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Ciliatoxicity in human primary bronchiolar epithelial cells after repeated exposure at the air–liquid interface with native mainstream smoke of K3R4F cigarettes with and without charcoal filter

Michaela Aufderheide[a,*], Stefanie Scheffler[a], Shigeaki Ito[b], Shinkichi Ishikawa[b], Makito Emura[a]

[a] Cultex Laboratories GmbH, Feodor-Lynen-Str. 21, 30625 Hannover, Germany
[b] Japan Tobacco Inc., 6-2, Umegaoka, Aobaku, Yokohama 227-8512, Kanagawa, Japan

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ABSTRACT

Mucociliary clearance is the primary physical mechanism to protect the human airways against harmful effects of inhaled particles. Environmental factors play a significant role in the impairment of this defense mechanism, whereas cigarette smoke is discussed to be one of the clinically most important causes.

Impaired mucociliary clearance in smokers has been connected to changes in ciliated cells such as decreased numbers, altered structure and beat frequency. Clinical studies have shown that cilia length is reduced in healthy smokers and that long-term exposure to cigarette smoke leads to reduced numbers of ciliated cells in mice.

We present an *in vitro* model of primary normal human bronchiolar epithelial (NHBE) cells with *in vivo* like morphology to study the influence of cigarette mainstream smoke on ciliated cells. We exposed mucociliary differentiated cultures repeatedly to non-toxic concentrations of mainstream cigarette smoke (4 cigarettes, 5 days/week, 8 repetitions in total) at the air–liquid interface. Charcoal filter tipped cigarettes were compared to those being equipped with standard cellulose acetate filters.

Histopathological analyses of the exposed cultures showed a reduction of cilia bearing cells, shortening of existing cilia and finally disappearance of all cilia in cigarette smoke exposed cells. In cultures exposed to charcoal filtered cigarette smoke, little changes in cilia length were seen after four exposure repetitions, but those effects were reversed after a two day recovery period. Those differences indicate that volatile organic compounds, being removed by the charcoal filter tip, affect primary bronchiolar epithelial cells concerning their cilia formation and function comparable with the *in vivo* situation. In conclusion, our *in vitro* model presents a valuable tool to study air-borne ciliatoxic compounds.

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1. Introduction

In the healthy lung, inhaled particles, pathogens and allergens are trapped by airway mucus and removed from the lung by beating cilia. This defense mechanism, namely mucociliary clearance, can be impaired in several conditions due to abnormal mucus production or ineffective cilia clearance.

Environmental factors play a significant role in the impairment of effective mucociliary clearance, whereas cigarette smoke is discussed to be the clinically most important one (Weinberger et al., 2014). Mucus is known to be overproduced and ineffectively cleared in smokers (Irwin and Rippe, 2007).

In previous studies, impaired mucociliary clearance in smokers has been linked to decreased numbers of ciliated cells, changes in cilia structure and beat frequency. Several investigations have shown that cilia are shortened in healthy smokers compared to healthy non-smokers, which is likely to contribute significantly to impaired mucociliary clearance (Lee et al., 2004; Verra et al., 1995; Simet et al., 2010; Leopold et al., 2009).

Further investigations demonstrated that smokers with COPD have even shorter cilia than healthy smokers and that these changes are present in the distal as well as in the proximal airways (Hessel et al., 2014).

In vitro studies exhibited similar results, when human airway cells or mouse nasal septal epithelium cells grown at the air–liquid interface (ALI) were exposed to non-toxic concentrations

* Corresponding author. Tel.: +49 511 563586 0; fax: +49 511 563586 69.
E-mail address: m.aufderheide@cultex-laboratories.com (M. Aufderheide).

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of cigarette smoke condensate or extract (Brekman et al., 2014; Tamashiro et al., 2009). Although these studies did not analyze the smoke components that contribute to cilia shortening, several components of the gas vapor phase, such as formaldehyde, acetone, acrolein and acetaldehyde, are hypothesized to be cytotoxic (Nara et al., 2013).

Early in vitro investigations of cigarette smoke were performed by using for example mussel cilia or extirpated ciliated rabbit lung. In the 1960th, several independent studies reported that cigarette mainstream smoke was found to be ciliatoxic in cellular based systems (Kensler and Battista, 1963; Wynder et al., 1963). However, ciliatoxicity here was defined as an inhibition of the ciliary beat.

