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Test Report

TOBACCO SMOKE VS. E-LIQUID VAPOUR

Toxic effects on cultured human lung cells after long-term exposure

BACKGROUND

An electronic cigarette or e-cigarette is a battery-powered vaporiser which simulates tobacco smoking by producing an aerosol which resembles smoke. It generally uses a heating element that vaporises a liquid solution known as e-liquid. 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.
Prompted by this background, the present study was performed to compare the toxic effects after ***long-term exposure*** of tobacco smoke with the vapour of three e-liquids from Happy People GmbH, D-80337 München, Germany.
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.

TOBACCO CIGARETTE AND E-LIQUID

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, and the following e-liquids of the brand Happy Liquid produced by Happy People GmbH, D-80337 München, Germany: (1) „Menthol“ with 18 mg/ml nicotine, (2) „Apfel“ with 6 mg/ml nicotine, and (3) „Erdbeer-Menthol“ with 6 mg/ml nicotine.

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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 was presumed. The same conditions were applied for the e-cigarette (EVOD, EU version, vaporiser 2,2 Ω and rechargeable battery 3,7 V; KangerTech). The smoke of the cigarette and the vapour of the e-cigarette 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 vapour. 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 in the next chapter.

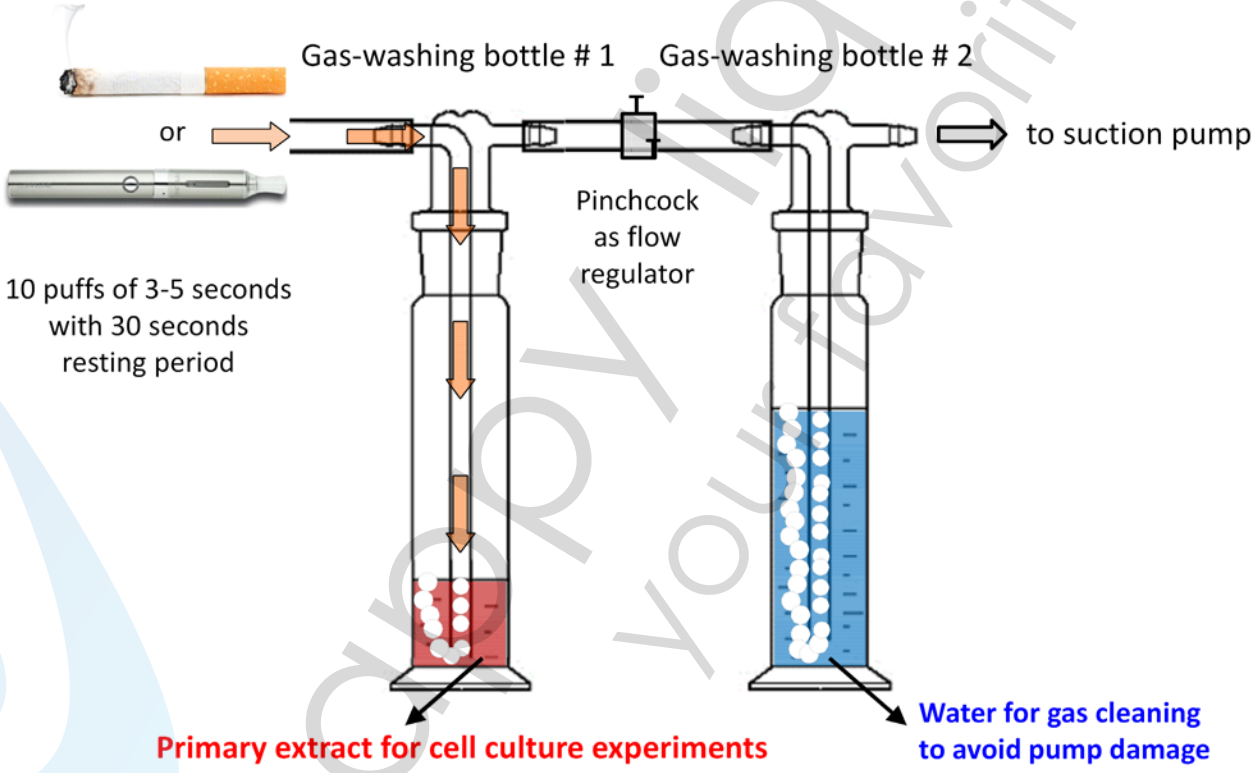

Figure 1: Experimental setup for a realistic simulation of cigarette smoking or vaping. The suction pump on the right generates an adjustable underpressure which aspirates the smoke or vapour and bubbles it into the culture medium in the left gas-washing bottle # 1. This yields the primary extract which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

