Tobacco smoke vs e-liquid vapour

Diverging toxic effects on human lung cells in culture

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An electronic cigarette or e-cigarette is a battery-powered vaporiser that simulates tobacco smoking by producing an aerosol that resembles smoke. It generally uses a heating element that vaporises a liquid solution known as e-liquid. Even though invented in China in 2004, it was introduced only recently to the market worldwide (Pauly et al. 2007; Henningfield and Zaatari 2010) as an alternative to tobacco cigarettes. Since then, e-cigarettes have achieved a worldwide popularity with increasing sales every year. E-liquids usually contain a mixture of propylene glycol, vegetable glycerol, and flavourings with or without nicotine. In contrast to tobacco smoking, the vapour of an e-cigarette is not the result of a combustion process and is believed to have much lower health effects. 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). Moreover, 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).

Prompted by this background, the present study was performed to compare the short-term and long-term toxic effects of tobacco smoke with those vapour of various e-liquids from Happy People GmbH, 80337 Munich, Germany.

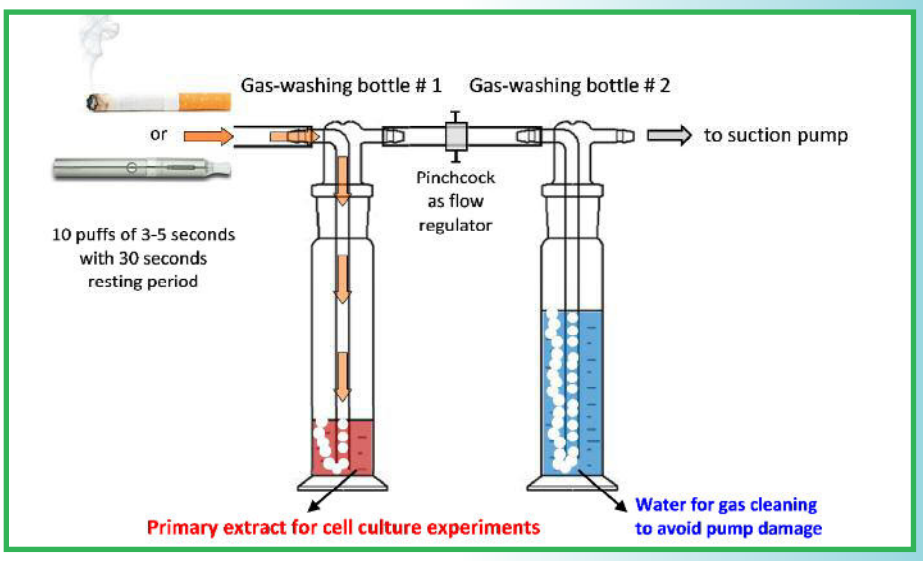

Figure 1: Experimental setup for a realistic simulation of cigarette smoking or vaping. The suction pump on the right generates an adjustable underpressure which aspirates the smoke or vapour and bubbles it into the culture medium in the left gas-washing bottle # 1. This yields the primary extract which is used either undiluted or diluted for the further cell culture experiments. The right gas-washing bottle # 2 is only for gas cleaning to avoid pump damage.

Cells

The investigations were done with human lung carcinoma cells (cell line A549; ECACC, Salisbury, UK) which are widely used in current scientific research all over the world (for example, see Cervellati et al. 2014; Misra et al. 2014; Jorgensen et al. 2010; Zhao et al. 2009; Ramage et al. 2006; Kode et al. 2006). The human lung cells were routinely cultured as mass cultures in a Binder CO_2 incubator at 37 °C with a moist atmosphere of 5 % CO_2 and 95% air. Culture medium was DMEM/Ham's F12 (1:1) supplemented with 10% fetal bovine serum and 100 Units/ml of penicillin and 100 µg/ml of streptomycin. All cell culture reagents were from GE Healthcare Life Sciences, 35091 Cölbe, Germany.

