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Efficacy of Two Breath Condensers

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Background: Examination of Exhaled Breath Condensate has been suggested to give information about inflammatory airway diseases. *Objectives*: The aim was to compare efficacy and variability in gain of two commercially available exhaled breath condensers, ECoScreen® and RTubeTM in an in vitro set up. *Methods*: Test fluids containing myeloperoxidase (MPO) or human neutrophil lipocalin (HNL) in addition to saline and bovine serum albumin were nebulized and aerosols were transferred by a servo ventilator to either of the two condensers. Analyses of MPO, HNL, or chlorine were done by means of ELISA, RIA, or a modified adsorbed organic halogen technique (AOX), respectively. *Results*: Recoveries of HNL were higher when using ECoScreen than RTube ($P < 0.05$). In contrast, there were no significant differences between the two condensers in recoveries of MPO or chlorine. The spread of data was wide regarding all tested compounds. *Conclusion*: Variability in gain was large and ECoScreen was more efficacious then RTube in condensing the tested solutes of HNL, but not those of MPO or chlorine J. Clin. Lab. Anal. 24:219–223, 2010. © 2010 Wiley-Liss, Inc.

Key words: chlorine; HNL; MPO; exhaled breath condensate; efficacy

INTRODUCTION

Exhaled air contains water vapor and small amounts of non-volatile and volatile compounds, suggested to originate from airway epithelial lining fluid (ELF) (1). Content of exhaled breath may be condensed on a cold surface and collected as condensate. Inflammation markers in exhaled breath condensate (EBC) can be analyzed and used as research tools. The clinical utility of EBC may, however, be limited owing to a number of confounding factors, such as the fact that concentrations of most biomolecules in EBC are too low to allow accurate measurement. Also, other methodological problems concerning collection and analysis of EBC have been identified (2–3). Proteins, such as albumin, were less efficiently condensed than the eicosanoid 8-isoprostane (4), suggesting polarity/electrical charge, molecular configuration, and/or other properties to be of importance for the outcome. Furthermore, operating temperatures of condensers, effective surface areas, and/or coating material differs between various types of condensers (4–7), and these factors tend to interfere with results. Also, a number of additional factors, such as different characteristics and condensability of various biomolecules, may add to variability of results, and differences in volatility and water solubility or hydrophilicity may further add to variation of results as suggested earlier (8). Each separate biomolecule may, as suggested earlier (4), perform in a specific way during condensation. If these factors are important for efficacy of retrieval of condensates, it may be important to stress the need of standardized collection procedures for each individual biomolecule (9).

The aim of this study was to compare two types of condensers, regarding efficacy and variability of gain in collecting and preserving inflammatory markers in an in vitro set up.

Myeloperoxidase (MPO), human neutrophil lipocalin (HNL), and chlorine were selected as markers of interest because of their presence in ELF found in chronic obstructive pulmonary disease (10–12), a chronic disease affecting most tobacco smokers. Test fluids containing MPO or HNL together with saline and bovine serum albumin (BSA) were nebulized and condensed.

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Chlorine, an inert constituent of the solvent, i.e., saline, was used as a reference molecule.

MATERIALS AND METHODS

Aerosols were produced by a jet nebulizer (Aiolos no 10550, Aiolos Medical AB, Karlstad, Sweden), fed by room-tempered air. Aerosols were intermittently transferred via a 30λcm long tubing system (Hytrel, Siemens, Solna, Sweden) by means of a modified servo ventilator (Servo Ventilator 900 C, Siemens) to either of the two separate condensers [ECoScreen® (Jaeger, Würzburg, Germany) or RTubeTM (Respiratory Research, Charlottesville, VA). Ventilator setting was chosen so as to mimic normal tidal breathing by a cycle frequency of 10 per minute, "tidal volume" 0.6 L, and "inspiration time" 50%.

ECoScreen is an electrically cooled condenser which is equipped with a one-way tubing system, a saliva trap, a condensing area consisting of a double lumen lamellar Teflon-coated aluminum tube and a disposable polypropylene collecting cup. Pretest cooling periods were at least 40 min.

Cooling of RTube is done by a precooled aluminum sleeve and the collecting area consists of a disposable polypropylene tube equipped with two one-way valves composed of silicone rubber; one of these is also serving as saliva trap. The cooling sleeve was kept frozen at −70°C for at least 1 hr before use.

Extensive cleaning procedures followed all experiments and all contaminated parts were flushed in hot water, repeatedly rinsed in deionized water, finally rinsed in Milli-Q® water (Millipore, Bedford, MA) and air-dried.

