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Electret filter collects more exhaled albumin than glass condenser

A method comparison based on human study

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Abstract

In recent years, noninvasive diagnosis based on biomarkers in exhaled breath has been extensively studied. The procedure of biomarker collection is a key step. However, the traditional condenser method has low efficacy in collecting nonvolatile compounds especially the protein biomarkers in breath. To solve this deficiency, here we propose an electret filter method.

Exhaled breath of 6 volunteers was collected with a glass condenser and an electret filter. The amount of albumin was analyzed. Furthermore, the difference of exhaled albumin between smokers and nonsmokers was evaluated.

The electret filter method collected more albumin than the glass condenser method at the same breath volume level ($P < .01$). Smokers exhaling more albumin than nonsmokers were also observed ($P < .01$).

The electret filter is capable of collecting proteins more effectively than the condenser method. In addition, smokers tend to exhale more albumin than nonsmokers.

Abbreviations: EBC = exhaled breath condensate, VOCs = volatile organic compounds.

Keywords: collection method, electret filter, exhaled albumin, smoker

1. Introduction

In recent years, noninvasive diagnosis based on biomarkers in exhaled breath has triggered an emerging interest in biomedical fields.[1] Biomarkers in exhaled breath consist of volatile compounds, which are generally named as volatile organic compounds (VOCs), the semivolatile compounds and the nonvolatile compounds, which are mainly in the form of droplets and particles in breath (e.g., NH_4^+, protein, etc.).[2] Analyzing biomarkers in exhaled breath can aid in diagnosing and monitoring human diseases. With the development of GC/MS technique, a lot of studies have been focused on examining VOCs in human breath.[3–6] Research on nonvolatile biomarkers in breath only emerges in recent decades.

The study of nonvolatile biomarkers in breath is limited by the unsatisfactory collection methods. Traditional condensation method has been used for collecting nonvolatile compounds in breath in the form of exhaled breath condensate (EBC). However, this method is not ideal and has prompted many complaints.[7–10] On the one hand, nonvolatile compounds would be diluted approximately 20,000 folds by condensed vapor in exhaled breath, rendering that biomarkers in EBC often fall below the detection limit of commercially available equipment.[7,11] On the other hand, it has been reported that liquid-based collection method wasted 90% of submicron particles, which may contain biomarkers in breath.[12,13]

Given the deficiency of the condenser method, other alternative methods have also been proposed. For instance, Almstrand and coworkers[14] designed a 3-stage impactor to collect exhaled breath particles. This method had been successfully applied to collect surfactant-A and albumin in breath.[15,16] However, complexity of the method (e.g., a pump is always needed) limits its application.

Thus, we propose a collection method based on electret filter. Breath particles containing nonvolatile biomarkers can be readily collected by simple electret filters with the electrostatic forces.[17] Our previous study has confirmed that the electret filter method was more effective in collecting exhaled albumin than the commercial EcoScreen method.[11] However, according to Rosias et al, the glass condenser was among the best apparatus for collecting exhaled albumin even when comparing to some commercial available devices (e.g., EcoScreen, Rtube, etc.).[18,19] Therefore, to further validate the effectiveness of the electret filter method, a glass condenser was designed to collect exhaled albumin in this paper.

Albumin was selected as the target protein in this paper because this protein is a widely used reference marker of dilution in

Editor: Eleonore Fröhlich.

Funding: This work was supported by the National Key Technologies R&D Program (No. 2013BAI03B04 and 2015BAI01B14).

Author Disclosure Statement: The authors declare no competing interests exist.

The authors have no conflicts of interest to disclose.

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Medicine (2018) 97:5(e9789)

Received: 31 May 2017 / Received in final form: 9 November 2017 / Accepted: 13 January 2018

http://dx.doi.org/10.1097/MD.0000000000009789

bronchoalveolar lavage fluid, that is to say, the leakage level of albumin from blood to breath is relatively stable. In this study, the breath of 6 volunteers, including both smokers and nonsmokers, was collected. We measured the amount of albumin collected by the glass condenser method and the electret filter method. It is reported that smoking may cause airway injury and increase permeability of respiratory epithelium, which may cause the leakage of albumin.[20–22] Thus, the amount of albumin exhaled by smokers was also compared with that by nonsomkers.

