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TITLE

***In vitro* Method to Control Concentrations of Halogenated Gases in Cultured Alveolar Epithelial Cells**

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KEYWORDS

Cell culture, sevoflurane, isoflurane, halogenated gases, alveolar epithelial cells, lung, ARDS, air-liquid interface, chromatography

SUMMARY

We describe an easy protocol specifically designed to reach precise and controlled concentrations of sevoflurane or isoflurane *in vitro* in order to improve our understanding of mechanisms involved in the epithelial lung injury and to test novel therapies for acute respiratory distress syndrome.

ABSTRACT

Acute respiratory distress syndrome (ARDS) is a syndrome of diffuse alveolar injury with impaired alveolar fluid clearance and severe inflammation. The use of halogenated agents, such as sevoflurane or isoflurane, for the sedation of intensive care unit (ICU) patients can improve gas exchange, reduce alveolar edema, and attenuate inflammation during ARDS. However, data on the use of inhaled agents for continuous sedation in the ICU to treat or prevent lung damage is lacking. To study the effects of halogenated agents on alveolar epithelial cells under "physiologic" conditions, we describe an easy system to culture cells at the air-liquid interface and expose them to halogenated agents to provide precise controlled "air" fractions and "medium" concentrations for these agents. We developed a sealed air-tight chamber in which plates with human alveolar epithelial immortalized cells could be exposed to a precise, controlled fraction of sevoflurane or isoflurane using a continuous gas flow provided by an anesthetic machine circuit. Cells were exposed to 4% of sevoflurane and 1% of isoflurane for 24 hours. Gas mass spectrometry was performed to determine the concentration of halogenated agents dissolved in the medium. After the first hour, the concentrations of sevoflurane and isoflurane in the medium were 251 mg/L and 25 mg/L, respectively. The curves representing the concentrations of both sevoflurane and isoflurane dissolved in the medium showed similar courses over time, with a plateau reached at one hour after exposure.

This protocol was specifically designed to reach precise and controlled concentrations of sevoflurane or isoflurane *in vitro* to improve our understanding of mechanisms involved in epithelial lung injury during ARDS and to test novel therapies for the syndrome.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a clinical syndrome characterized by diffuse alveolar injury, lung edema, and hypoxemic respiratory failure. Although ARDS represents more than 10% of intensive care unit (ICU) admissions and nearly 25% of ICU patients requiring mechanical ventilation, it is still an under-recognized challenge for clinicians, with a hospital mortality rate of 35-45%[1]. Despite intense research, the identification of an effective ARDS pharmacologic therapy or prevention has failed to date. Two major features contribute to mortality in ARDS: impaired alveolar fluid clearance (AFC) (*i.e.,* the altered resorption of alveolar edema fluid from distal lung airspaces) and severe inflammation[2]. Since ARDS mortality remains high, current initiatives should also include primary prevention; however, a key challenge is to identify at-risk patients in whom ARDS is likely to develop and who would benefit if ARDS were prevented.

Volatile halogenated anesthetics, such as sevoflurane and isoflurane, are widely used to provide general anesthesia in the operating room. Worldwide, more than 230 million patients undergoing major surgery each year require general anesthesia and mechanical ventilation[3], and postoperative pulmonary complications adversely affect clinical outcomes and healthcare utilization[4]. The use of sevoflurane instead of propofol was associated with improved lung inflammation in patients undergoing thoracic surgery and significant decreases in adverse events, such as ARDS and postoperative pulmonary complications[5]. Similarly, pretreatment with

isoflurane had protective effects on respiratory mechanics, oxygenation, and hemodynamics in experimental animal models of ARDS[6,7]. Although further studies are warranted to address the impact of inhaled agents on outcomes in noncardiac surgery, a similar decrease in pulmonary complications has been recently observed in a meta-analysis, demonstrating that inhaled anesthetic agents—as opposed to intravenous anesthesia—are significantly associated with a reduction in mortality for cardiac surgery[8].

Specific prospective data about the use of volatile agents for the sedation of ICU patients to prevent or treat lung damage is lacking. However, several trials now support the efficacy and safety of inhaled sevoflurane for the sedation of ICU patients, and preclinical studies have shown that inhaled sevoflurane and isoflurane[7,9] improve gas exchange, reduce alveolar edema, and attenuate inflammation in experimental models of ARDS. Additionally, sevoflurane mitigates type II epithelial cell damage[10], whereas isoflurane maintains the integrity of the alveolar-capillary barrier through modulation of tight junction protein[11]. However, further studies are needed to verify to what extent the experimental evidence of organ protection from inhaled sevoflurane and isoflurane could be translated to humans. A first single-center randomized controlled-trial (RCT) from our group found that early use of inhaled sevoflurane in patients with ARDS was associated with improved oxygenation, reduced levels of some pro-inflammatory markers, and reduced lung epithelial damage, as assessed by the levels of the soluble form of the receptor for advanced glycation end-products (sRAGE) in plasma and alveolar fluid[12].