Condensing temperatures were measured by means of a thermistor (Digital thermometer GTH 1200, Greisinger Electronic, Germany) positioned in the center of condensing tubes at approximately half the tube length while condensing aerosolized saline (245 mg/l). The temperature measured inside the collecting tubes of ECoScreen reached −6°C or −14°C after 10 or 20 min, respectively, whereas temperature inside the collecting tubes of RTube increased successively and reached + 2°C or +7°C after 10 or 20 min, respectively.

Experimental Procedures

Biomolecules

Pretest experiments resulted in the choice of suitable concentrations of MPO (400 μg/l) or HNL (240 μg/l) put in the nebulizer cup, allowing measurement of concentrations in condensates well above limit of detection (LOD). Saline (245 mg/l) was used as solvent and BSA (0.25 mg/ml) was added to stabilize the solutions of biomolecules in all experiments. The concentration of BSA was selected according to preceding pre-test experiments in order to minimize foam formation in the jet nebulizer. Volumes of condensates were measured by means of a pipette and dispensed to three 1.5 ml plastic tubes (Sarstedt AG & Co, Nümbrecht, Germany). Condensates were kept frozen at −70°C until measurements of MPO, HNL or chlorine.

Experiment 1

Aliquots of 3 ml of test solution were nebulized during 10 min and the procedure was repeated ten times with each solution, using either ECoScreen or RTube in random order. Concentrations of MPO, HNL, or chlorine were measured in the condensates as well as in the test solutions placed in the nebulizer cup. Samples were taken from the nebulizer cup before and after each completed 10 min nebulization period for calculation of mean concentrations in the nebulizer cup. Efficacy of recovery of each molecule was defined as the concentration in the condensate divided by the calculated mean concentration in the nebulizer cup in each experiment, and expressed as percentage of the latter. Three milliliters of 0.5% Cetyl-Trimethyl Ammonium Bromide (CTAB) dissolved in saline was sprayed into the condensers and collecting cups by means of a spray catheter (PW-6P-1, Olympus, Solna, Sweden) after each completed session, in order to estimate the extent of adherence of MPO or HNL to the walls of the test system during experiments. Lavage fluids were collected and analyzed for MPO or HNL.

Experiment 2

A separate experiment was performed to assess whether efficacy of ECoScreen or RTube depended on concentration levels of chlorine in the nebulizer cup. Aliquots of 4 ml of saline with either of two different concentrations (245 or 6.4 mg/l) were placed in the nebulizer cup and nebulized for 20 min. This procedure was repeated 16 times with each concentration, using either of the two condensers in an alternating way.

Laboratory Analyses

Biomolecules

Concentrations of MPO were measured by means of MPO ELISA (Diagnostics Development, Uppsala, Sweden). LOD was 1.56 μg/l and coefficient of inter-assay variability was 0.06 ($n = 5$). Concentrations of HNL were measured by means of HNL radioimmunoassay (13). LOD was 1.0 μg/l and inter-assay coefficient of variation was 0.08 ($n = 10$).

Chlorine

Chlorine was measured in a titration cell (DIN 34809, Euro glass, Delft, The Netherlands) by means of a

modified adsorbed organic halogen technique (AOX) as described earlier (13). The results were validated by measuring standard solutions with known concentrations of chlorine [Titrisol sodium chloride 0.1 mol/l (5.844 g/l; Merck, Darmstadt, Germany) diluted with Milli-Q® water (Millipore) to concentrations of 20, 40, 200, 1,000, 2,000, and 10,000 µmol/l]. Milli-Q® water was used as blank. LOD was set to 3 µmol/l and the coefficient of intra-assay variability was 0.097 ($n = 10$). All data were transformed from molar entities to mg/l.

Statistical Analysis

Data were expressed as median (minimum to maximum) values. Mann–Whitney *U*-tests were used to compare groups of data (Statistica 7.0; Stat Soft, Inc., Tulsa, OK).

Spread of data, as defined by standard deviation (SD)/median value, was used to describe variability of gain.

RESULTS

Efficacy and Variability

Although MPO and the higher concentration of chlorine tended to be more efficiently recovered by ECoScreen rather than by RTube during the 10 min nebulization periods, only HNL was significantly more efficiently collected by ECoScreen rather than by RTube ($P < 0.05$; Fig. 1).

