Further *in vitro* studies on the cytotoxic effects of cigarette smoke vs. e-liquid vapour

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In contrast to cigarette smoke, the vapour of e-cigarettes is not the result of a combustion process. However, the risks of e-cigarette use are uncertain which is due to the limited amount of scientific data regarding their health effects related to the variability of vaporisers, e-liquid ingredients and their quality (Dawkins and Corcoran 2014; Farsalinos and Polosa 2014; Grana et al. 2014). There are also limited amounts of studies looking on the *in vitro* toxicity profile of e-liquids and e-cigarettes by using cultured cells of the lung (Misra et al. 2014), mammalian fibroblasts (Romagna et al. 2013) and myocardial cells (Farsalinos et al. 2013).

Here, we present further data on the toxicity of cigarette smoke and e-liquid vapour after long-term exposure of cultured human lung cells. Moreover, we demonstrate the effect of both conditions on the activity of freshly isolated ciliated human epithelial cells of the upper respiratory tract.

Simulation of smoking or vaping

In order to simulate the conditions in reality, a special smoking apparatus was constructed which allows to vary the frequency, length and the depths of the puffs. For smoking a cigarette, 20 puffs with a duration of 3 seconds and a pause of 15 seconds between two puffs was presumed. The same conditions were applied for the e-cigarette (EVOD, vaporiser 2,2 Ω and rechargeable battery 3,7 V; KangerTech) which was used with an electrical power of 6.2 W. The smoke of the cigarette and the vapour of the e-cigarette were aspirated by a suction pump and passed into 20 ml of cell culture medium. The resulting primary extracts had a neutral pH value of 7.4 ± 0.3. For a more detailed description of the apparatus, see Dartsch et al. (2015).

Cytotoxicity after long-term exposure

Background

Chronic toxicity is the development of adverse effects as the result of a long term exposure to toxic substances. Adverse effects associated with chronic toxicity can be directly lethal but are more commonly sublethal, including changes in growth and vitality. In contrast, acute toxicity occurs after a shorter period of time at higher concentrations.

In previous investigations, e-liquid vapour extracts did not cause an acute toxic effect after an exposure time of 24 hours. Thus, we additionally examined the cytotoxic effect after a continuous exposure period of 12 days. During that exposure period cultured human lung cells had divided at least 5-6 times which is adequately to the division ratio of that cell type *in vivo* over several years. This allows a prediction of the chronic toxicity of cigarette smoke and e-liquid vapour.