Tobacco cigarettes and e-liquids

The investigations were done by using two common cigarette brands of medium strength with 10 mg tar, 0,8 mg nicotine und 10 mg carbon monoxide. For comparison, the following e-liquids of the brand Happy Liquid produced by Happy People GmbH, 80337 Munich, Germany were used: (1) "Menthol" with 18 mg/ml nicotine, (2) "Apfel" (apple) with 6 mg/ml nicotine, and (3) "Erdbeer-Menthol" (strawberry/menthol) with 6 mg/ml nicotine.

Simulation of smoking & vaping to obtain the primary extract

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 (Figure 1). For smoking a cigarette, 10 puffs with a duration of 3-5 seconds and a pause of 30 seconds between two puffs were presumed. The same conditions were applied

for the e-cigarette (EVOD, EU version, vaporiser 2.2 W and rechargeable battery 3.7 V; KangerTech). The puff duration was in the time range described by Farsalinos et al. (2013) in their study on e-cigarette vaping and tobacco cigarette smoking habits. The smoke of the cigarettes and the vapour of the e-cigarettes were aspirated by a suction pump and bubbled into 20 ml of cell culture medium. The resulting primary extracts had a neutral pH value of 7.4 ± 0.3. This extract was brownish-yellow for cigarette smoke and colourless for e-cigarette vapours. Both primary extracts were filtrated sterile by pressing them through a sterile porous membrane (porous size 0.45 μm) and added to the lung cells cultures at the concentrations described below to evaluate short-term and long-term toxicity.

At the test conditions used in this study, an approximately calculated smoke or vapour concentration in the lung of 10 vol% was assumed as the result of the relation of tidal volume (500 ml) to vital lung capacity (5,000 ml). However, this approach may vary depending on the individual smoking or vaporising habits and should be taken only as a rough value for the evaluation of the effects as described in this study.

Short-term exposure to extracts of tobacco smoke or e-liquid vapour

For quantification of cell vitality after exposure to the extract concentrations, an enzymatic test using XTT was used. XTT is the sodium salt of 2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide and has a yellowish colour. Mitochon-drial dehydrogenases of viable cells cleave the tetrazolium ring of XTT to yield orange formazan crystals which are soluble in aqueous solutions. The intensity of the resulting orange solution correlates directly with cell vitality and metabolic activity (Roehm et al. 1991; Brosin et al. 1997).

Cells were taken from 80 to 90 % confluent mass cultures and were seeded into 96-well plates. Seeded cell densities were adapted so that cell cultures did not reach confluency during the total experimental and exposure period. 24 hours after seeding, cells were completely attached and spread to the bottom of the wells. Then, culture medium was discarded and replaced by fresh culture medium containing the primary extract of tobacco smoke or e-liquid vapour "Menthol" with 18 mg/ml nicotine to yield the test concentrations of the primary extract ranging from 0 to 100 vol% with 0 vol% as control (= only culture medium without primary extract) and 100 vol% as undiluted primary extract. The exposure time was 24 hours. Thereafter, culture medium of the 96-well plates was discarded and replaced by fresh culture medium and XTT. Multiwell plates were incubated with XTT for another hour at 37 °C in the incubator and optical density of each well was examined by a difference measurement at $\Delta OD = 450 - 690$ nm using a double-wavelength ELISA reader (BioTEK Elx 808). Experiments were done in triplicate and results were expressed graphically as relative values in comparison to untreated controls.

As can be seen from Figure 2, even a strong dilution of 10 vol% of the primary extract of tobacco smoke caused a loss in lung cell vitality by 95 % in comparison to untreated controls. Higher concentrations increased cell death to 100 %, thus demonstrating the high toxic potential of tobacco smoke at short-term exposure. The primary extract of e-liquid vapour "Menthol" with 18 mg/ml nicotine caused only a slight and dose-dependent reduction of cell vitality at primary extract concentrations above 50 vol%. The maximum loss in cell vitality was only 27 % and was achieved with the undiluted primary extract. This decrease might be due to the nicotine present in the vapour, although recent studies of our group have shown that nicotine alone does not affect vitality of lung cells even at 36 mg/ml in an experimental e-liquid.

