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TEST REPORT

Mutagenic effect of tobacco smoke in comparison to the vapour of two e-liquids manufactured by Happy People GmbH

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.

Especially the amount of mutagenic substances should be much lower in the vapour than in tobacco smoke – provided that an e-cigarette is used at „normal conditions“, i.e. with a maximum power of 6.5 watts for vapour production. The fact that a higher power of e-cigarette use causes an increase in vaporising temperature and, thus, a significant higher amount of mutagenic substances has been shown recently by Kosmider et al. (2014) and Jensen et al. (2015).

Prompted by this background we examined the mutagenic potential of the vapour of two different e-liquids from Happy People GmbH, D-80337 München, Germany, in comparison to the tobacco smoke of a common cigarette by using an Ames MPF Aqua test.

Kosmider L, Sobczak A, Fik M, Knysak J, Zaciera M, et al. (2014): Carbonyl compounds in electronic cigarette vapors - effects of nicotine solvent and battery output voltage. Nicotine Tob Res 16:1319–1326).

Jensen RP, Wentai L, Pankow JF, Strongin RM, Peyton DH (2015): Hidden formaldehyde in e-cigarette aerosols. Letter to the editor. N Engl J Med 372: 392-394.

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TOBACCO CIGARETTE AND E-LIQUID

The examinations 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 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 and (2) „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 specially designed smoking apparatus was used which allows to vary the frequency, length and the depths of the puffs (Figure 1). For smoking 5 cigarettes, 5 x 10 puffs with a duration of 3 seconds and a pause of 15 seconds between two puffs was presumed (Vansickel et al. 2010).

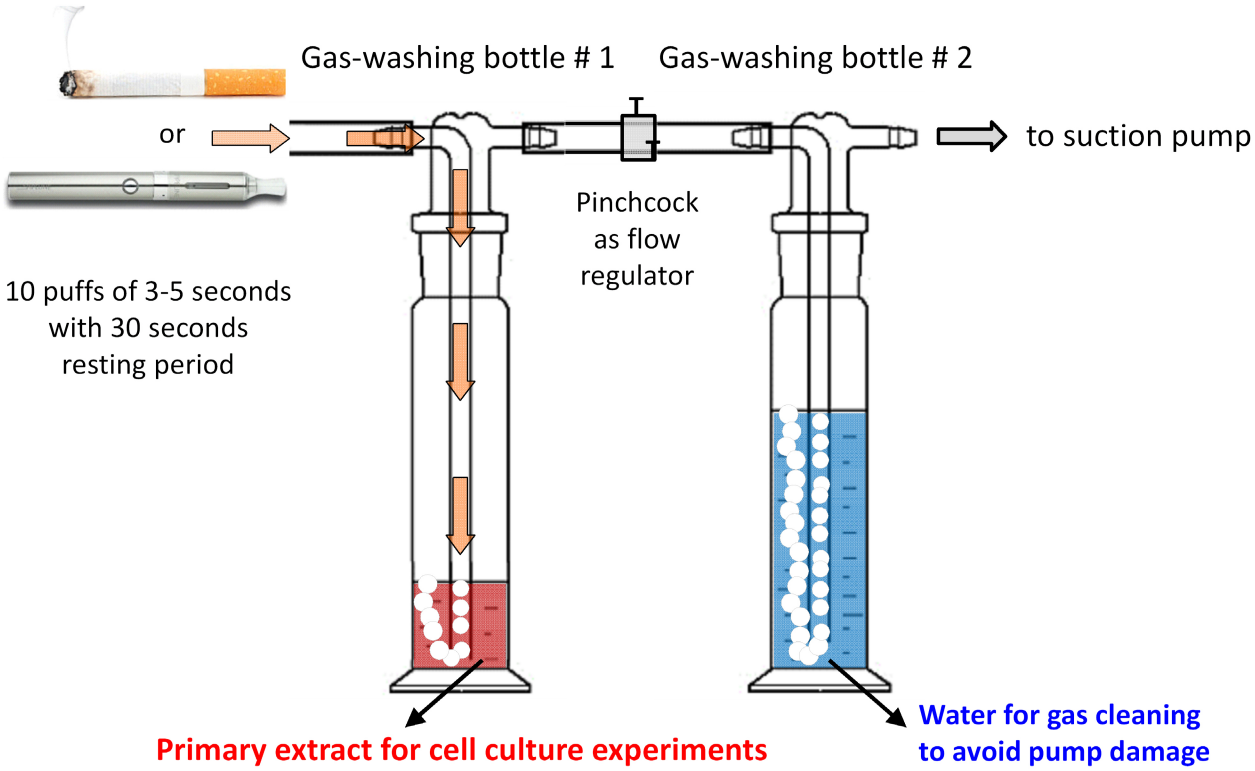

Figure 1: Experimental setup for a realistic simulation of cigarette smoking or vaping. The suction pump on the right generates an adjustable underpressure which aspirates the smoke or vapour and passes it into the culture medium in the left gas-washing bottle # 1. This yields the primary extract which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

Vansickel AR et al. (2010): A clinical laboratory model for evaluating the acute effects of electronic “cigarettes”: Nicotine delivery profile and cardiovascular and subjective effects. Cancer Epidemiology, Biomarkers, and Prevention 19:1945–1953.