2. Material and methods

2.1. Study population

Six volunteers including smokers and nonsmokers were recruited from Chongqing, China. Smokers, who worked in the lab, came from a group of manual workers named the "Bang-Bang," and nonsmokers were the laboratory colleagues. All the volunteers received a physical examination to exclude pulmonary diseases. All signed the informed consent after the study was explained in detail. Protocols including any relevant details of this study were carried out in accordance with the relevant guidelines and approved by Medical Ethics Committee of Chongqing University. The information of the volunteers was listed in Table 1.

2.2. EBC and exhaled particles collection

A glass condenser was designed to collect EBC in this study (Fig. 1). The device was consisted of 3 parts: the condensation part, the exhalation nozzle, and the collecting bottle. The condensation part was composed of inner and outer layers. Ice was placed in the inner glass layer and a breath outlet connecting to a flow meter (Siargo Inc., Santa Clara, CA) was located in the outer glass. A saliva filter was fixed at the end of exhalation nozzle. Condensation happened between these 2 glass layers, and the formed EBC was collected by a 5 mL collection bottle.

The workflow for EBC collection was as follows:

1. All volunteers rinsed their mouth for 3 minutes with purified water and breathed deeply for 1 minute.
2. Volunteers wore a nose clip and then inhaled the ambient air to their vital capacities.
3. Volunteers exhaled the breath into the condensation device using a disposable mouthpiece.
4. Volunteers repeated steps 2 and 3 to exhale 100 L, 150 L, and 200 L breath, respectively.
5. The device was tapped to make the droplets hanging on the inner layer to flow into the collection bottle when collection stopped.

EBC was then transferred into 5 mL centrifuge tubes (Eppendorf, Hamburg, Germany) and stored at 4°C. Average time needed for exhaling 100 L breath was about 10 to 15 minutes. Volunteers were asked to rest for 10 minutes after they had exhaled to each volume level. Smoking was not allowed in 2 hours before and during collection. EBC collection was performed at the same place for 3 times at each breath volume level in 1 week. The 3 volume levels used in EBC collection were chosen to facilitate comparison with electret filter method. According to our previous study, a breath volume between 100 and 200 L was optimal for particle collection using electret filter method.[11] In addition, to obtain an optimal condensation effect, ice was replaced constantly during EBC collection.

Twenty four hours after EBC collection, exhaled particles collection was performed at the same place. A portable device as previously reported [11] was used for this purpose. This device was mainly comprised of a circular electret filter (North, Honeywell Inc., # 7506N99, 2 cm in diameter), an annular sealer and a saliva trap. Procedure of collecting exhaled particles was the same as in previous report.[11] Process after collection was shown in Figure 2. Volunteers were asked to rest for 10 minutes after exhaling every desired volumes of breath. Smoking was also not allowed in 2 hours before and during the collection. Particle collection was repeated in triplicate at each breath volume level in one week.

Totally, 108 samples were collected from these 6 volunteers.

2.3. Albumin concentration measurement

Amylase alpha 1 activity was firstly tested using ELISA kit (Cloud-Clone Corp., Houston, TX, # SEB482Hu, detection limit 1.3 ng/mL) to exclude saliva contamination before the albumin concentration measurement. The albumin concentration was quantified with ELISA kit (Cloud-Clone Corp., Houston, TX, # HEB028Hu) according to the manufacturer's instructions. The lowest detectable limit (LOD) of the kit was 0.61 ng/mL. Absorbance of samples at 450 nm was measured on a microplate reader (Tecan, Mannedorf, Switzerland) with the reference wavelength at 620 nm. Albumin concentrations were calculated from a fitted four-parametric standard curve using OriginPro V9.0.0 (OriginLab Corporation, Northampton, MA).