In order to examine the dynamics of the morphological alterations due to tobacco smoke extract and in direct comparison to e-liquid vapour extract, we used a time-lapse video system with an acA1600-20uc digital camera (resolution 1600 x 1200 pixels; Basler, Ahrensburg, Germany) and an incubator system for the Olympus IX50 inverted microscope from ibidi, Munich, Germany. The ideal frame rate was one frame every 30 seconds. From this dynamic tracking, the alterations of lung cell morphology due to tobacco smoke extract were visualised and showed a dramatic rounding and detachment of the cells within 10 hours at a test

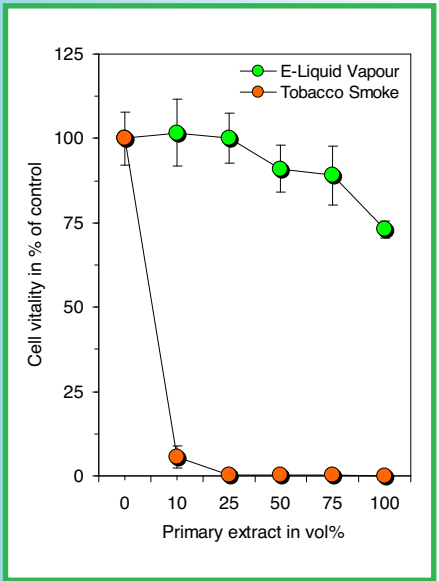

Figure 2: Graphical presentation of the summarised experimental results on the short-term toxic effect of tobacco smoke extract in comparison to e-liquid vapour extract "Menthol" of the brand Happy Liquid containing 18 mg/ml of nicotine. The 1:10 diluted primary extract of tobacco smoke causes death of nearly all cultured human lung cells, whereas the undiluted (!) primary extract of e-liquid vapour does not cause a marked loss in cell vitality. Thus, tobacco smoke is considerably more toxic to the lung cells than e-liquid vapour. However, the vapour itself is not harmless as lung cells are also slightly affected in a dose-dependent manner at concentrations above 50 vol%. Data represent mean value ± standard deviation of three experiments.