Walker and Kiefer (1966) separated cigarette smoke in its components by gas chromatography and found that acetaldehyde, acrolein and hydrogen cyanide strongly inhibited the ciliary beat.

At the same time, first studies also revealed that charcoal-containing filter tips reduce the ciliatoxic activity of cigarette smoke markedly (Kensler and Battista, 1963; Kaminski et al., 1968). Walker and Kiefer (1966) described that 80% of hydrogen cyanide, 92% of formaldehyde and more than 95% of acrolein and acetaldehyde were removed by filter tips containing 200 mg carbon.

In 2008, Polzin et al. presented comparable results. Charcoal containing filter tips remove volatile organic compounds including acrolein, acetaldehyde, benzene and styrene from the cigarette mainstream smoke. However, the charcoal filter size and design contributes significantly to the amount of volatile organic compounds removed, varying between 9 and 93% (Polzin et al., 2008).

A clinical study with 39 smokers demonstrated that the induction of exposure biomarkers was significantly reduced after smoking cigarettes with charcoal filters in comparison to cigarettes with standard cellulose acetate filters. Metabolites of crotonaldehyde, 1,3-butadien, benzene and, to a lower extent, acrolein were found to be reduced when smoking charcoal filter tipped cigarettes (Scherer et al., 2006).

In our experiments, we investigated the influence of repeated cigarette smoke exposure at non-toxic doses on cilia length in primary normal human bronchiolar epithelial cells. In contrast to other *in vitro* studies (Brekman et al., 2014; Tamashiro et al., 2009) in which the cells were exposed to cigarette smoke extracts or condensates from the basal side, we exposed them directly at the air–liquid interface in a CULTEX®RFS module. Native cigarette mainstream smoke of four cigarettes was introduced at five days per week, eight repetitions were done in total.

Furthermore, we compared the effects of standard cellulose acetate filter tipped cigarettes to those of cigarettes equipped with charcoal filters. The cells were analyzed immunohistochemically to determine the cilia length quantitatively after exposure.

2. Materials and methods

2.1. Cell cultivation

Normal human bronchial epithelial (NHBE) cells were isolated from bronchus samples of a male patient (age 67) with a lung adenocarcinoma in the right upper lobe.

The sample was obtained from the KRH Klinikum Oststadt-Heidehaus (Hannover, Germany). In accordance with the Declaration of Helsinki, the subject gave the informed consent to the research use of the removed lung tissue samples.

In our studies, we were interested in the anatomical region of bronchioles to investigate toxicological effects. In comparison to other regions of the airways, this region is characterized by a greater amount of stem/progenitor cells differentiating to club cells with potent drug-metabolizing capacity (Emura et al., 2015).

Upon arrival in our laboratory, the bronchus samples were incubated for 24 h at 4 °C on a rocking platform in incubation medium (MEM medium containing dithiothreitol (0.5 mg/mL), DNase (10 μg/mL) and antibiotics (40 μg/mL tobramycin, 50 μg/mL vancomycin, 50 μg/mL ceftazidime, 2.5 μg/mL amphotericin B, 50 U/mL penicillin/streptomycin)). Afterwards, the samples were transferred into a PBS containing petri dish, isolated from residual parenchyma and cut into smaller pieces of approximately 8 × 5 mm. The bronchus pieces were then placed into cryovials, containing DMEM with 10% FCS and 10% DMSO and frozen to −80 °C. After storage at −80 °C overnight, the vials were moved to a liquid nitrogen tank and stored until needed.

For cell isolation, the samples were thawed in a water bath at 37 °C, transferred into petri dishes and rinsed with PBS after removal of the freezing medium. Incubation medium containing 0.1% protease XIV was added and the samples were incubated for 24 h at 4 °C on a rocking platform. Afterwards, bronchial epithelial cells were isolated by thoroughly scraping the luminal surface of the bronchus pieces with a scalpel.

The cell suspensions were homogenized, pipetted into centrifugation tubes and centrifuged for 10 min at 170 × *g*. The resulting cell pellets were resuspended in 4.5–9 mL BEGM medium. The cell suspensions of each sample were then equally divided to two collagen IV/fibronection coated wells of a 6-well plate to grow in culture.