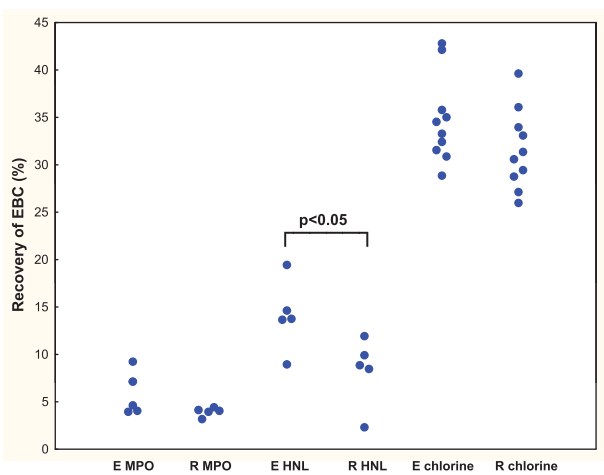

Fig. 1. Recovery of HNL, MPO, and chlorine after 10 min nebulization and condensation by either of two separate condensers; ECoScreen (**E**) or RTube (**R**), in experiment 1 (see text for details). Solutions of MPO and HNL were nebulized five times to each condenser resulting in 10 condensates of chlorine of each condenser (E) and (R) (see text for details). Recovery was defined as concentration of each molecule in the condensate divided by the calculated mean concentration in the nebulizer cup and expressed as percentage of the mean.

Contrasting results were found in nebulization of the lower concentration of saline, which resulted in significantly more chlorine recovered by RTube than by ECoScreen. This was demonstrated in a separate experiment nebulizing two different concentrations of saline for 20 min, thus showing concentration dependency of recovery favoring RTube ($P < 0.001$; Fig. 2)

Calculation of efficacy was based on the assumption that changes in concentrations of solutes in the nebulizer cup were linear and time dependent. Increase of concentrations of saline in the nebulizer cup ranged from 6 to 12% during the 10 min long nebulizations. In contrast, concentrations of MPO as well as of HNL fell during the same time periods of nebulizations by approximately 43 and 13%, respectively. Discrepancy in behavior of the various solutions made us suspect adherence to surfaces and/or degradation of MPO and HNL. Washing the walls of the condensing system with CTAB revealed presence of MPO in all lavage fluids tested [5.2 (1.7–10.1) µg/l], suggesting adherence of MPO to exposed surfaces. In contrast, HNL was not measurable in any of the lavage fluids, suggesting no major adherence to surfaces. Significant degradation (18–25%) of HNL was observed in samples of test solutions that were left in vitro at room temperature for 2 or 3 hr. Degradation of MPO during similar conditions was approximately 7%.

Variability in measured concentrations of MPO or HNL in condensates were wide using any of the devices; the spread of MPO retrieval ranged from 36 to 38% using ECoScreen and from 10 to 44% using RTube. Corresponding figures for HNL ranged from 12 to 30%

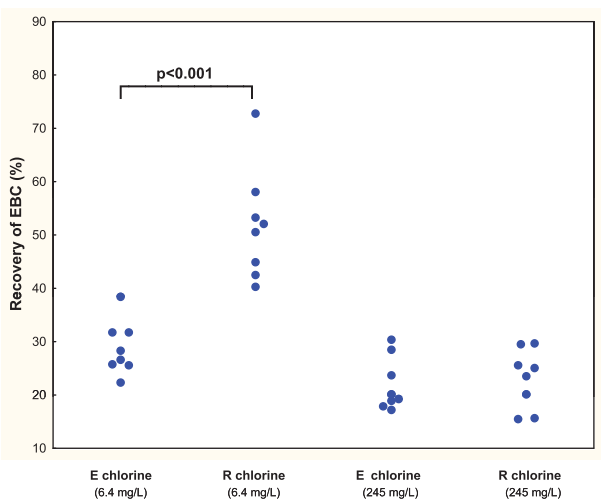

Fig. 2. Recovery of chlorine of two different concentrations (245 or 6.4 mg/l) after 20 min nebulization and condensation by either of two separate condensers; ECoScreen (**E**) or RTube (**R**), experiment 2 (see text for details). Recovery was defined as concentration of each molecule in the condensate divided by the calculated mean concentration in the nebulizer cup and expressed as percentage of the latter.

for ECoScreen and from 24 to 45% for RTube. Variability of chlorine tended to be lower and ranged from 19–26% using ECoScreen and 19–23% using RTube.

EBC Volumes

Volumes of condensates collected during 10 min of condensation by ECoScreen or RTube, respectively, did not differ significantly (E: 430 [330–500] μl vs. R: 395 [350–475] μl, $P > 0.05$). In contrast, significantly higher volumes of condensates were recovered by ECoScreen rather than by RTube during the 20 min nebulizations (E: 865 [770–1025] μl vs. R: 750 [700–825] μl, $P < 0.001$).