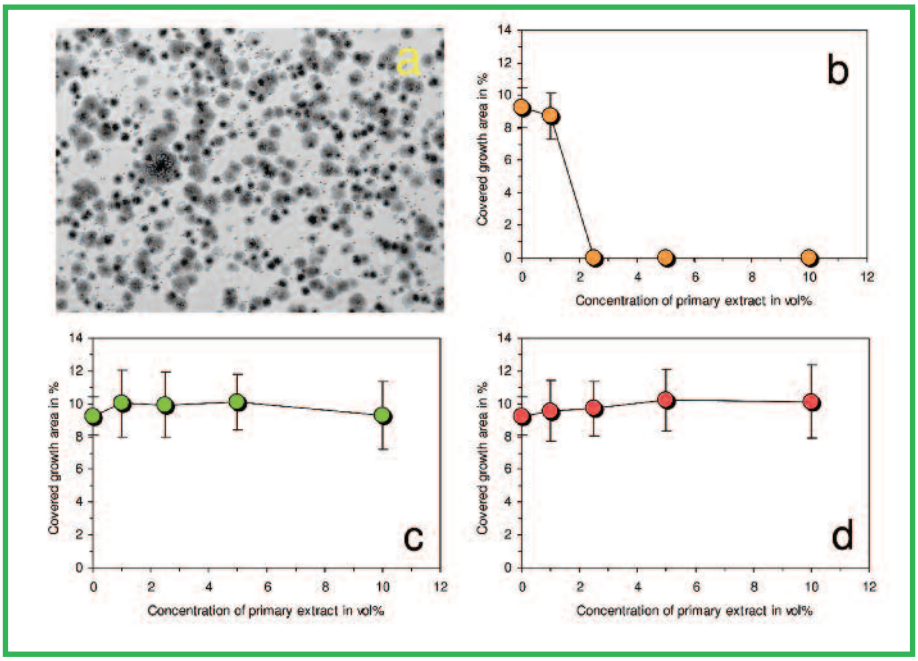

Fig. 1: (a) Representative example of the human lung cell cell growth in the reagent control after 12 days of continous incubation. The cell-covered growth area was examined by digital image analysis (Wimasis Image Analysis). (b-d) Graphical presentation of the results of long-term exposure to cigarette smoke exctract (b), and two e-liquid vapour extracts named "Apple" (c) and "Strawberry-Menthol (d) of the brand Happy Liquid. Note the strong dose-dependent decrease in cell growth for cigarette smoke extracts and the complete loss of cell vitality at concentrations ≥ 2.5 vol%. In contrast, both e-liquid vapour extracts do not affect cell vitality at all concentrations tested up to 10 vol%. Data represent mean value ± standard error of the mean of three experiments.

Materials and methods

The investigations were conducted by using one common cigarette brand of medium strength with 10 mg tar, 0,8 mg nicotine und 10 mg carbon monoxide, and two e-liquids of the brand Happy Liquids: (1) "Apple" with 6 mg/ml nicotine, and (2) "Strawberry-Menthol" with 6 mg/ml nicotine.

For examination of cytotoxicity after long-term exposure, human lung cells (cell line A-549) were seeded as low-density mass cultures (seeding density: 1,000 cells/flask with 25 cm^2 of growth area). Cells were allowed to attach and spread for 48 hours and were then exposed to the primary extract at 0 to 10 vol% for another 12 days. Cells were incubated in DMEM/Ham's F12 culture medium supplemented with 10% fetal bovine serum and containing 100 µg/mL penicillin and 100 IU/mL streptomycin in an incubator at 37°C gassed with 5% CO_2 and 95% air.

After this time period the culture medium was discarded and the cells were fixed with methanol for 2 minutes and stained with Coomassie-Giemsa solution (Romanowsky staining). The stained cultures were air-dried and photographed with a Nikon D300 digital SLR and a macro lens at a magnification of 1:2 (Fig. 1a). The photographs were processed by a digital image analysis system (Wimasis Image Analysis, ibidi GmbH, München). The results expressed as percentage of cell-covered area in the flasks were taken for evaluation.

Results and conclusions

As can be seen in Fig. 1b, the two highest test concentrations of cigarette smoke extracts completely inhibited cell growth at concentrations ≥ 2.5 vol%. Moreover, at these concentrations no viable lung cells could be detected in the flasks demonstrating that exposure to cigarette smoke extracts caused a complete death of the cells. Only at a concentration of 1 vol% we observed a lung cell growth which did not differ significantly from the untreated controls.

Long-term exposure of cells to the vapour extracts of both e-liquids resulted in completely different outcomes (Figs. 1c and 1d). At all concentrations tested the cell covered growth area in the flasks was in the same range as the untreated controls or even slightly higher.

Thus, the results clearly indicate that vaping should be preferred for health reasons when having the choice to smoke a tobacco cigarette or to use an e-cigarette.

Effect on ciliary beat frequency

Background

Ciliary beat is one of the most important defense mechanisms in the respiratory tract with its frequency and coordination depending on many factors. A chronic inhalation of cigarette smoke is associated with a decrease or even paralysation in ciliary beat activity (Dalhamn 1959; Stanley et al. 1986; Dye and Adler 1994; Cohen et al. 2009). Epithelial cells line the lumen in a unique position to interact directly with inhaled cigarette smoke. This study was undertaken to examine freshly isolated human nasal epithelial cells for alterations of ciliary beat frequency (CBF) after exposure to cigarette smoke and e-liquid vapour extracts.