After the first passage, NHBE cells were cultivated in collagen IV/fibronectin coated culture flasks using BEGM Medium. After reaching 80-90% confluence, the cells were seeded on collagen IV coated cell culture inserts (seeding density: 1–1.5 × 10^5/cm^2). The cells were cultivated under submerged conditions and supplied with BEGM medium until reaching 100% confluency before the apical medium was removed and the basal medium was replaced by differentiation medium (AEGM/DMEM 1:1, +Supplements Mix + 2% Ultroser G). After 10 days of cultivation at the air–liquid interface, the cells were transferred to the exposure module. The exposure experiments were performed with cells in passage 2.

BEGM medium was obtained from Lonza (Basel, Switzerland); PBS, Penicillin/Streptomycin, DMEM from Biochrom (Cambridge, UK) and AEGM medium from Promocell (Heidelberg, Germany). All other cell culture reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cigarette smoke exposure

For the cigarette smoke exposure, the cell culture inserts were transferred into a CULTEX® RFS module (Cultex Laboratories, Hannover, Germany). Two modules were operated in parallel: one module contained the smoke exposed cell culture inserts, the other one held cell culture inserts which were exposed to synthetic air (process control), both were located under a laminar flow hood to maintain a sterile environment.

K3R4F research cigarettes (University of Kentucky, Lexington, KY, USA), containing a standard cellulose acetate filter tip, were used for the exposure experiments as well as cigarettes equipped with a charcoal filter. Here, the cellulose acetate filter was replaced by a tip containing 100 mg charcoal.

The cigarettes were smoked by a smoking robot according to ISO 3308 (35 ml puff volume/2 s, 1 puff per minute). The freshly generated main stream smoke was diluted with synthetic air (1 L/min) and sucked into the module at a rate of 5 mL/min/insert *via* a vacuum pump. The flow rate as well as the flow above the cells (5 mL/min) was controlled by mass flow controllers controllers (IQ + Flow and EL-Flow Select, Bronckhorst, Ruurlo, Netherlands). The exhaust air was directed back to the fume hood.

In each experiment, the cell cultures were exposed to mainstream smoke of four K3R4F cigarettes (with or without charcoal filter) as well as clean air (for the same time period). The exposure

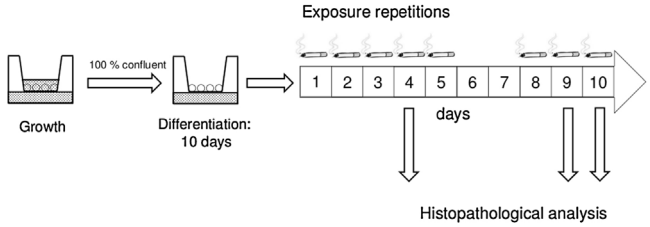

Fig. 1. Scheme of exposure and analyze.

was performed daily for five days and after a recovery phase of two days again on three subsequent days (Fig. 1). After the 4th, 6th and 8th exposure repetition, three cell culture inserts each were analyzed histopathological. As a reference control, cells cultivated air-lifted in the incubator were additionally analyzed (incubator control). Cells exposed to clean air for the time of cigarette smoke exposure were used as process control.

2.3. Histopathological analysis

After exposure, the cultures were post-incubated for 24 h in differentiation medium (air lifted) and subsequently fixed with 10% formalin for 1 h. Control cultures were processed at the same time as exposed cultures. Following the fixation, the membranes were washed twice with water, released from the inserts and embedded in paraffin. Using a microtome, sections of 5 μm thickness were prepared, beginning at the middle of the membrane. After deparaffinization, sections were stained with hematoxylin and eosin.

The histological samples were analyzed microscopically (Axiophot, Zeiss, Oberkochen, Germany) and the length of the cilia was measured by using the integrated image analyzing software (AxioVision Imaging Software, Zeiss, Oberkochen, Germany), allowing a semi-automatic quantification of the cilia length.

2.4. Statistical analysis

Significant differences between two experimental groups were evaluated by Student's unpaired *t*-test, whereas the symbols (asterisks) are defined as followed: **** $p < 0{,}0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

3. Results

Throughout the whole exposure experiment, the incubator control cultures showed a pronounced mucociliary differentiation (Fig. 2). Cilia bearing as well as mucus secreting cells were distributed homogeneously within the culture. The clean air control cultures (process controls) revealed a comparable morphological differentiation pattern until the end of the experiment (Figs. 3–5).