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EXPERIMENTAL PROCEDURE

Cells were routinely cultured as mass cultures in a Binder CO2 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 & 100 µg/ml of streptomycin. All cell culture reagents were from GE Healthcare Life Sciences, D-35091 Cölbe, Germany.

For the experiments, cells were taken from 80 to 90 % confluent mass cultures and were seeded in 9 ml culture medium into new cell culture dishes (diameter 10 cm and 55 cm^2 of growth area) at a density of 1,000 cells/dish to yield a single cell distribution.

Two days after seeding, cells were completely attached and spread to the bottom of the wells. Then, 1 ml of undiluted or diluted primary extract was added to yield a tobacco smoke concentration of 0 – 1 – 2.5 – 5 vol% and an e-liquid vapour concentration of 0 – 1 – 2.5 – 5 – 10 vol% in the test. „0" is the internal control with pure culture medium without primary extract.

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 osmolarity 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. The number of clones and their size are related to the culture conditions and will be 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 an exposure period of only one day (see test report on acute toxicity of e-liquids vs. tobacco smoke).

After 14 days, culture medium was discarded and the cell clones were fixed with methanol for 2 minutes and stained with coomassie-giemsa solution according to Romanowsky. By this dye combination, cell nuclei are stained red and cytoplasm is stained blue. The stained cultures were air-dried and photographed with a Nikon D300 digital SLR and a macro lens at a magnification of 1:2 (Figure 2). The photos were processed by a digital image analysis system (Wimasis Image Analysis, ibidi GmbH, München). The results such as cloning efficiency (number of developed clones x 100/number of seeded cells), clone size and amount of cell-covered area in the dishes are presented in tabular and graphical form. The investigations were done in triplicate (n = 3).

RESULTS AND CONCLUSIONS

As can be seen in Table 1 and Figure 3, 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

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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 test report on acute toxicity). Only at a concentration of 1 vol% we observed cloning parameters which did not differ significantly from the untreated controls.

The long-term exposure of human lung cells to the vapour of the three e-liquids gave completely different results (Table 2 and Figure 3). Two of the e-liquids, namly „Apfel“ and „Erdbeer-Menthol“ with the low nicotine concentration 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 of the e-liquid „Menthol“ with 18 mg/ml resulted in a concentration-dependent reduction of cloning efficiency and cell-covered area. However, the reduced values seem to be not related to a toxic effect, but to a reduced mitotic activity of the cells due to the high nicotine content of 18 mg/ml. Further investigations on the effect of nicotine on lung cell viability and proliferation should clarify this point.

In summary, all three e-liquids of the brand Happy Liquid produced by Happy People GmbH in D-80337 München, Germany, had no toxic effect on cultured human lung cells at 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. However, reducing the nicotine concentration in his e-liquid is the decision of each user.

In contrast, the smoke of both tobacco cigarette brands caused a marked toxic effect in long-term exposure even at 10 times lower concentrations than the vapour of the e-liquids.

Thus, the results indicate that vaping of one of the 3 e-liquids should be preferred when having the choice to smoke a tobacco cigarette or to use an e-cigarette.

Investigator and responsible for the correctness of the presented experiments and results.