Taken together, the beneficial effects of sevoflurane and isoflurane on lung injury could point to multiple biological pathways or functional processes that are dependent on the RAGE pathway, namely alveolar fluid clearance (AFC), epithelial injury, translocation of nuclear factor (NF)-κB, and macrophage activation. In addition, sevoflurane may influence the expression of the RAGE protein itself. Since previous research by our research team and others supports pivotal roles for RAGE in alveolar inflammation and lung epithelial injury/repair during ARDS, we designed an experimental model to provide a translational understanding of the mechanisms of sevoflurane in lung injury and repair[13–15]. The *in vitro* effects of sevoflurane and isoflurane were investigated in a novel human alveolar epithelial primary cell line specifically designed to study the air-blood barrier of the peripheral lung, hAELVi (human Alveolar Epithelial LentiVirus immortalized), with alveolar type I-like characteristics including functional tight junctions[16].

While preparing the design of our *in vitro* investigations (*e.g.,* cultures of alveolar epithelial cells at the air-liquid interface with exposure to "inhaled" sevoflurane or isoflurane, we understood from previously published studies that fractions of sevoflurane have only been assessed in the "air" interface[17–19] using standard monitors (similar to those used in a clinical setting). Halogenated agent concentrations were usually chosen according to the minimum alveolar concentration (MAC) values (*e.g.,* in humans, for sevoflurane, 0.5, 1.1, and 2.2 vol%, representing 0.25, 0.5, and 1 MAC, respectively; for isoflurane, 0.6, 0.8, and 1.3 vol% representing 0.25, 0.5, and 1 MAC, respectively)[20]. Indeed, sevoflurane and isoflurane concentrations have never been investigated in the culture medium itself, thus limiting the validity of previous experimental models/instruments. Furthermore, most experiments used an anaerobic jar that was sealed after the air mix containing sevoflurane had been flushed inside. As our goal was to study alveolar

epithelial cells under "physiologic" conditions, we believed that such an anaerobic state may not be optimal and would not be compatible with long experimental durations. Therefore, we developed our own system to culture cells at the air-liquid interface and expose them to halogenated agents (sevoflurane and isoflurane) with the aim of providing precise controlled "air" fractions and "medium" concentrations for these agents. In our opinion, this experimental step, which has not been reported to date in the literature, is mandatory prior to any further *in vitro* investigations of sevoflurane and isoflurane.

PROTOCOL

1. Culture of Alveolar Epithelial Cells (hAELVi)

1.1. Thawing

1.1.1. Pipette 4 mL of cultivation ready-to-use human alveolar epithelial (huAEC) medium in a 15 mL plastic tube and quickly thaw the vial in a preheated water bath (37 °C).

1.1.2. Transfer the thawed cell suspension to a 15 mL plastic tube containing 4 mL of the medium before centrifuging the tube at 200 x g for 5 min.

1.1.3. Aspirate the supernatant and resuspend the cell pellet with 5 mL of the cultivation medium. Then, transfer the cells to a T25 flask.

1.1.4. Cultivate the cells under standard conditions (5% CO_2, 95% humidified air, 37 °C)

1.2. Splitting

1.2.1. Check the status of the cells microscopically. When the cells are 80-90% confluent, split the cells, following steps 1.2.2 through 1.2.10.

1.2.2. Aspirate the cultivation media of the cells with a sterile pipette.

1.2.3. Wash the cells once with 4 mL of Dulbecco's Phosphate Buffered Saline (DPBS) and aspirate the DPBS.

1.2.4. Add 1 mL of Trypsin/EDTA solution (TE) to the cells prior to incubating at 37 °C for 3 min until the cells start to detach; check for detachment under a microscope.

1.2.5. Resuspend the cells with 2 mL of the culture medium and centrifuge the cells at 200 x g for 5 min. Then, aspirate the supernatant and resuspend the cell pellet again with 3 mL of the cultivation medium.