Fig. 3 shows the morphological changes of the cells after four exposure repetitions. The number of cilia bearing cells as well as the cilia length was comparable in control cultures that remained in the incubator (incubator control, IC) and cultures, which were exposed to clean air (clean air control, CA). Cultures exposed to cellulose acetate filtered cigarette smoke (hereafter referred to as cigarette smoke, CS) showed fewer cilia bearing cells. Furthermore, the cilia were shortened compared to the control cultures. In the presence of a charcoal filter (CCS), more cilia bearing cells were visible whereas the cilia length was decreased compared to the control cultures, but longer compared to cigarette smoke exposed cells.

After six exposure repetitions (day eight of the experiment), the morphological pattern of the control cultures was similar to that found after four exposure repetitions. In cigarette smoke exposed cells, no cilia bearing cells were detectable anymore and mucus

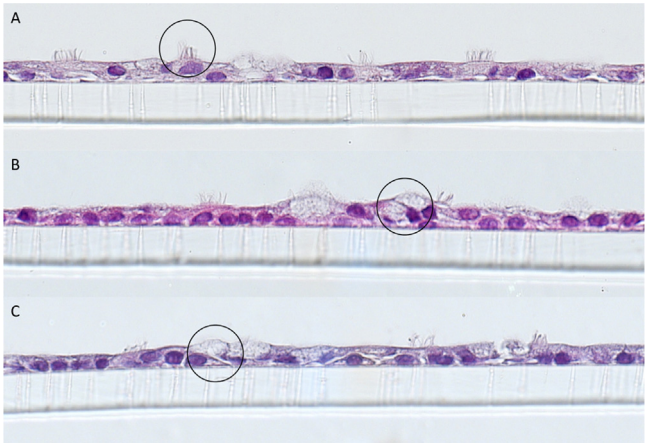

Fig. 2. HE stained histological sections of primary normal bronchial epithelial cells. Incubator control cultures corresponding to repeatedly exposed cultures after 4 (A), 6 (B) and 8 (C) smoke exposure repetitions. Circles indicate areas of ciliated and mucus secreting cells.

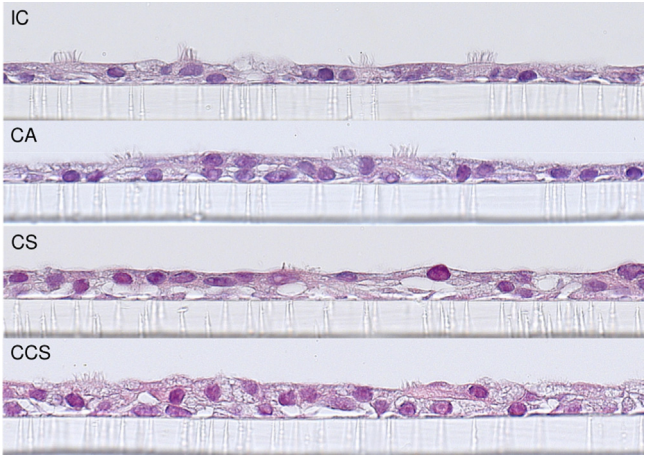

Fig. 3. HE stained histological sections of primary normal bronchial epithelial cells. IC: incubator control: cells remained air-lifted in the incubator for the exposure time. CA: cultures exposed four times to clean air. CS: cultures exposed *4 times* to K3R4F cigarette smoke (cellulose acetate filtered). CCS: cultures exposed four times to K3R4F cigarette smoke (charcoal filtered) Magnification: 630×.

