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Test Report

TOBACCO SMOKE VS. E-LIQUID VAPOUR
ACUTE TOXIC EFFECTS ON CULTURED HUMAN LUNG CELLS

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 acute toxic effects of tobacco smoke with the vapour of an e-liquid 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 (for example, see Ramaga et al. 2006; Kode et al. 2006; Zhao et al. 2009; Jorgensen et al. 2010; Cervellati et al. 2014).

Ramage L, Jones AC, and Whelan CJ (2006). Induction of apoptosis with tobacco smoke and related products in A549 lung epithelial cells in vitro. J Inflammation 3: 3-14.

Kode A, Yang SR, and Rahman I (2006). Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. Respiratory Res 7: 132-152.

Zhao H, Albino AP, Jorgensen E, Traganos F, and Darzynkiewicz Z (2009). DNA damage response induced by tobacco smoke in normal human bronchial epithelial and A549 pulmonary adenocarcinoma cells assessed by laser scanning cytometry. Cytometry A 75: 840–847.

Jorgensen ED, Zhao H, Traganos F, Albino AP, and Darzynkiewicz Z (2010). DNA damage response induced by exposure of human lung adenocarcinoma cells to smoke from tobacco- and nicotine-free cigarettes. Cell Cycle 9: 2170–2176.

Cervellati F, Muresan XM, Sticozzi C, Gambari R, Montagner G, Forman HJ, Torricelli C, Maioli E, Valacchi G (2014): Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells. Toxicology in Vitro 28: 999-1005.

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TOBACCO CIGARETTE AND E-LIQUID

The investigations were done by using (1) a common cigarette brand of medium strength with 10 mg tar, 0,8 mg nicotine und 10 mg carbon monoxide, and (2) an e-liquid of the brand Happy Liquid produced by Happy People GmbH, D-80337 München, Germany, and containing menthol and 18 mg/ml nicotine.

SIMULATION OF SMOKING & VAPORISING 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. For smoking a cigarette, 10 puffs with a duration of 3 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 pump and piped 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 as described below.

EXPERIMENTAL SETUP

At the test concentrations 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 on cultured human lung cells as described in this study.
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 into 96-well plates (for enzymatic test; 200 µl/well) and 12-well plates (for morphological examination; 2 ml/well). 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 to yield the following concentrations of the primary extract in the test: 0 – 10 – 25 – 50 – 75 – 100 vol% with 0 vol% as control (= only culture medium without primary extract) und 100 vol% as undiluted primary extract. The exposure time to the different concentrations of the primary extracts was 24 hours.

Thereafter, culture medium of the 96-well plates was discarded and replaced by 180 µl/well of culture medium and 20 µl/well of XTT. Multiwell plates were incubated for another hour at 37 °C in the incubator and the optical density of each well was examined by a difference measurement at OD = 450 – 690 nm using a double-wavelength elisa reader (BioTEK Elx 808). XTT is the sodium salt of 2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide and has a yellowish colour. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of XTT yielding orange formazan crystals which are soluble in aqueous solutions. The intensity of the resulting orange solution is directly correlated with cell vitality and metabolic activity. The results were expressed graphically as relative values in comparison to untreated controls. Experiments were done in triplicate.
For further information on the use of XTT, see Roehm et al (1991) and Brosin et al (1997).

Roehm NW, Rodgers GH, Hatfield SM, and Glasebrook AL (1991). An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Meth 142: 257-265.
Brosin A, Wolf V, Mattheus A, and Heise H (1997). Use of XTT-assay to assess the cytotoxicity of different surfactants and metal salts in human keratinocytes (HaCaT). A feasible method for in vitro testing of skin irritants. Acta Dermato-venereologica 77:26-28.