Schongau – December 1, 2014

CELL-BIOLOGICALLY TESTED PRODUCT
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PRODUKT ZELLBIOLOGISCH GEPRÜFT

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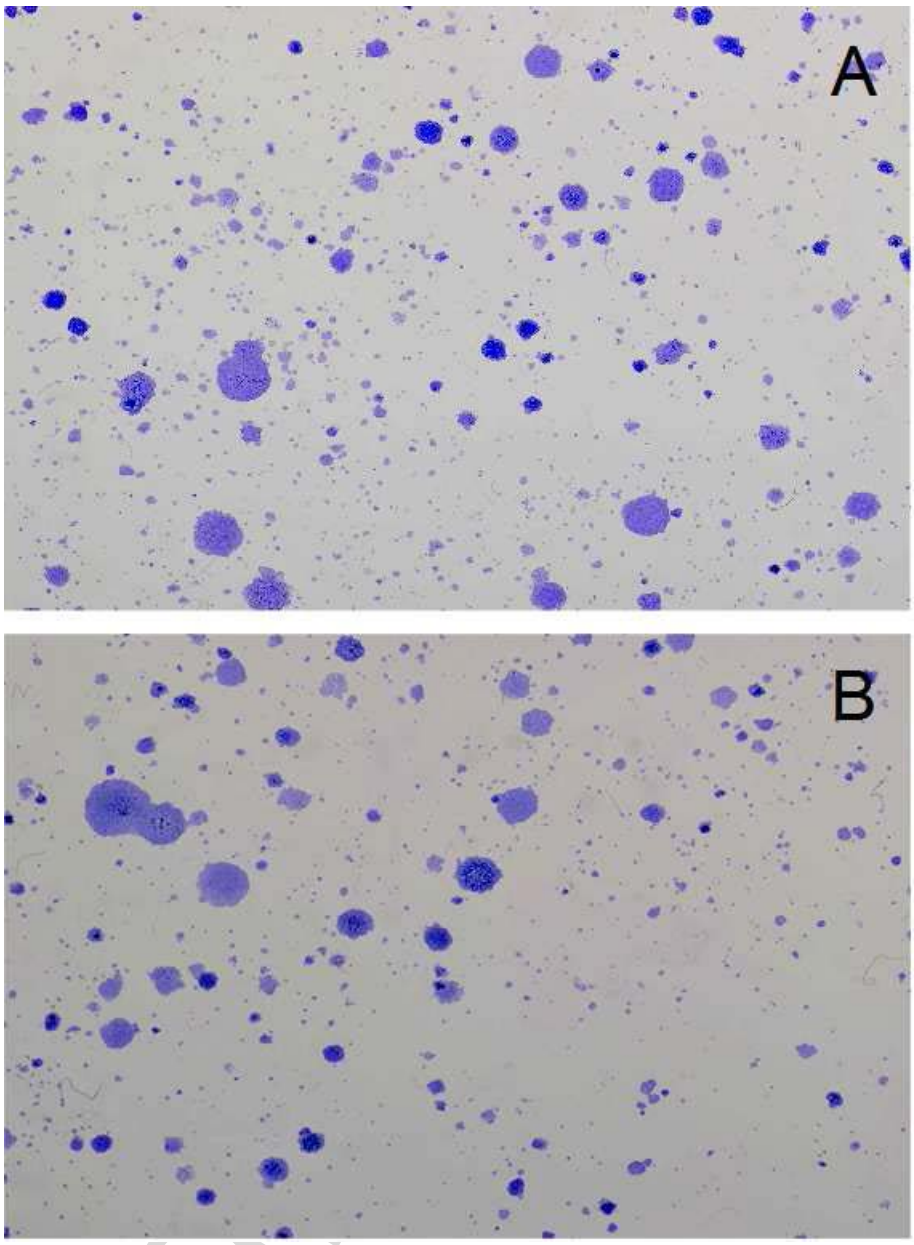

Figure 2: Representative macroscopic images of the stained clone cultures of e-liquid „Erdbeer-Menthol“ with 6 mg/ml nicotine at a concentration of 10 vol% (A) and the smoke of a tobacco cigarette at a concentration of 1 vol% (B). It can be seen that the cloning efficiency at 10 vol% of vapour extract is slightly higher than for 1 vol% of smoke extract. Images like these were taken for digital image analysis using Wimasis software.

Table 1: Presentation of the results for two different tobacco cigarettes of medium strength. For more explanations, see text.