3. Calculations

Collection bottles were weighed using an analytical balance (Sartorius AG, Gottingen, Germany) when EBC was collected. If the mass of the bottle before and after collection were m_G (g) and m_G' (g), respectively, albumin collected by the glass condenser could be calculated as

$$M_G = \frac{m_G' - m_G}{\rho_G} \times C_G \quad (1)$$

where MG (ng) is the mass of albumin collected by the glass condenser and ρ_G is the density of the condensate. As the

Table 1

Study population eligible in this study.

No.	Age, years	Sex	Status	Smoking history, years	Smoking amount, packs/day
1	26	Male	Smoker	2	1
2	34	Male	Smoker	11	2
3	57	Male	Smoker	30	2
4	29	Male	Nonsmoker	N/A	N/A
5	31	Male	Nonsmoker	N/A	N/A
6	27	Male	Nonsmoker	N/A	N/A

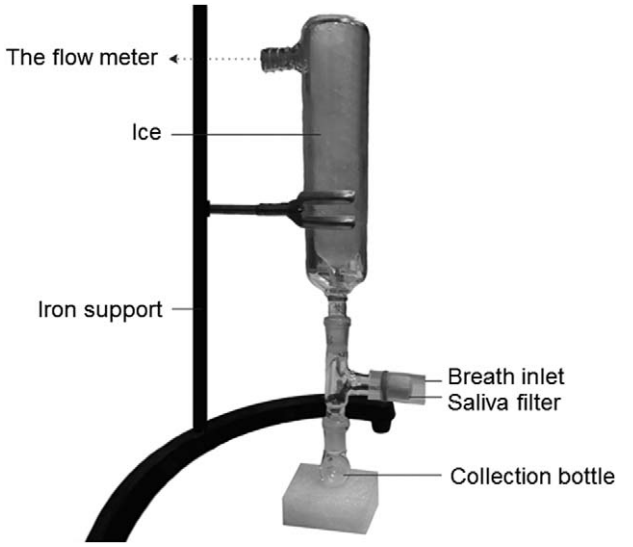

Figure 1. Glass condenser for EBC collection. EBC = exhaled breath condensate.

component of the condensate is mainly water, ρ_G is roughly estimated as 1 g/mL. C_G is albumin concentration of stored EBC measured using ELISA.

Electret filters were also weighed using the analytical balance (Sartorius AG, Gottingen, Germany) when exhaled particles were collected. If the mass of the electret filter before and after collection were m_P (g) and m_P' (g), respectively, albumin collected by the electret filter could be calculated as

$$M_P = (V_E + \frac{m_P' - m_P}{\rho_P}) \times C_P \quad (2)$$

where M_P (ng) is the mass of albumin collected by the electret filter and ρ_P is the density of the droplets adsorbed by the electret filter. As the component of the droplets is mainly water, ρ_P can be estimated as 1 g/mL. V_E (2 mL) is the volume of eluent added. C_P is concentration of albumin in the stored supernatant.

For albumin in both EBC and the supernatant measured below LOD, the concentration was defined as follows[15]:

$$C = \frac{\mathrm{LOD}}{\sqrt{2}} = 0.43\mathrm{ng/mL} \quad (3)$$

3.1. Statistical analysis

Significant difference during comparison was analyzed using nonparametric Kruskal–Wallis ANOVA and Mood's Median Test in OriginPro V9.0.0 (OriginLab Corporation, Northampton, MA). Data visualization was drawn using Anaconda (CONTINUUM Analytics, TX) based on Python programming.

4. Results

4.1. Smoking has little effect on the volume of EBC

Amylase alpha 1 in all samples in this study was not detectable (<1.3 ng/mL), which indicates that none sample collected was contaminated by saliva. The condensation efficiency of the glass condenser was 1.45 ± 0.16 mL EBC/100 L breath (mean ± SD). Small variance may indicate that smoking or not has little effect on EBC volumes. To verify this deduction, the effect of smoking (or not) on collected EBC volumes was investigated. As shown in Figure 3, no significant difference was observed in collected EBC volumes between smokers and nonsmokers ($P > .1$).

