measurements speculatively have a role in monitoring disease status in the clinic and home or in predicting safe hospital discharge on exacerbation.

Leukotrienes

Another compound studied in EBC is the neutrophil chemoattractant LTB₄. In multiple diseases, this mediator is found in elevated levels relative to controls (Table I). In addition, the cysteinyl leukotrienes are identified in EBC, raising the as-yet-untested possibility, proposed by P.J. Barnes (oral communication, June 2001), that EBC cys-LT

levels might predict response to leukotriene antagonists. The levels in EBC are readily assayable with ELISA.

Cytokines

Cytokines reported in EBC include TNF- α , IL-1 β ,¹ IL-8,³⁴ and IL-10.³⁵ There are insufficient published EBC data regarding these and other highly relevant cytokines, though this is an area of active investigation. EBC might be a particular useful tool for assay of lung cytokines, and it has the as-yet-untested potential to discriminate between T_H1 and T_H2 cytokine profiles in human beings.

Nitrogen oxides,³⁶ including nitrotyrosine,^{37,38} have been investigated, and the findings mesh well with our evolving understanding of the airway chemistry of asthma and other lung diseases. The utility and relevance of these and the many other EBC assays, intelligently used for studying mechanisms of disease in research protocols, seems essentially assured. Table I summarizes some highlights in the literature.

FUTURE DIRECTIONS

Routine pulmonary function tests cannot contribute confidently to our assessment of the degree of inflammation and redox disturbance in the lung. To an increasing extent, however, our thinking and our therapies are directed to precisely those unmeasured disturbances. EBC assays expand on the promise of fractional exhaled NO concentration (FeNO) measurements to provide clinically useful methods by which to measure and monitor the biochemical and inflammatory processes underlying alterations in airflow characteristics. Yet like FeNO, the precise anatomic and molecular source of the compounds present in EBC remains debated or obscure.

The concern of variable dilution and the absence of a confident dilution marker currently prevents confident assessment of ALF solute concentrations based on EBC assays. This does not limit the utility of EBC, however, any more than the same difficulties have limited the utility of BAL, induced sputum, or FeNO. Ratios of solutes can or will provide important redox information (ratios of nitrite to nitrate, nitrotyrosine to total tyrosine, acids to bases [pH]) and important information about inflammation (leukotriene to prostaglandin ratio, IL-4 to interferon- γ ratio) that do not depend on dilution factors. Furthermore, the usefulness of EBC in capturing exhaled volatiles, though in need of further assessment, will not be diminished by dilution.

The great utility in these assays comes from their completely noninvasive nature and their ease of use. The wide array of biologically relevant compounds in the fluid and their predictable alterations during pathologic processes raise the possibility of significant new findings resulting from the expanded use of EBC assays for investigation into the mechanisms of lung disease, particularly when it comes to assessing acute exacerbations of illnesses such as asthma and COPD, for which invasive assays are rarely performed. Moreover, because markers of inflammation are increasingly needed to evaluate the efficacy of new medications and identify occupational diseases, EBC can readily supply needed data. Finally, because several of the reported assays have been or can be performed in the home, there is a very reasonable possibility that in the near future EBC will become useful for monitoring therapy and anticipating inflammatory lung disease exacerbations in individual patients.

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