RESULTS

As can be seen in Figures 1 and 2, the alterations of lung cell morphology were dramatic after exposure to the primary extract of tobacco smoke for only 24 hours. Even the lowest test concentration of 10 vol% caused a rounding and detachment of the vast majority of the cells. Moreover, numerous detached cells were formed like thorn-appled erythrocytes after loss of water or showed vacuolisation. At higher concentrations of primary extract, most cells lost vitality and resembled „cell ghosts“ which had lost their cytoplasm and nuclei after cell membranes were destroyed. For comparison, lung cells which were exposed to the primary extract of e-liquid vapour did not show such prominent alterations in cell morphology. The only signs of an exposure to e-liquid vapour was a dose-dependent increase in the number of detached cells at concentrations above 50 vol%.
The observed morphological alterations correlated well with the cell vitality data as presented in Table 1 and Figure 3. In the case of the primary extract of tobacco smoke, the lowest test concentration of 10 vol% 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 acute toxic potential of tobacco smoke. The primary extract of e-liquid vapour cause 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.
When evaluating the acute toxic effect by extrapolation of the measurement data, it can be stated that tobacco smoke was approximately 50 to 100-fold more toxic than the e-liquid vapour of Happy Liquid as tested with the simulation used here.

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SUMMARY & CONCLUSION

The results of the present investigation have demonstrated that smoking of only one single tobacco cigarette causes a marked acute toxic effect when using a test system with human lung cells. Within an exposure period of 24 hours, the vast majority of the cells lost their vitality. In contrast, the vapour of the e-liquid of Happy People GmbH exhibited no such toxic effects in the same concentration range. However, the three highest concentrations of the vapour caused a dose-dependent detachment of a minority of the cells resulting in a reduced vitality of the total lung cell population.

As a matter of fact, e-liquid vaporising is not harmless for the health, but far less toxic than inhaled tobacco smoke. A long-term investigation using lung cells for the evaluation of chronic or long-term effects of the vapour could give further insights. In addition, a chemical analysis of the primary extract after simulation of e-liquid vaporising in reality might help to persue the behaviour of the basic ingredients and the resulting compounds present in the vapour and finally entering the lung.

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

Schongau: August 8, 2014

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PRODUKT ZELLBIOLOGISCH GEPRÜFT