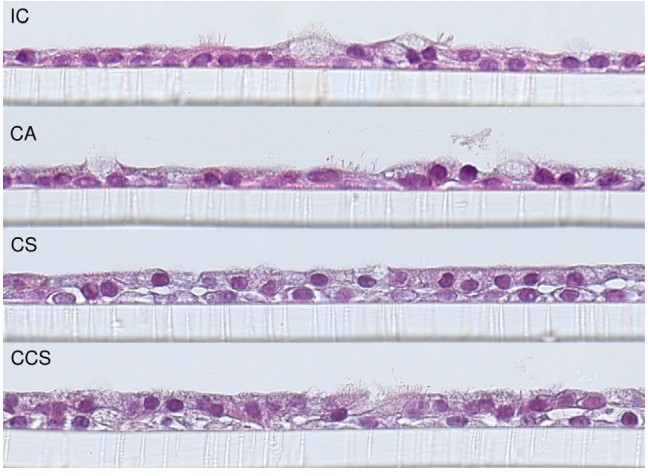

Fig. 4. HE stained histological sections of primary normal bronchial epithelial cells. IC: incubator control: cells remained air-lifted in the incubator for the exposure time. CA: cultures exposed six times to clean air. CS: cultures exposed *6 times* to K3R4F cigarette smoke (cellulose acetate filtered). CCS: cultures exposed six times to K3R4F cigarette smoke (charcoal filtered) Magnification: 630×.

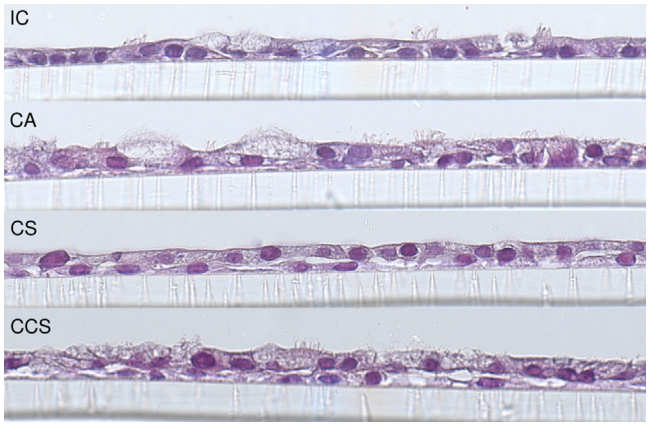

Fig. 5. HE stained histological sections of primary normal bronchial epithelial cells. IC: incubator control: cells remained air-lifted in the incubator for the exposure time. CA: cultures exposed eight times to clean air. CS: cultures exposed *8 times* to K3R4F cigarette smoke (cellulose acetate filtered). CCS: cultures exposed eight times to K3R4F cigarette smoke (charcoal filtered) Magnification: 630×.

secretion seemed to be reduced. In charcoal filtered cigarette smoke exposed cells, cilia were not significantly shorter than those in control cultures (Fig. 4).

After eight exposure repetitions (day 10 of the experiment), no significant differences of cilia length were found between incubator control and clean air control cultures. Cilia of charcoal filtered cigarette smoke exposed cells were not significantly shorter than the cilia of control cultures. In cigarette smoke exposed cells, no cilia bearing cells could be detected. Furthermore, the number of mucus secretion seemed to be reduced (Fig. 5).

The cilia length of the cultures were measured and the results were statistically evaluated using an unpaired *t*-test (Fig. 6); images with measurements are shown in Suppl. Figs. 1–3. The quantitative analysis confirmed the histopathological findings. K3R4F cigarettes equipped with the normal cellulose acetate filter induced a significant reduction in cilia length already after 4 smoke repetitions. With ongoing exposure cycles cilia disappeared completely, whereas the charcoal filter cigarettes showed only a clear reduction in cilia length after 4 smoke repetitions. After a recovery period of two days, no statistically significant difference in cilia length could be estimated in comparison with the control cultures. These results showed clearly that the integrated charcoal filter tips reduced cilia toxicity of K3R4F cigarette smoke in normal bronchial epithelial cells.

4. Discussion

The repeated exposure of normal human bronchial epithelial cell (NHBE) cultures with mainstream cigarette smoke led to a reduction of cilia bearing cells as well as shortening of existing cilia and finally to the disappearance of all cilia. The stability of the cultures as demonstrated by the incubator controls allows the quantification of the morphological changes of the cilia besides the qualitative analysis of the cultures.

The presence of a charcoal filter reduced the effects of mainstream cigarette smoke significantly. After four exposure repetitions, cilia length was reduced compared to clean air control cultures, but after six and eight exposure repetitions (day 8 and 10 of the experiment), no significant change in cilia length could be observed. Due to the fact that there was a recovery phase of two days after the fifth exposure repetition (Fig. 1) the increased cilia length after six and eight exposures could be the result of cell recovery. In contrast, no recovery is seen in cultures exposed to mainstream smoke of cigarettes with a cellulose acetate filter.