1.2.6. Use the trypan blue dye exclusion assay to determine the viability of the cells. Take 30 µl of the cell suspension and add 30 µl of a 0.4% solution of trypan blue in a tube. After that,

effectively mix the solution 3-5 times using a 100 µl pipette. Take 10 µl of the solution and put it under the coverslip of the hemocytometer.

1.2.7. Count both the total number of viable cells and the number of dead cells (blue) in the areas of the hemocytometer. Then, calculate the cell viability [%] using the equation: cell viability = 100 – (100 / total number of cells x number of dead cells)

1.2.8. Transfer the aliquot with the resuspended cell pellet to a new cell culture T25 flask or a 6 well-plate. Add the cultivation medium to the cells to achieve a total of 5 mL of cultivation medium in the T25 flask, or 1 mL for each well of the 6 well-plate.

1.2.9. Cultivate the cells in an incubator under standard conditions (5% CO_2, 95% humidified air, 37 °C)

1.2.10. Once the cells are completely confluent, they are ready for the experiment.

2. Preparation of an Air-tight Chamber

Note: The construction plan for the air-tight chamber is depicted in **Figure 1.**

2.1. Use a hermetic polypropylene box with a capacity of 6.5 L. The length, width, and height are 30 x 20 x 15.5 cm, respectively. Please note, the volume of the box is lower than the theoretical volume because of the rounded corners.

2.2. Drill a 2.5 cm diameter hole on the bottom side of the lateral wall.

2.3. Insert a corrugated tube with a green mark, which will serve as the gas-air mixture input pipe, and seal it with silicon.

2.4. Drill a second 2.5 cm diameter hole on the top side of the opposite lateral wall.

2.5. Insert another corrugated tube with a red mark and connect it with a charcoal filter, which will serve as the gas-air mixture output pipe, and seal it with silicon.

2.6. Drill a tight 4 mm diameter hole at the center of the mean wall of the box.

2.7. Insert short infusion tubing that is connected at a manifold with a rotating male luer-lock, which will be plugged into a gas analyzer, and seal it with silicon.

2.8. Place a digital thermometer/hygrometer inside the air-tight chamber.

3. Expose Alveolar Epithelial Cells to Halogenated Agents (Sevoflurane and Isoflurane)

Note: A schematic drawing of the device is depicted in **Figure 2.**

CAUTION: Although animal studies have revealed no evidence of fetal harm or impaired fertility, and a very small study during cesarean sections did not show any untoward effects on the mother or fetus, the safety of using halogenated agents (*e.g.,* sevoflurane or isoflurane) during labor and delivery has not been demonstrated to date. Furthermore, no controlled data has been collected during human pregnancies. Therefore, performing experiments using sevoflurane or isoflurane while pregnant should be strongly discouraged.

3.1. Work under a laboratory extractor hood.

3.2. Customize an anesthetic machine circuit to switch the gas line of nitrous oxide by carbon dioxide (CO_2).

3.3. Plug the air-tight chamber with the green-marked, corrugated tube into the customized anesthetic machine circuit. Insert a heated humidifier (such as those used on ICU ventilators) into the pipe between the anesthetic machine and the air-tight chamber to warm the gas flow mixture to approximately 37 °C

3.4. Install the air-tight chamber on a hot plate, providing a heating plate temperature of 37°C.

3.5. Put the 6 well-plate containing the hAELVI cells into the air-tight chamber and seal the lid.

3.6. Regulate the gas flow rates (*i.e.,* the mixture of air and CO_2) to quickly obtain the standard conditions, defined as 5% of CO_2 and 95% of humidified air.

3.7. Open the halogenated agent evaporator and choose the percentage desired (in the present study, the tested concentrations of sevoflurane and isoflurane were 4% and 1%, respectively).

3.8. Note the composition of gas mixture and the sevoflurane or isoflurane concentration as measured by an external gas analyzer and displayed on the screen.

3.9. Once the target values are achieved, reduce the fresh gas flow rate to 1 L/min.

3.10. The air-tight chamber can be maintained with this gas flow rate as long as necessary for the experiment.

4. Measure Sevoflurane or Isoflurane by Chromatography

4.1. Preparation of samples

4.1.1. At different time points, very briefly open the chamber to take out the studied samples (in the present study, we used a 6 well-plate) and close the lid. Keep the other samples in the box. Then, aspirate 1 mL of the medium contained in each sample with a multi-volume adjustable micropipette.

4.1.2. Put the medium into 10 mL headspace chromatography vials, which should be screwed hermetically tight with a Teflon-sealed cap. Freeze the chromatography vials at -20 °C if you do not use them immediately.