DISCUSSION

We have shown that variability of recoveries by both test systems were large and that ECoScreen tended to be more efficacious than RTube in sampling of HNL. Focusing on chlorine, which is presumed not to be degraded or adherent to surfaces, revealed that more than 50% of chlorine was lost in both test systems. These massive losses are assumed to pertain at least partly to losses in the tubing system, and hence are attributed to the test system per se. Furthermore, a fraction of the air containing aerosolized biomolecules may also have been lost owing to the design of the condensers, which permits an unknown fraction of the aerosols to escape from the devices. The technique of harvesting the condensates from the devices, and low concentration levels of inflammation markers relative to sensitivity of analysis kits, may further add to the variability of results. In addition, a number of other confounding factors may contribute to massive loss and/or variability. The tendency of some molecules to adhere to surfaces has earlier been reported (4,14), and this was also suspected by the finding of a much larger fall of concentrations of biomolecules than of chlorine in condensates vs. the nebulizer cup (10–40:1 vs. 3:1). Furthermore, although concentrations of chlorine in the nebulizer cup increased during nebulization, as foreseen by earlier reports (15), levels of MPO or HNL in the nebulizer cup decreased during identical procedures, suggesting degradation and/or adhesion to exposed surfaces. Adherence to surfaces was confirmed by washing the devices after the main experiments, using a solution of CTAB, which is known to be a functional surfactant. Substantial amounts of MPO but not of HNL were found in the lavage fluids confirming adherence of MPO to exposed surfaces. In contrast, HNL was not found in lavage fluid, suggesting none or minimal adherence of HNL. On the other hand, degradation of HNL in vitro was evident by the fact that concentrations fell around 20% in samples of test solutions left in vitro at room temperature for up to 3 hr. In contrast with HNL, we found degradation of MPO in vitro to be less than 10%. We conclude that adherence to exposed surfaces of the device as well as degradation occurred despite efforts to stabilize test solutions by adding BSA.

We also examined if efficacy of condensation of saline was dependent on the concentration level of solutes in the nebulizer cup, by nebulizing two separate concentrations of saline. It was confirmed that the lower concentration (that was similar to the one recorded earlier in EBC recovered from healthy volunteers and asthmatics) (16,17) was more efficiently collected by RTube than by EcoScreen (17). This advantage of RTube over ECoScreen was, however, not true when nebulizing a higher concentration of chlorine. We do not know whether the demonstrated concentration dependent differences in efficacy also pertain to MPO or HNL in our test system.

Influence of temperature of the device and duration of condensation has earlier been reported (6,18) and is now also supported by our results. As in earlier reports (19), we found that volumes of condensates did not differ in the 10 min experiments, whether recovered by ECoScreen or RTube. By contrast, higher volumes were recovered by ECoScreen rather than by RTube during longer condensations, also shown by Soyer et al. (6). This difference is presumed to depend on the fact that while ECoScreen is capable of maintaining a constant low temperature during longer periods of time, temperature in RTube successively increases during a 20 min session and ultimately reaching a temperature well above 0°C. The higher temperature is presumed to result in less efficient condensation.

True concentrations of MPO or HNL in ELF are not known, even if levels in bronchial lavage (BL) or bronchoalveolar lavage (BAL) fluids has been reported to be around 750 (MPO) or 200 μg/l (HNL) (10,12,20), respectively. Dilution of ELF by saline during a BAL process may vary (21) and a useful dilution marker has not been identified despite several trials (22,23). The lack of feasible reference substances incapacitates evaluation of the true local concentration of inflammation products in BL or BAL as well as in EBC. Furthermore, local concentrations of inflammation markers may depend on the actual state of activity in the corresponding cellular sources of the studied compartment. This, in turn, may lead to levels of certain molecules exceeding the one in other compartments, as suggested earlier, by a significantly higher level of 8-isoprostane, nitrogen oxides, and phospholipids in EBC rather than in BAL or proteins to be higher in BAL rather than in EBC (8). Such data emphasizes the need to collect samples from identifiable parts of the airways, which is impossible by means of

the EBC method because exhaled air passes many contributing parts of the airways, including the oral cavity (24).

We conclude that despite large variability in gain of MPO or HNL, the results tend to support the notion of ECoScreen being more efficacious then RTube concerning some tested solutes. In agreement with earlier reports (4),) we suggest that each separate biomolecule may perform in a specific way during condensation, possibly owing to characteristics, such as differences in adherence, rate of degradation in vitro, polarity, volatility, hydrophilicity, or thermolability. It is important to emphasize that results of this in vitro study can not be directly extrapolated to be valid for EBC collected in clinical studies. The concept of measuring inflammation markers in secretions from an inflamed locus of the airways in a noninvasive and patient-friendly set-up, however, seems desirable and further studies on exhaled air are still warranted.

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