Due to the fact that charcoal filter tips remove volatile organic compounds such as acrolein and acetaldehyde (Walker and Kiefer, 1966) our findings support the evidence that such compounds are responsible for the damaging effect of native cigarette mainstream smoke on cilia bearing cells. They also match the results of a clinical study from Leopold et al. (2009) who have shown that cilia are shortened in healthy smokers compared to healthy non-smokers.

Long-term cigarette smoke exposure has furthermore been linked to the loss of ciliated cells in mice (Simet et al., 2010), correlating to our results found after six and eight exposure repetitions.

However, although our experiments point to the cytotoxic effects of cigarette smoke on ciliated cells *in vitro*, the mechanisms of these effects have to be clarified in future studies. From our

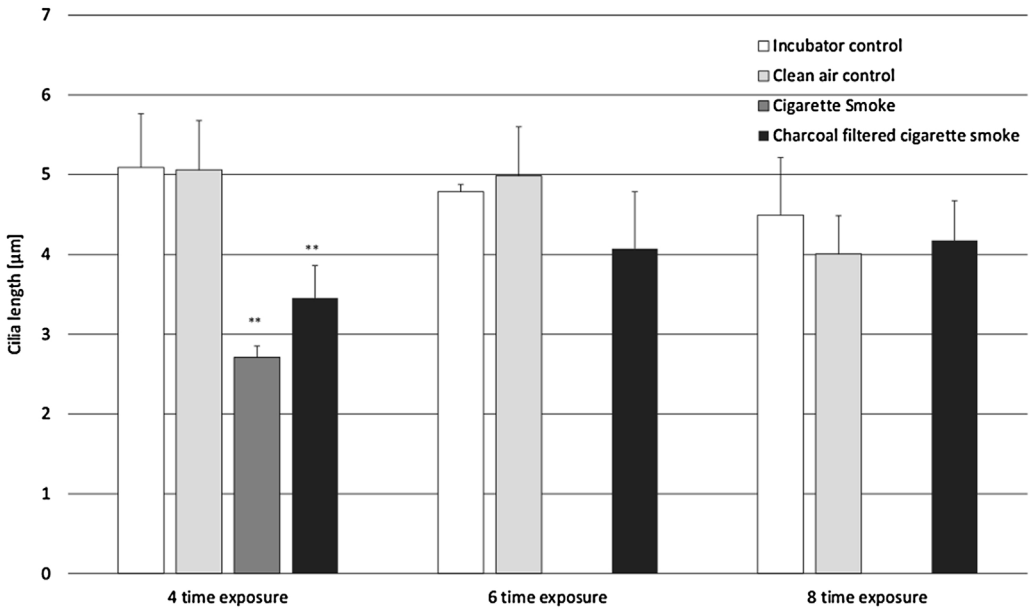

Fig. 6. Cilia length in primary normal bronchial epithelial cultures. Values are shown for cultures, which were exposed repeatedly to clean air, whole cigarette smoke or charcoal filtered cigarette smoke as well as for incubator control cultures. Exposure was performed five times/week with four cigarettes. Values are given as mean + SD. No values are given for cells exposed to cigarette smoke six and eight times, since no cilia could be detected.

findings, it is not clear, whether cigarette smoke affects only existing cilia or if it also interfere with cilia regeneration. Studies of Brekman et al. (2014) revealed the inhibition of ciliated cell-related gene expression, which could potentially also influence the regeneration of cilia.

In conclusion, we demonstrated a model of normal human bronchiolar epithelial cell cultures with *in vivo* like differentiation pattern for the examination of ciliatoxic effects caused by the exposure with native cigarette mainstream smoke. It could be shown that, in accordance with clinical and animal studies, ciliated cells are affected especially by volatile organic compounds of cigarette smoke. The described system represents a stable and relevant model for *in vitro* studies with inhalable compounds. Accordingly, the use of normal human bronchial epithelial cells might be an alternative method in the field of inhalation toxicology by taking into consideration the 3R principles for reducing animal testing. (Russell and Burch, 1959) A functional system may reduce or even replace animal tests and might give more reliable data due to the lack of interspecies differences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.etp.2015.04.006

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