4.1.3. Prepare a stock solution of sevoflurane and another stock solution of isoflurane, both at 50 g/L in methanol. Simultaneously, prepare a stock solution of chloroform (internal standard, IS) at 2 g/L in methanol. Store all standard solutions at -20 °C.

4.1.4. Prepare working solutions of sevoflurane and isoflurane at 50, 500, and 5000 mg/L in ultrapure water/dimethyl sulfoxyde (50/50; v/v). For internal standardization, the working solution is fixed at 100 mg/L in methanol.

4.2. Analysis of cellular samples

Note: The extraction procedure is based on the previously validated method of gas chromatography and mass spectrometry from Bourdeaux *et al.*[1] and uses the same parameters of sensibility and specificity. Sevoflurane and chloroform (IS) were used in this protocol, and isoflurane was associated with the multiparametric analytical method. Briefly, for mass spectrometry acquisition, the method was developed after pure solution injection. Then, m/z was confirmed with reference standards and literature data. Three m/z were selected for each analyte (except IS): one m/z for quantification (the most abundant and the higher), for which abundance was calculated by integrating the area under the curve for quantification, and two m/z for confirmation. Using three m/z, analytes could be specifically identified because all m/z had the same retention time, as well as because all m/z amounts (relative of ion confirmation *vs.* quantification) were the same in the pure standard and in all samples. With this acquisition mode, analytes could be identified and quantified with good specificity.

4.2.1. Construct a calibration 8-point curves with the concentration ranges of 0.5-400 mg/L and multiple quality controls (0.5, 1, 5, 10, 20, 75, 200, and 400 mg/L).

Note: To validate each calibration, four quality controls were used: the lower limit of quantification (C1 = 0.5 mg/L), two intermediate levels (C2 = 20 mg/L and C3 = 75 mg/L) and the final level (C4 at 400 mg/L; upper level of quantification). All standards and controls were analyzed in cultured cell matrices to avoid the matrix effect. For each calibration curve, a blank matrix was analyzed to validate that there was no interference with cultured cellular and internal standards.

4.2.2. Prepare a stock solution of sevoflurane and another stock solution of isoflurane, both at 50 g/L in methanol. Simultaneously, prepare a stock solution of chloroform (internal standard, IS) at 2 g/L in methanol. Store all standard solutions at -20 °C. Then, prepare working solutions of mixed sevoflurane/isoflurane at 50, 500, and 5000 mg/L in ultrapure water / dimethyl sulfoxyde (50/50; v/v). For IS, the working solution is diluted at 100 mg/L in methanol.

4.2.3. For calibration curves and controls, prepare the samples by spiking 50 µL of IS (100 mg/L) into 1 mL of the cellular sample matrices.

4.2.4. Prepare sample solutions with 200 µL of saturated sodium chloride water solution in a 10 mL headspace tube, screwed hermetically tight with a Teflon-sealed cap.

4.3. Gas chromatography and mass spectrometry

4.3.1. For sample analyses, use headspace injections in a gas chromatography, coupled with the mass detection method.

4.3.2. Carry headspace tubes for 10 min at 80 °C with a heater shaker. Then, withdraw and inject 1.5 mL of the gas sample into the gas chromatograph. Set the parameter of the injector at 260 °C with a split flow at 100 mL/min for 2 min at the start of the chromatography run.

4.3.3. Use Split/splitless injector with a carrier mode-programmed pressure. First, keep the gas pressure at 40 kPa for 0.15 min. Then, increase the rate program pressure to 150 kPa at 125 kPa/min before setting a rate of 16 kPa/min to 300 kPa pressure for 5 min.

4.3.4. Simultaneous to the injection, start with an oven temperature of 60 °C for 1 min and increase until a temperature of 140 °C is reached at a rate of 20 °C/min. Then, increase the temperature again until 250 °C is achieved. The total time of the run is 7 min.

4.3.5. Carry out the chromatography separation using a fused-silica capillary column (30 m x 1.4 µm, 0.25 mm ID). Perform mass experiments with a single ion monitoring (SIM) condition, and monitor ion quantification m/z 181, and ion qualifications m/z 151 and 51 simultaneously at a retention time (RT) of 2.30 min for sevoflurane, m/z 149 (ion quantification), m/z 115 and 87 (ion qualifications) for isoflurane at RT 2.8 min, and m/z 83 (ion quantification) for chloroform at RT 3.70 min.