Materials and methods

Cells were obtained from 3 male and healthy volunteers at the age from 35 to 59 years on different experimental days. Volunteers had been free of respiratory infection for at least 2 weeks. Ciliated human nasal epithelial cells were obtained with a cytology brush from the inferior turbinate of the volunteers by an otorhinolaryngologist and dispersed in Airway Medium (Promocell, Heidelberg, Germany) containing 100 µg/mL penicillin and 100 IU/mL streptomycin and were buffered with 10 mM HEPES buffer to avoid pH changes during transportation. Isolated epithelial cells were transported to the laboratory immediately after retrieval within 60 minutes (Fig. 2). The investigations were done by using one common cigarette brand of medium strength with 10 mg tar, 0.8 mg nicotine und 10 mg carbon monoxide, and two e-liquids of the brand Happy Liquid: (1) "Apple" with 6 mg/ml nicotine, and (2) "Strawberry-Menthol" with 6 mg/ml nicotine. For measurement of ciliary beat frequency, increasing amounts of cigarette smoke and e-liquid vapour extracts were pipetted to 250 µl of suspended nasal epithelial cells and incubated for 15 minutes in a temperature controlled chamber at 37°C (ibidi, München, Germany) mounted on the stage of an Olympus IX50 inverted microscope. The ciliary beat was recorded by a Basler high-speed video camera acA640-120um operated by pylon camera software 4.2 from Basler (Rauscher, Olching, Germany) at a speed of 100 frames per second with an Olympus 40x planachromate objective. Beat frequency was calculated afterwards by visual examination of the beats from the single pictures recorded by the high-speed camera.

Results and conclusions

Ciliary beat frequency of freshly isolated human nasal epithelial cells was in the range of 10 beats per second for untreated cells. Treatment with cigarette smoke extracts caused a dose-dependent decrease in beat frequency which became significant ($p < 0.01$) at a primary extract concentration > 25 vol% according to the two-tailed Wilcoxon-Mann-Whitney U test as a nonparametric test for non-normal distributions (Fig. 3a). Both e-liquid vapours did not cause a statistically significant decrease at all tested concentrations of the primary extract (see Fig. 3b, 3c).

The results show that vaping has much less harmful effects on epithelial cells located in the respiratory tract and their defense and clearing function than cigarette smoke which accounts for a number of frequently observed respiratory infections and airway diseases.

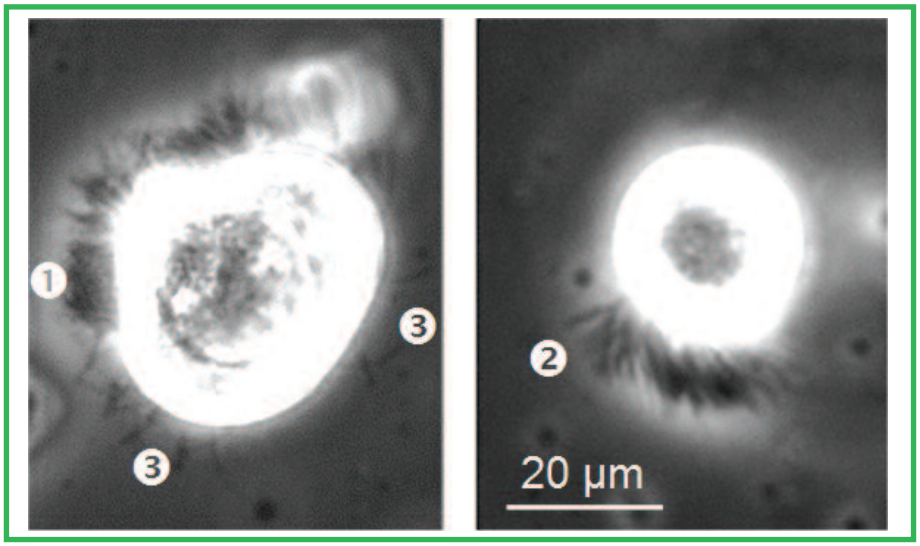

Fig. 2: Examples for the cell morphology of freshly isolated and free-floating human nasal epithelial cells with cilia which can be observed in arrays (1), bundles (2) or in singular form (3). Phase contrast microscopy of vital cells with beating cilia.