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Biochemist

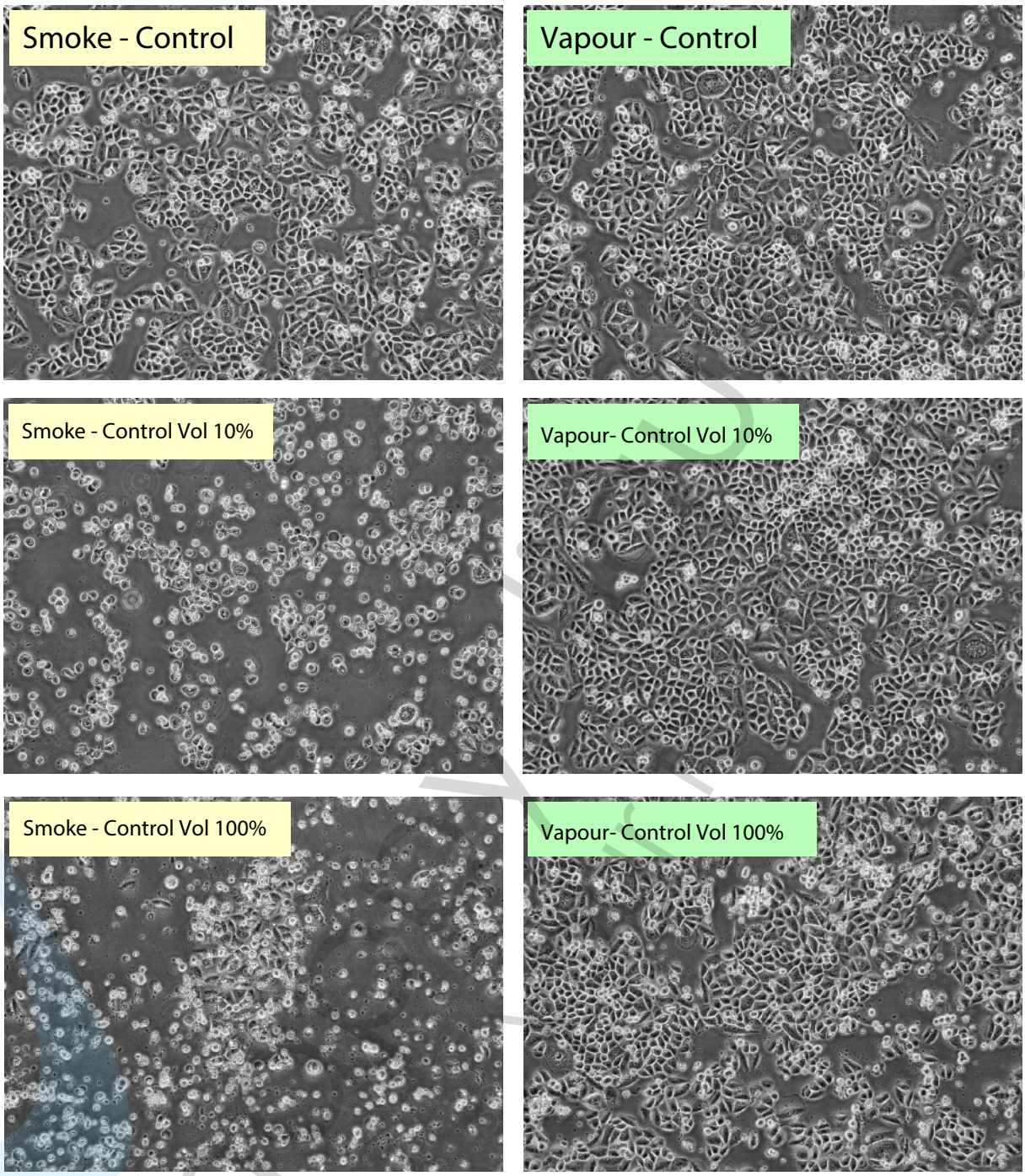

Figure 1: Effect of different concentrations of tobacco smoke (left column) and e-liquid vapour (right column) on morphology of cultivated human lung cells. Control = pure culture medium without any extract; primary extracts either diluted 1:10 (= 10 vol%) or undiluted (= 100 vol%). Note the pronounced acute toxic effect of tobacco smoke even at concentrations of only 10 vol% by rounding and detachment of dead cells as well as the almost unaltered cell morphology after exposure to e-liquid vapour of the brand Happy Liquid at 100 vol%. The small rounded cells present in controls and vapour samples are mitotically active cells and are hardly to distinguish from dead and floating cells in tobacco smoke samples. Inverted microscope Olympus IX50 at phase contrast with Olympus E-10 digital camera and Olympus Planachromate 10x lens.