4.3.6. Determine the concentrations of sevoflurane and isoflurane in the cell culture by their area ratios to that of the IS using a weight quadratic fit. The lower limit of quantification (LLOQ) for sevoflurane and isoflurane was at 0.5 mg/L and the upper limit of quantification (ULOQ) was 400 mg/L.

REPRESENTATIVE RESULTS

The concentrations of the sevoflurane and isoflurane, which dissolved in the medium over time, are reported in **Table 1** and **Table 2**, respectively.

The courses of the sevoflurane and isoflurane concentrations in the medium were similar over time. Immediately after the required concentration of halogenated agent was set, concentrations rose over the first hour. A plateau was then reached, which persisted until the administration of

the halogenated agent was stopped. After administration interruption, concentrations decreased within one hour (**Figure 3**).

After the first hour, the median concentrations of sevoflurane and isoflurane in the medium were 251 mg/L and 25 mg/L, respectively. No significant difference was found between the different experiments.

FIGURES & TABLES

Figure 1: **Construction plan of the air-tight chamber**

Figure 2: **Schematic drawing of the device**

Figure 3: **Concentration of sevoflurane (n = 5) and isoflurane (n = 5) over time**. **A)** Concentration of halogenated agent over time. Values are expressed in mg/L. Values are expressed in mean and SEM. **B)** Concentration of halogenated agent over time for each experiment. Value are expressed in mg/L. **C)** Fraction of halogenated agent over time in the air-tight chamber measured by the gas analyzer. Values are expressed in percentages.

Table 1: **Concentrations of sevoflurane dissolved in the medium over time**. Numerical data are expressed as a median value with interquartile range for the concentration and as percentage for the fraction. IQR (for interquartile range)

Table 2: **Concentrations of isoflurane dissolved in the medium over time**. Numerical data are expressed as a median value with interquartile range for the concentration and as percentage for the fraction. IQR (for interquartile range)

DISCUSSION

Our protocol describes an easy method to expose cells to a precise fraction of a halogenated anesthetic agent, such as sevoflurane or isoflurane. Furthermore, we report here—for the first time—a rigorous correlation between both the gas fraction and the concentration of sevoflurane and isoflurane inside the culture medium itself. This fundamental step now allows us to safely use our air-tight chamber to study the effects of these halogenated agents in a cultured monolayer of human alveolar epithelial cells.

Currently, most research teams studying the effects of sevoflurane in alveolar cells use a jar that is first saturated with halogenated gas and then sealed. In this case, sevoflurane may be metabolized, and it could be speculated that the fraction of volatile agent may decrease linearly over time, leading to an unstable gas concentration. However, the correlation between the gas fraction of sevoflurane and its concentration in the culture medium is not clearly reported in the literature. Usually, the concentration of sevoflurane used in these experiments is chosen based on a simple relationship between the gas fraction and the MAC. MAC was introduced in 1965 and is the concentration of a vapor in the lungs that is needed to prevent a motor response (movement) in 50% of subjects in response to a surgical stimulus (pain)[22]. MAC is used to compare the strength, or potency, of anesthetic vapors. In ICU patients, MAC is correlated to

FeSevo and the clinical Richmond Assessment Agitation-Sedation Scale (RASS)[23]. Although it is a useful indicator in daily clinical practice, the relevance of this parameter has never been investigated in the setting of experimental *in vitro* research. In our protocol, using chromatography analyses of the medium, we determined the precise correlation between the sevoflurane contained in the gas fraction and the sevoflurane dissolved into the medium. With this method, the specific effect of a volatile agent is expressed according to the real concentration in the medium rather than based on the approximation of a clinical effect. This important element allows the study of the specific effect of a precise concentration of a halogenated agent on cells growing in a medium, in order to compare the effects of different concentrations of inhaled agents. Furthermore, as the air-tight chamber is very easy to use, this method allows researchers to replicate the experiment with precision.

Another important point that may preclude the use of the correlation between gas fraction and MAC in experimental research is that a halogenated agent has low solubility in blood (blood/gas partition coefficient at 37°C = 0.63 to 0.69 for sevoflurane). A minimal quantity of sevoflurane is mandated to dissolve in the blood before the pressure in the alveoli achieves equilibrium with the pressure in the arterial. Thus, during the induction of anesthesia, the alveolar (end-tidal) concentration (AF, alveolar fraction) of sevoflurane rapidly increases around the inspired concentration (FI, inspired fraction). However, *in vitro* culture conditions do not allow such mechanisms, and usual cell media mainly consist of aqueous solutions. Furthermore, the solubility coefficient between water/gas (partition coefficient at 37°C = 0.36 for sevoflurane) is lower than between blood and gas, underlying the critical importance of performing chromatography analyses.