Tobacco smoke brand # 1

Sample	Clon.eff. [%] M.W.	±	Clon.eff. [%] S.E.M.	Clone size [px] M.W.	±	Clone size [px] S.E.M.	Cov. area [%] M.W.	±	Cov. area [%] S.E.M.
Control (= 0 vol%)	3.82	±	0.30	11,216	±	974	9.25	±	1.18
Primary extract 1 vol%	3.75	±	0.28	11,343	±	894	10.36	±	2.01
Primary extract 2.5 vol%	0			0			0		
Primary extract 5 vol%	0			0			0		

Tobacco smoke brand # 2

Sample	Clon.eff. [%] M.W.	±	Clon.eff. [%] S.E.M.	Clone size [px] M.W.	±	Clone size [px] S.E.M.	Cov. area [%] M.W.	±	Cov. area [%] S.E.M.
Control (= 0 vol%)	3.82	±	0.30	11,216	±	974	9.25	±	1.18
Primary extract 1 vol%	3.95	±	0.41	10,440	±	935	7.63	±	1.04
Primary extract 2.5 vol%	0			0			0		
Primary extract 5 vol%	0			0			0		

Clon.eff. = Cloning efficiency for clones > 5.000 px
Cov. area = Covered area in culture dish
M.W. = Mean value
S.E.M. = Standard error of the mean
px = Pixel

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Table 2: Presentation of the results for three different e-liquids of the brand Happy Liquid. For more explanations, see text.

E-Liquid "Menthol" with 18 mg/ml nicotine

Sample	Clon.eff. [%] M.W.	±	Clon.eff. [%] S.E.M.	Clone size [px] M.W.	±	Clone size [px] S.E.M.	Cov. area [%] M.W.	±	Cov. area [%] S.E.M.
Control (= 0 vol%)	3.82	±	0.30	11,216	±	974	9.25	±	1.18
Primary extract 1 vol%	3.84	±	0.24	10,549	±	696	9.83	±	0.96
Primary extract 2.5 vol%	3.64	±	0.31	9,996	±	791	8.77	±	1.39
Primary extract 5 vol%	3.41	±	0.17	9,282	±	683	7.89	±	1.24
Primary extract 10 vol%	3.26	±	0.29	8,750	±	720	7.53	±	1.02

E-Liquid "Apfel" with 6 mg/ml nicotine

Sample	Clon.eff. [%] M.W.	±	Clon.eff. [%] S.E.M.	Clone size [px] M.W.	±	Clone size [px] S.E.M.	Cov. area [%] M.W.	±	Cov. area [%] S.E.M.
Control (= 0 vol%)	3.82	±	0.30	11,216	±	974	9.25	±	1.18
Primary extract 1 vol%	3.85	±	0.27	9,776	±	847	10.02	±	2.02
Primary extract 2.5 vol%	3.79	±	0.33	10,383	±	943	9.94	±	1.99
Primary extract 5 vol%	3.76	±	0.31	9,297	±	735	10.1	±	1.68
Primary extract 10 vol%	4.62	±	0.28	8,859	±	945	9.29	±	2.06

E-Liquid "Erdbeer-Menthol" with 6 mg/ml nicotine

Sample	Clon.eff. [%] M.W.	±	Clon.eff. [%] S.E.M.	Clone size [px] M.W.	±	Clone size [px] S.E.M.	Cov. area [%] M.W.	±	Cov. area [%] S.E.M.
Control (= 0 vol%)	3.82	±	0.30	11,216	±	974	9.25	±	1.18
Primary extract 1 vol%	3.95	±	0.41	10,437	±	996	9.57	±	1.84
Primary extract 2.5 vol%	4.63	±	0.35	10,381	±	664	9.71	±	1.67
Primary extract 5 vol%	4.80	±	0.32	9,543	±	891	10.22	±	1.89
Primary extract 10 vol%	4.92	±	0.42	9,144	±	927	10.13	±	2.21

Clon.eff. = Cloning efficiency for clones > 5.000 px
Cov. area = Covered area in culture dish
M.W. = Mean value
S.E.M. = Standard error of the mean
px = Pixel