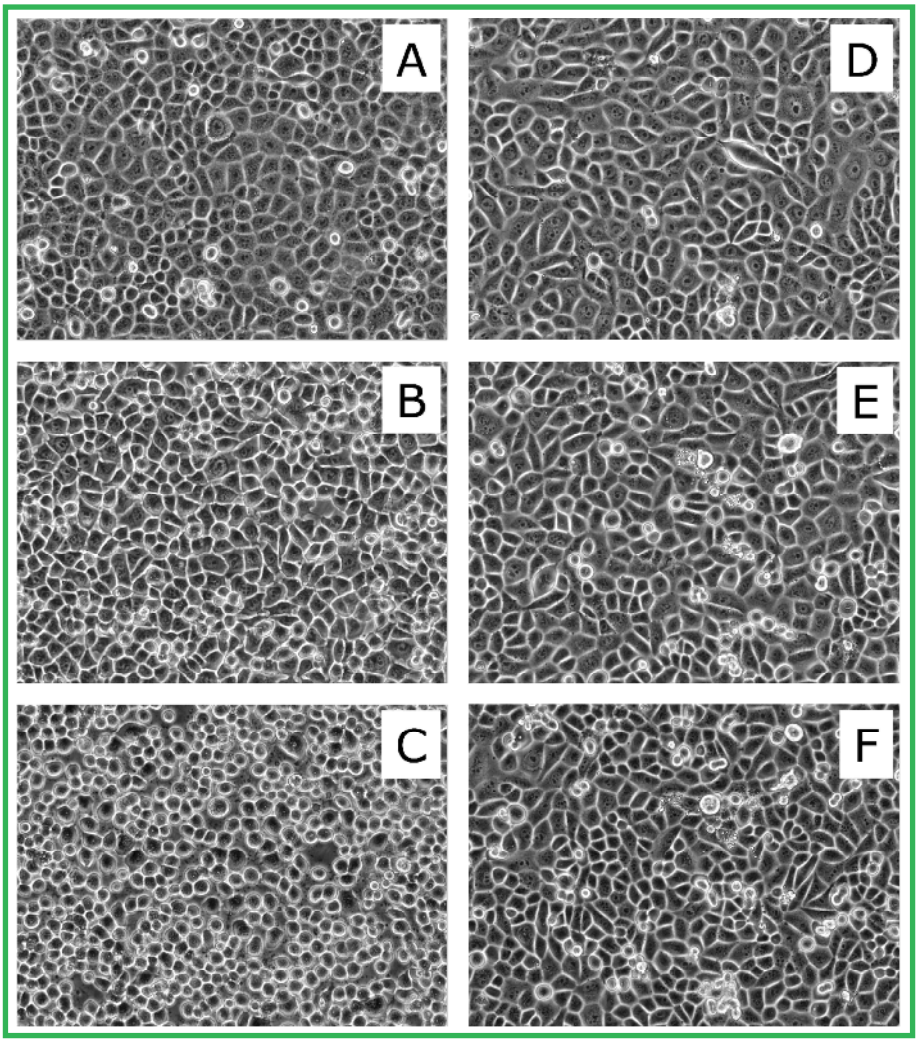

Figure 3: Effect of 10 vol% tobacco smoke extract (A-C; left column) and undiluted e-liquid "Erdbeer-Menthol" vapour extract (D-F; right column) on morphology of cultured human lung cells after 0 (A, D), 4 (B, E) and 10 (C, F) hours of exposure. Note that even the diluted tobacco smoke extract causes a rounding and detachment of cells and, finally, cell death. In contrast, undiluted e-liquid vapour extract causes only an increased amount of rounded cells indicating its low acute toxicity. Single cells from a time-lapse recording at phase contrast microscopy. The field of view is always the same for each recording series A-C and D-F.

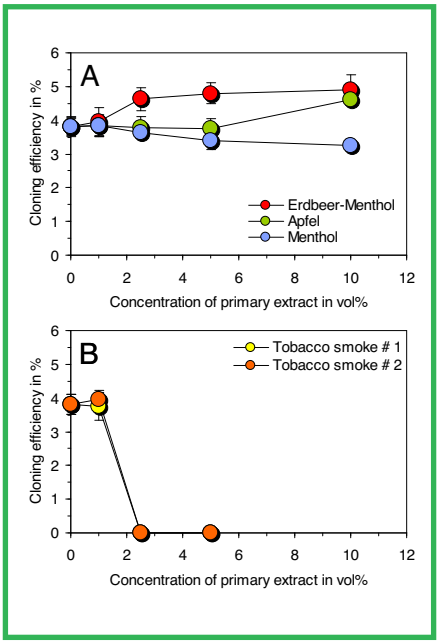

Figure 4: Graphical presentation of the results for the vapour extracts of three e-liquids of the brand Happy Liquid (A) and the smoke extract of two tobacco cigarette brands (B). Only the cloning efficiency as the most meaningful value for vitality and proliferation of human lung cells after long-term exposure is depicted (detailed explanation in the text). Data represent mean value ± standard error of the mean of three experiments.

concentration of 10 vol% (Figure 3, left column). Finally, cells did no longer show any signs of vitality and resembled „cell ghosts" that had lost their cytoplasm and nuclei after cell membranes were destroyed. In contrast, lung cells that were exposed to the undiluted primary extract of e-liquid vapour "Erdbeer-Menthol" with 6 mg/ml nicotine did not show any prominent alterations in cell morphology (Figure 3, right column). The only signs of an exposure to the e-liquid vapour extract was a higher amount of rounded cells which was not observed in controls.