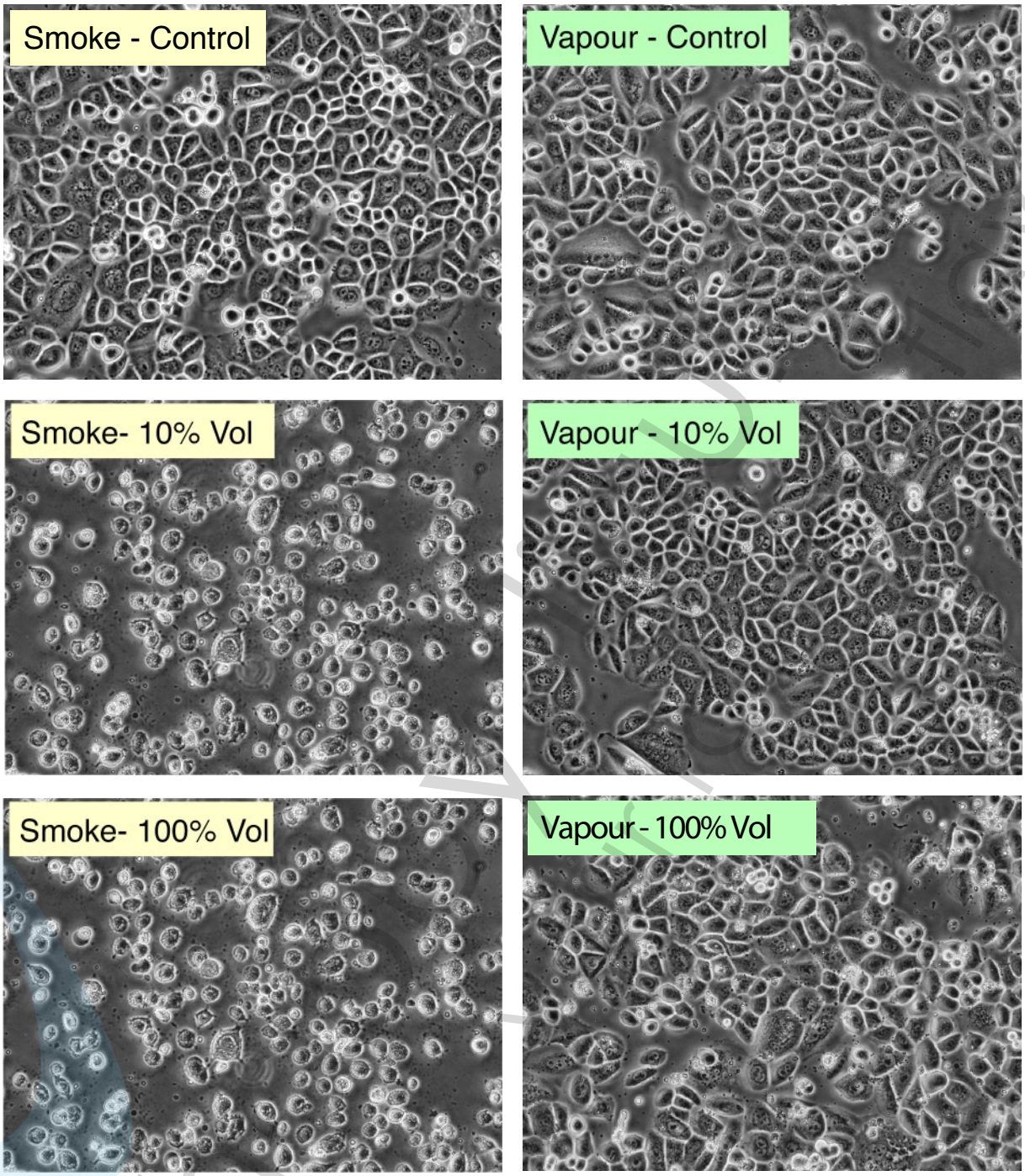

Figure 2: Effect of different concentrations of tobacco smoke (left column) and e-liquid vapour (right column) on morphology of cultivated human lung cells. Control = pure culture medium without any extract; primary extracts either diluted 1:10 (= 10 vol%) or undiluted (= 100 vol%). Note the pronounced acute toxic effect of tobacco smoke even at concentrations of only 10 vol% by rounding and detachment of dead cells as well as the almost unaltered cell morphology after exposure to e-liquid vapour of the brand Happy Liquid at 100 vol%. The small rounded cells present in controls and vapour samples are mitotically active cells and are hardly to distinguish from dead and floating cells in tobacco smoke samples. Inverted microscope Olympus IX50 at phase contrast with Olympus E-10 digital camera and Olympus Planachromate 20x lens.

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Tobacco Smoke

Sample	Measured optical density (single values)			Mean value	±	S.D.	Rel. cell vitality in % vs. control	±	S.D. in %
Primary extract 0 vol% (= control)	720	616	662	666	±	52	100.0	±	7.8
Primary extract 10 vol%	58	16	39	38	±	21	7.8	±	3.2
Primary extract 25 vol%	5	1	2	3	±	2	0.4	±	0.3
Primary extract 50 vol%	2	3	1	2	±	1	0.3	±	0.2
Primary extract 75 vol%	1	2	2	2	±	1	0.3	±	0.1
Primary extract 100 vol% (= undiluted)	0	0	1	0	±	1	0.1	±	1.5

E-Liquid Vapour

Sample	Measured optical density (single values)			Mean value	±	S.D.	Rel. cell vitality in % vs. control	±	S.D. in %
Primary extract 0 vol% (= control)	705	714	662	694	±	28	100.0	±	4.0
Primary extract 10 vol%	757	627	731	705	±	69	101.6	±	9.9
Primary extract 25 vol%	746	645	692	694	±	51	100.1	±	7.3
Primary extract 50 vol%	682	585	628	632	±	49	91.1	±	7.0
Primary extract 75 vol%	581	687	585	618	±	60	89.0	±	8.7
Primary extract 100 vol% (= undiluted)	496	497	528	507	±	18	73.1	±	2.6

Table 1: Tabular presentation of the results of the single experiments (n = 3) as well as the calculated values for cell vitality. S.D. = standard deviation. Further explanations can be found in the legend to Figure 3.