4.2. Electret filter is more effective than the glass condenser in collecting exhaled albumin

For each collection method, samples of 6 volunteers were collected at 3 breath volume levels, and collection at each volume level was repeated triplicate in one week. A total number of 108 samples ($2 \times 6 \times 3 \times 3$) were obtained in this study. Albumins in these samples were not all detectable. Actually, only 6/54 of EBC samples could detect albumin (0.63–0.97 ng/mL). In contrast, 49/54 of samples collected using electret filter method had positive detections of albumin (0.66–25.28 ng/mL). For albumin concentrations lower than LOD, they were assigned to 0.43 ng/mL according to Eq. (3).

Then, amounts of albumin collected by both methods were calculated based on Eq. (1) and (2). Significant difference of collected albumin using these 2 methods was observed at every breath volume level ($P < .01$) (Fig. 4). Therefore, the electret filter was more effective in collecting exhaled albumin than the condenser method.

4.3. Smokers tend to exhale more albumins

Finally, given the excellent performance of electret filter method in collecting exhaled albumin, the difference of exhaled albumin between smokers and nonsmokers was assessed. As shown in Figure 5, smokers exhaled more albumins than nonsmokers based on the collection results of electret filter method ($P < .01$).

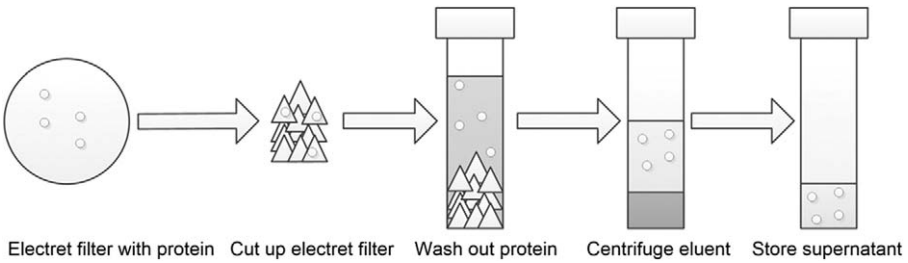

Figure 2. Process flowchart after exhaled particles collection.

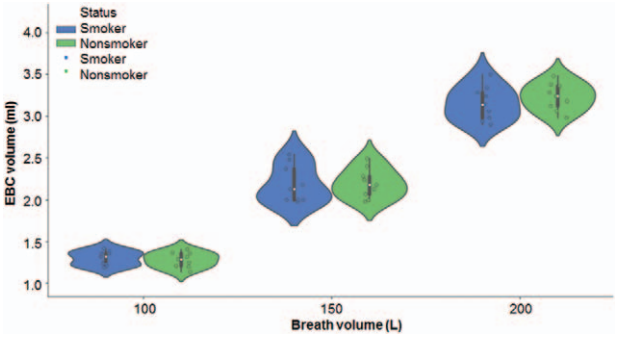

Figure 3. Effect of smoking on EBC collection. Both scatter plot and violin plot are shown. EBC = exhaled breath condensate.

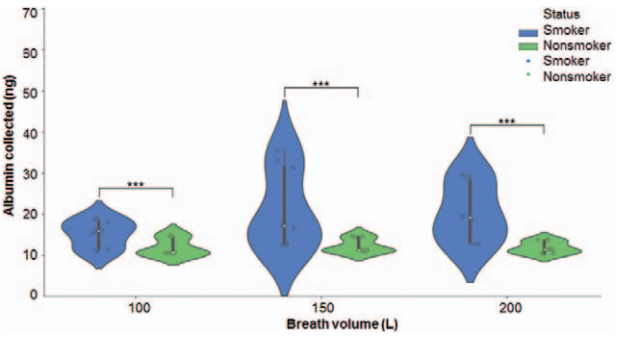

Figure 5. Effect of smoking on exhaled albumin. Both scatter plot and violin plot are shown. *** indicates significance at $P < .01$.