Additionally, when a sealed jar is used, the atmospheric oxygen in the jar is absorbed by the cells with the simultaneous generation of carbon dioxide. This effect is probably insignificant in short experimental procedures, but for longer experimental durations, cells that are deprived of oxygen would switch to an anaerobic metabolism; this change in metabolism may induce a certain degree of bias in experimental analyses. In contrast to the sealed jar, when using our air-tight chamber, both oxygen and halogenated agent flows are adjustable over time to maintain the targeted level. This major characteristic of our protocol therefore allows the design of *in vitro* experiments for long time periods (*e.g.,* more than one day), making it an interesting tool to study the cellular mechanisms involved in lung epithelial injury and repair over time, especially when halogenated agents are used. Indeed, the effects of inhaled anesthetic agents on lung cells or tissue during alveolar injury remain poorly investigated to date while this alternative therapy seems to show very encouraging results[12].

However, there are limitations to this technique. First, an anesthetic machine circuit is needed to provide oxygen, carbon dioxide, and halogenated agent gas flows. Using such a device is mandatory to set the flow rate and maintain stable concentrations over time. Second, to sample the medium prior to chromatography analyses, the air-tight chamber is briefly opened, which induces a transient decrease in the gas concentrations. As we use an anesthetic machine circuit, gas flows are thereafter increased until expected concentrations are achieved again on the gas analyzer. Third, we have measured the concentration in the medium for only one fraction of each

halogenated agent, chosen *a priori* based on previous study. Fourth, to stabilize the cell medium to the growth of alveolar epithelial cells, we need to use carbon dioxide at a concentration of 5%. Indeed, no anesthetic machine circuit provides such a concentration of carbon dioxide. Therefore, the anesthetic machine circuit needs to be customized to allow the connection of carbon dioxide gas flow in place of nitrous oxide. Such a connection should be used exclusively in the setting of experimental research and should cautiously be unplugged after each experiment. Furthermore, to avoid any risk for humans, we invite researchers to use a devoted anesthetic machine circuit to perform this protocol and not to use a machine dedicated to clinical anesthesia.

The main advantages of this technique are that it is relatively inexpensive and very easy to adopt, even when researchers have never manipulated an airtight chamber before. Moreover, with our protocol, the results of dissolved sevoflurane and isoflurane concentrations are reproducible, which represents a major quality criterion for experimental research. In addition, our system could allow for the study of other volatile halogenated agents, such as desflurane. Indeed, a simple change of the type of gas evaporator device would be sufficient in this case. Similarly, our system could provide a means to study the concentrations of sevoflurane or isoflurane dissolved in any type of medium with different solubilities, such as water, blood, or oil.

Our experiment represents a fundamental step that is part of a larger project designed to test the hypothesis that sevoflurane and isoflurane may exert beneficial effects on lung injury, inflammation, and AFC through RAGE-mediated pathways. A primary culture of human alveolar epithelial cells will be used for mechanistic investigations of transepithelial fluid transport, channel-specific fluid transport (*e.g.,* using pharmacological antagonism), epithelial paracellular permeability, wound repair, cell migration and proliferation, with or without a halogenated anesthetic agent (sevoflurane or isoflurane), alone or combined with cytomix (*in vitro* model of alveolar injury)[24].

In conclusion, this protocol was specifically designed to reach precise and controlled concentrations of sevoflurane or isoflurane *in vitro* to improve our understanding of mechanisms involved in epithelial lung injury during ARDS and to test novel therapies for this frequent and life-threatening syndrome.

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DISCLOSURES

The authors declare that they have no competing financial interests.