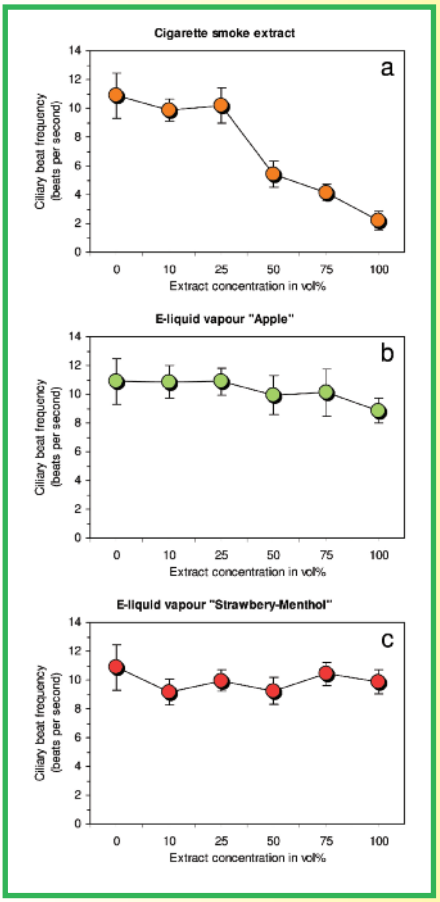

Fig. 3: Graphical presentation of the ciliary beat frequency (CBF) with different extract dilutions of cigarette smoke and both e-liquids after 15 min of application. Note the dose-dependent decrease in CBF for cigarette smoke extract, but nor for both e-liquid vapour extracts. Data represent mean values ± standard deviations of 3 donors.

References

Cohen NA, Zhang S, Sharp DB, Tamashiro E, Chen B, Sorscher EJ, and Woodworth BA (2009): Cigarette smoke condensate inhibits transepithelial chloride transport and ciliary beat frequency. Laryngoscope, 119: 2269–2274.

Dalhamn T (1959): The effect of cigarette smoke on ciliary activity in the upper respiratory tract. AMA Arch Otolaryngol 70:166-168.

Dartsch PC, Mrva T, and Okle O (2015): Tobacco smoke vs e-liquid vapour. Diverging toxic effects on human lung cells in culture. Innov Food Technol 66: 37-39 (2015).

Dawkins L and Corcoran O (2014): Acute electronic cigarette use: nicotine delivery and subjective effects in regular users. Psychopharmacology 231: 401-407.

Dye JA and Adler KB (1994): Effects of cigarette smoke on epithelial cells of the respiratory tract. Thorax 49: 825-834.

Farsalinos KE and Polosa R (2014): Safety evaluation and risk assessment of electronic cigarettes as tobacco cigarette substitutes: a systematic review. Ther Adv Drug Saf 5: 67-86.

Farsalinos K, Romagna G, Allifranchini E, Ripamonti E, Bocchietto E, Todeschi S et al. (2013): Comparison of the cytotoxic potential of cigarette smoke and electronic cigarette vapour extract on cultured myocardial cells. Int J Environ Res Public Health 10: 5146–5162.

Grana R, Benowitz N and Glantz SA (2014): E-Cigarettes. A Scientific Review. Circulation 129: 1972-1986.

Misra M, Leverette RD, Cooper BT, Bennett MB and Brown SE (2014): Comparative in vitro toxicity profile of electronic and tobacco cigarettes, smokeless tobacco and nicotine replacement therapy products: e-liquids, extracts and collected aerosols. Int J Environ Res Public Health 11: 11325-11347.

Romagna G, Allifranchini E, Bocchietto E, Todeschi S, Esposito M and Farsalinos KE (2013): Cytotoxicity evaluation of electronic cigarette vapor extract on cultured mammalian fibroblasts (ClearStream-LIFE): comparison with tobacco cigarette smoke extract. Inhal Toxicol 25: 354–361.

Stanley PJ, Wilson R, Greenstone MA, MacWilliam L, and Cole PJ (1986): Effect of cigarette smoking on nasal mucociliary clearance and ciliary beat frequency. Thorax 41: 519-523.

Part of the investigations shown here have been presented at the Global Forum on Nicotine, June 5-6, 2015 at Warsaw.

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