5. Discussion

The condensation performance of glass condenser designed in this study was comparable to that of the similar condenser used in other report.[18] That is to say, the glass condenser designed in this study was feasible. However, low positive detection of albumin in EBC collected by glass condenser was not only observed in this study, but also confirmed by other reports.[19,23] The reasons may be explained as follows. Albumin in exhaled breath is negatively charged molecule according to its isoelectric point, and the glass is also negatively charged. Thus, the repulsion between them may prevent the collection.

As described in the result, smoking or not had no significant effect on collected EBC volumes. This observation was also supported by the findings of Bloemen et al.[24] According to the report by Liu and Thomas,[25] tidal and minute lung volume could affect the amount of EBC that can be expected to be collected whereas smokers or subjects with airway disease have no significant effect on collected EBC volumes. However, the observation of this study was based on samples from 6 volunteers and future studies with more subjects are needed to validate this conclusion.

Our argument that electret filter could collect more albumins than condensation method was supported by Figure 4. The significantly higher collection efficiency of the electret filter can be easily explained. Firstly, as described above, collection based on condensation mechanism would lose and dilute the desired biomarkers severely. Loss of the biomarkers may lead to relatively low efficiency of the collection method. Massive dilution may also cause negative detection of biomarkers existed in EBC. However, electrostatic forces play a critical role in collecting nanoparticles and facilitate the collection. Charged submicron particles can be easily captured, and even neutral particles can also be polarized and finally be trapped by the electret filter.[17] In this study, isoelectric point of human albumin was situated at pH 4.8, but pH of human exhaled breath ranged from 6.5 to 7.8.[19,26] Therefore, albumin in exhaled breath was negatively charged and finally captured by the electret filter.

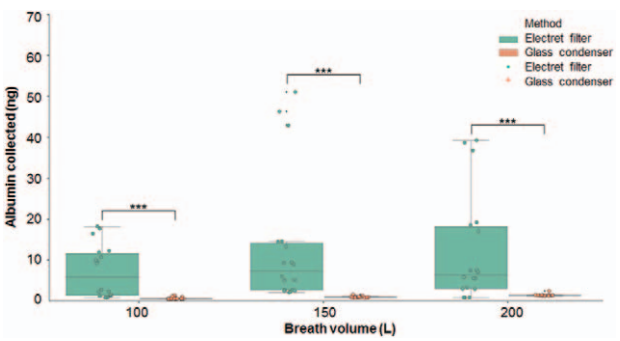

Figure 4. Comparison of 2 methods in collecting albumin. ***indicates significance at $P < .01$.

As concluded in the results part, smokers exhaled significantly more albumin than nonsmokers. This observation was consistent with other reports, for example, Morrison et al[27] also found increased albumin in broncho alveolar lavage fluid of smokers. This finding can be easily explained. As we know, smoking may cause airway injury and increase permeability of respiratory epithelium.[20–22] In this situation, blood albumin can penetrate capillaries of airway and finally leak into the breath more easily.[20,21,28] However, due to the small sample size of this study, this conclusion still needs further verification.

The changed permeability of vascular and respiratory epithelium of airway is related to many respiratory inflammations,[29–31] therefore, airway inflammations may be indirectly assessed by collecting and quantifying exhaled plasma proteins. And electret filter described in this study may show a direction of collection. However, the electret filter may be helpful in diagnosing airway inflammations and other respiratory diseases (e.g., lung cancer) noninvasively. Besides, the electret filter may be used as a convenient collector for some exhaled virus from lung and stomach.

In summary, electret filter was more effective in collecting exhaled albumin than condensation method. Based on this finding, we further suggest that electret filter is potentially more effective and helpful in collecting nonvolatile biomarkers in breath, and finally aids noninvasive detection of respiratory diseases.

Acknowledgments

The authors would like to thank all volunteers participated in this study.

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