Long-term exposure to extracts of tobacco smoke or e-liquid vapour

In order to simulate a long-term exposure of the cells by undergoing multiple cell divisions (at least 5 mitotic divisions), human lung cells were seeded into cell culture dishes with a diameter of 10 cm and a growth area of 55 cm^2 at a density of only 1,000 cells/dish to yield a single cell distribution. 9 ml of DMEM/Ham's F12 with 20 % fetal calf serum and antibiotics was used for one cell culture dish. Two days after seeding, cells were completely attached and spread to the bottom of the dishes. Then, 1 ml of primary extract was added to yield a tobacco smoke extract concentration of 0 to 5 vol% and an e-liquid vapour extract concentration of 0 to 10 vol% in the test. After another six days of continuous incubation, the evaporated liquid in the culture dishes was balanced by the addition of 3 ml of deionised sterile water to keep the osmotic concentration of the culture medium in the dishes as constant as possible. The lung cells were cultured for a total of 14 days after seeding, i.e. 12 days under the influence of the primary extracts. During this time period only a small amount of the single lung cells is mitotically active so that single cell clones (= cell clusters with genetically identical cells) can be obtained (Figure 4A). The number of clones and their size are related to the culture conditions and are directly influenced by any toxic substances present in the medium. Because of the long-term exposure, the toxic effect becomes more pronounced than in short-term cultures with a one-day exposure period (see previous chapter. Finally, cell cultures were fixed with methanol and stained with Coomassie-Giemsa solution for further examination. The stained cultures were air-dried and photographed with a Nikon D300 digital SLR and a macro lens at a magnification of 1:2. The photographs were processed by a digital image analysis system (Wimasis Image Analysis, ibidi, München). Although clone size and amount of cell-covered area in the dishes are also suitable evaluation parameters, only the data on cloning efficiency (= number of developed clones x 100/number of seeded cells) are presented here. The investigations were done in triplicate.

The long-term exposure of human lung cells to the vapour extracts of all three e-liquids resulted in different results which might be caused by the nicotine content in the e-liquids (Figure 4B). Two of the e-liquids, namly „Apfel" and „Erdbeer-Menthol" with the low nicotine concentration of 6 mg/ml had a cloning efficiency at all test concentrations which was in the same range as the untreated controls or even slightly higher. Exposure to the vapour extract of the e-liquid „Menthol" with 18 mg/ml resulted in a slight concentration-dependent reduction of cloning efficiency. However, the reduced values of "Menthol" do not seem to be related to a toxic effect, but to a reduced mitotic activity of the cells due to the high nicotine content. Additional investigations on the effect of increasing nicotine concentrations on lung cell vitality and proliferation did not show an effect at short-term exposure.

In contrast, tobacco smoke extracts gave completely different results. As can be seen in Figure 4C, the two highest test concentrations of tobacco smoke (2.5 and 5 vol%) completely inhibited the generation of cell clones. Moreover, at these concentrations no viable single cells could be detected in the dishes indicating that exposure to 2.5 and 5 vol% of tobacco smoke caused a complete death of lung cells and that this increased exposure period also increased the toxic effect (see previous chapter). Only at a concentration of 1 vol% we observed a cloning efficiency which did not differ significantly from the untreated controls.

Summary and conclusions

In summary, all three e-liquids of the brand Happy Liquid had no direct toxic effect on cultured human lung cells at short-term and long-term exposure. Only the e-liquid „Menthol" with its high nicotine concentration of 18 mg/ml resulted in a reduced mitotic activity of the cells in the long-term experiments. However, reducing the nicotine concentration in the e-liquid is the decision of each user. In contrast, the smoke extracts of both tobacco cigarette brands caused a marked toxic effect in the experimental setups presented here – even at 10 to 50 times lower concentrations than the vapour extracts of the e-liquids.

Moreover, the results presented here confirm and expand the detailed investigations of the Farsalinos group on fibroblasts and myocardial cells (Farsalinos et al. 2013; Romagna et al. 2013). In accordance to the concept of Farsalinos, we also conclude from our results that e-cigarettes may be less harmful for humans' health than tobacco cigarettes and could be useful products in tobacco harm reduction or smoking cessation.

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