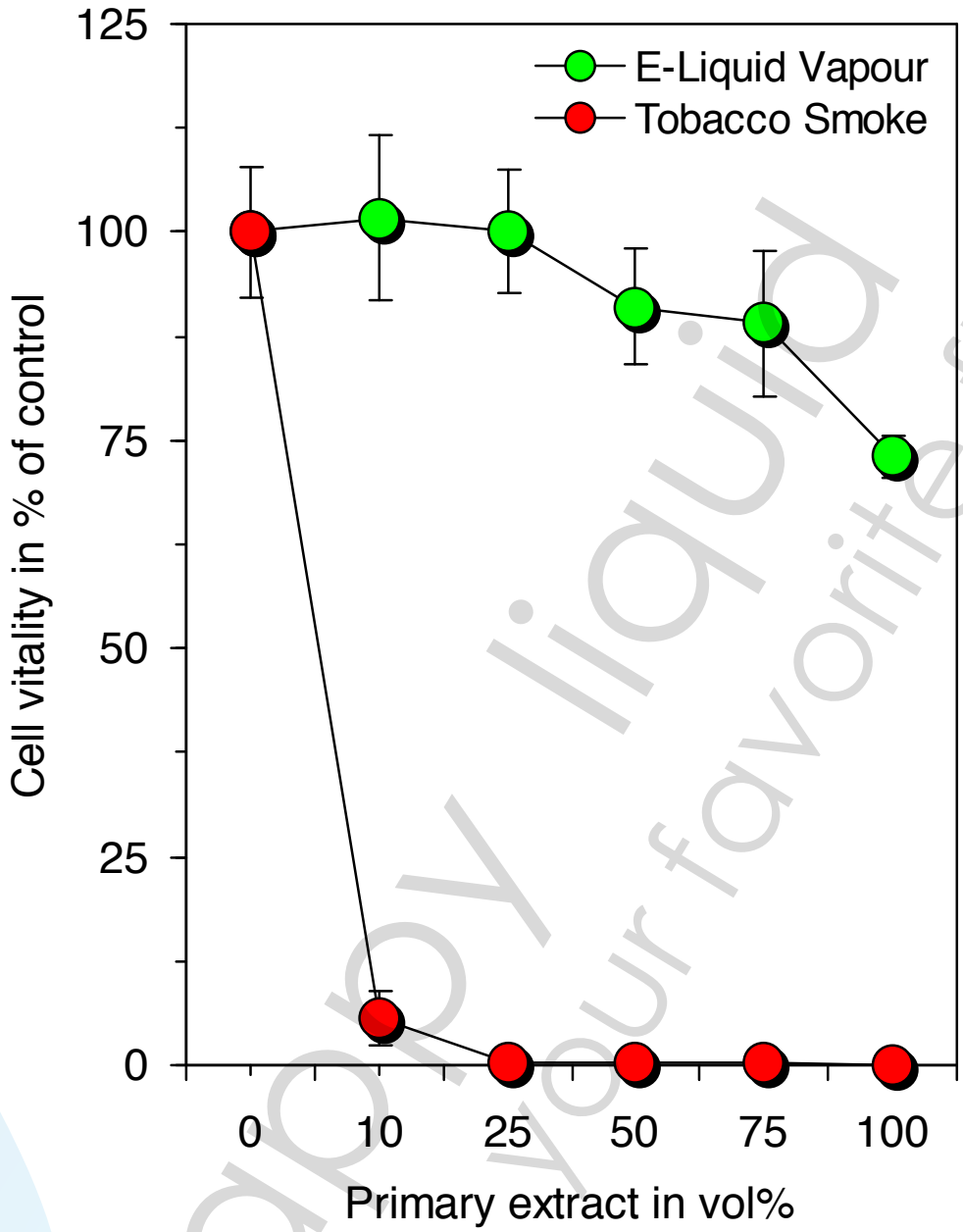

Figure 3: Graphical presentation of the summarised experimental results on the acute toxic effect of tobacco smoke in comparison to e-liquid vapour of the brand Happy Liquid containing menthol and 18 mg/ml of nicotine. The 1:10 diluted primary extract of tobacco smoke causes death of nearly all cultured 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 3 experiments.