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Figure 3

Click here to access/download;Figure;Figure 3_Concentrations over the time.tiff

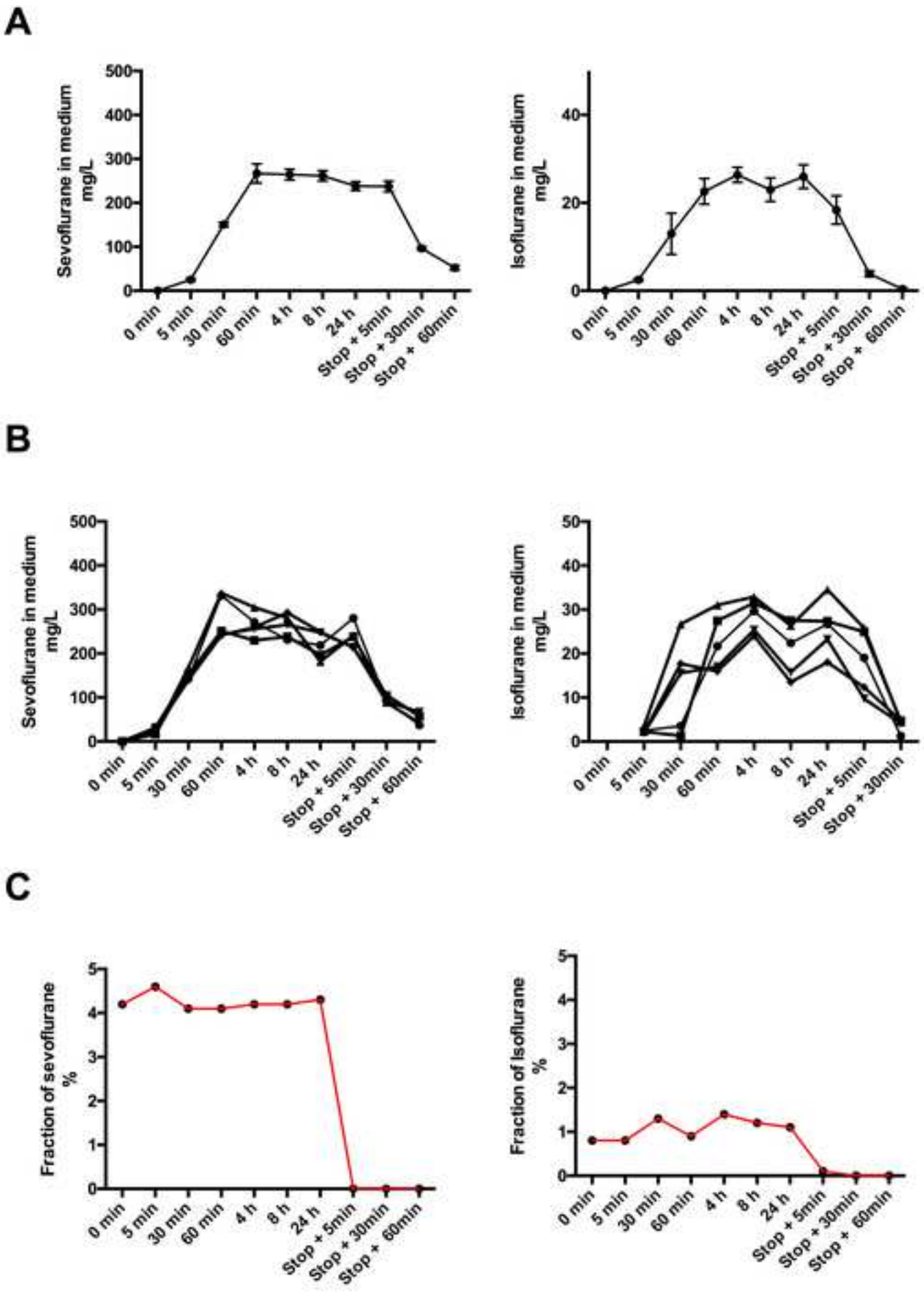
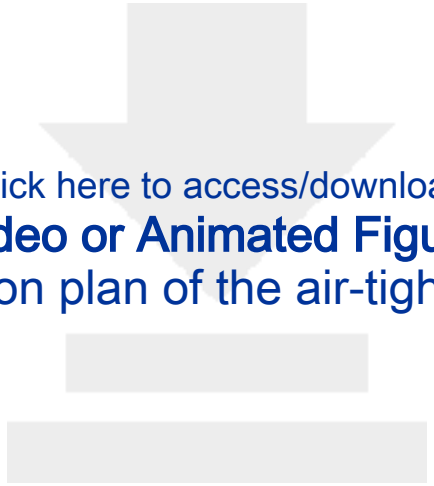


Figure 1



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Video or Animated Figure

R2_Construction plan of the air-tight chamber.svg

Figure 2

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Figure 2_Schematic drawing of the device.svg

Name of Material/ Equipment
Sevoflurane
Isoflurane
Human Alveolar Epithelial cells
huAEC Medium (ready-to-use)
Anesthetic machine circuit
Gas analyzer
Anesthetic gas filter
Heated Humifier
Chamber
Gas chromatography coupled with mass detection
Fused-silica column (30 m x 1.4 µm, 0.25 mm ID)

Company	Catalog Number
Baxter	
Virbac	
InScreenex	INS-CI-1015
InScreenex	INS-ME-1013-500ml
Drager	Fabius
Drageer	Vamos Plus
SedanaMedical	FlurAbsord
Fisher&Paykel	MR850
Curver	00012-416-00
Thermo Fisher Scientific, San Jose, CA, USA	Trace 1310 with TSQ 8000evo
Restek, Lisses, France	Rxi-624Sil MS

Comments/Description
Performing experiments using sevoflurane or isoflurane while being pregnant should be strongly discouraged

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RESPONSE TO EDITOR AND REVIEWER COMMENTS
R2

Manuscript ID: JoVE58554R1

Title: ***In vitro*** **method to control concentrations of halogenated gases in cultured alveolar epithelial cells**

Authors: Raiko Blondonnet[1,2], Bertille Paquette[1,2], Damien Richard[3], Rémi Bourg[2,4], Géraldine Laplace[2,4], Romain Segurel[2,4], Henria Pouvelle[2,4], Corinne Belville[2], Loic Blanchon[2], Thomas Godet[1,2], Jean-Michel Constantin[1,2], Jean-Etienne Bazin[1,2], Vincent Sapin[2], Matthieu Jabaudon[1,2]

We thank the Editor for their careful read and thoughtful comments on previous manuscript version. We have carefully taken their comments into consideration in preparing our revision, and we hope the manuscript has been improved. Please find below a point-by-point response to the comments and questions.

Editorial comments:

C1. Some parts of the manuscript are still hard to understand (see e.g. notes in text); please proofread, ideally by a fluent English speaker.
R1. As recommended, the manuscript had been proofread by an editing and proofreading service (www.scribendi.com). We added the marked proofread version of the manuscript during the submission process.

C2. Please revise 1.2.6 and 1.2.7 to avoid previously-published text.
R2. We apologize for this mistake and the text about this 2 steps had been changed.

C3. The highlighting is currently very scattered, and it's hard to pick out a narrative for most of it-e.g., are you intending to film the construction of the chamber? How much of the GC/MS are you intending to show? Please redo highlighting to make this clearer.
R3. We apologize if the narrative appears hard to understand. We did our best to highlight a new narrative and also to make it clearer.
If it could help the Editor, we took a video when we built the chamber. Of course we could give our movie to the Editor unless he prefers to shoot a new video of the construction of the chamber.
In order to bring more continuity and cohesion into the movie, we decided to removed all the part about GC/MS and to focus the video about the building of the chamber and it use with the anesthetic device.

C4. 2.1: The dimensions here do not agree with the dimensions in Figure 1; also, this appears to describe a container with a volume of 9.3 (or 9 in Figure 1) L, well over 6.5 L. Lastly, please include the box in the Table of Materials.

R4. We thank the Editor for this great comment. Indeed if we calculated the volume of the chamber using the dimensions provide in the manuscript we have 9.3 L. We checked again in the catalog of the provider (Please see the attached link - https://www.curver.com/fra/wp-content/uploads/multisites/2018/01/curver-cata-france-2018.pdf - *Page:* 106 *Reference:* 00012-416-00) and the container had a volume of 6.5 L. After contacted the provider, the volume was not of 9.3 L because the container was not a "perfect" rectangle but a rectangle with rounded corners. In order to avoid a misunderstanding, we added a sentence in the manuscript in step 2.1 to explain it to the readers.

We apologized for the figure 1 that it was a mistake. We changed the correct width (*i.e.*, 155mm) in figure 1.

The reference of the box had been added in the Table of Materials.

C5. 4.1.1: Is there a particular procedure for aspirating media? Are you, e.g., opening the chamber, and for how long?

R5. We thank the Editor for this comment. For aspirating media we used a multi-volume adjustable micropipette set to 1000 µL. We only opened very briefly the chamber to take the studied samples. After we immediately closed the lid of the container in order to keep the concentration of halogenated gases as stable as possible. As suggested, we added some details in the step 4.1.1 to improve the readability and the understanding.

C6. Figure 3B: What is an experiment here? One well? Were these taken during the same exposure to agent?

R6. For our experiment we analyzed 1 samples (*i.e.,* 1 well) at each time point during the same exposure to sevoflurane or isoflurane. We repeated the experiment five times. The figure 3B showed the variability between the different experiment. We added in sentence in the legend of the figure 3 to improve the understanding.