For vaping, 50 puffs with a duration of 5 seconds and a pause of 15 seconds were done by using an e-cigarette at 6.2 watts (EVOD, EU version, vaporiser 2,2 Ω and rechargeable battery 3,7 V; KangerTech). The smoke of the cigarette and the vapours 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. This extract was brownish-yellow for cigarette smoke and colourless for e-cigarette vapour.

Ames MPF test – Basic principle

The bacteria reversed mutation assay (Ames test) was developed in the 1970's as a fast and sensitive assay of the ability of a chemical compound or mixture to induce mutations in DNA (Maron and Ames, 1983). The test uses amino acid-dependent strains of *Salmonella typhimurium* and *Escherichia coli*. In the absence of an external histidine source, the cells cannot grow to form colonies. Colony growth is resumed if a reversion of the mutation occurs, allowing the production of histidine for bacterial growth. Spontaneous reversions occur with each of the strains; mutagenic compounds cause an increase in the number of revertant colonies relative to the basal background level. Because the assay does not use a live animal model, it is relatively inexpensive, easy and fast.

The Ames MPF Mutagenicity Assay corresponds to the Ames Fluctuation Assay and is based on the same principle as the traditional test, but it sets a new standard for this type of testing, offering several advantages over the traditional Ames test. The Ames Fluctuation Assay is cited in the guidelines of OECD and FDA. For further information, see Gee et al. (1998).

EXPERIMENTAL PROCEDURE

The test samples were assayed in the Ames MPF 98/100 Aqua reverse mutation assay (Xenometrix AG, Switzerland), using the strains TA100 for base-pair substitutions and TA98 for frameshift mutations, in the presence and absence of Aroclor 1254-induced S9. The strains are histidine auxotroph, and mutagenesis will lead to reversion of the strains to histidine prototrophy.

The aqueous test samples were diluted stepwise to yield the following test concentrations: 74, 37, 18.5, 9.25, 4.63 and 2.32 vol%. All doses were run in triplicate. The following positive control chemicals were used to assess the performance of the Ames MPF 98/100 Aqua

Maron DM, Ames BN (1983): Revised methods for the Salmonella mutagenicity test. Mut Res 113: 173-215.

Gee P, Sommers CH et al. (1998): Comparison of base-specific salmonella tester strains with the traditional strains for identifying mutagens: the results of a validation study. Mut Res 412: 115-130.

assay: 4-nitroqionoline-N-oxide, 0.5 µg/ml (TA100-S9); 2-nitrofluorene, 2 µg/ml (TA98-S9); 2-aminoanthracene, 2.5 µg/ml (TA100 and TA98+S9).
The MPF test, in brief, was as follows: 1. Growth of tester strains overnight in growth medium. 2. A 90-minute incubation in exposure medium with limiting histidine in the presence of test sample and S9 if employed. 3. Dilution and distribution of exposed bacteria into 384-well plates in a medium which selects for revertants. This medium is free of histidine and contains a pH indicator dye that turns from purple to yellow upon bacterial growth. 4. Incubation of the microplates for 48 hours to allow growth of revertant colonies. 5. Scoring of microplates for positive (yellow) wells.
The following criteria were used to evaluate the results: fold increase over the solvent control baseline and dose-dependency. The fold increase of revertants relative to the solvent control was determined by dividing the mean number of positive wells at each dose by that of the negative control baseline. The negative control baseline was derived from the mean number of positive wells in the negative control plus 1 standard deviation. If the baseline was below 1, it was set to 1. A fold increase equal to or greater than 2 times the baseline level is generally considered as an alert. Multiple responses of greater than 2-fold the baseline level with a dose-response will lead to the test compound being classified as a clear positive. A test compound is classified negative where no response greater than 2 times the baseline is recorded.

RESULTS & CONCLUSIONS

Examination of both e-liquid samples „Menthol“ and „Strawberry-Menthol“ of the brand Happy Liquid using the Ames MPF 98/100 Aqua reverse mutation assay showed no mutagenic effects for strain TA98 after a continuous exposure time of 90 minutes (Fig. 2, Ia und IIa). Exposure with cigarette smoke in the same assay resulted in a dose-dependent increase of revertants demonstrating its marked mutagenicity (Fig. 2, IIIa).When the assay was conducted with metabolic activation by S9, similar results were achieved. For both e-liquids, the critical threshhold was not reached (Fig. 2, Ib and IIb), whereas the critical threshhold was exceeded by cigarette smoke much more than without metabolic activation (Fig. 2, IIIb).
Examination of both e-liquids by using the strain TA100 ± S9-activation showed no mutagenic effect (Fig. 3, Ic, IIc, Id, IId). The higher critical threshhold for TA100 is caused by the presence of a higher number of spontaneous revertants. For cigarette smoke the critical threshhold was almost reached (Fig 3, IIIc and IIId). However, the highest concentrations of cigarette smoke were clearly cytotoxic as indicated by a partial lysis of the bacteria and a delayed development of the positive wells. In addition, the cytotoxic component had a volatile character as it delayed also the development of the yellow wells in the positive control

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chemicals which were positioned next to the highest concentrations in the plate. Thus, the mutagenic strength of this test sample might be even underestimated.
In summary, both tested e-liquids from Happy People GmbH did not show any mutagenic potential when vaped under normal conditions. In contrast, cigarette smoke caused a clear mutagenic effect which might be even underestimated by the partial bacterial lysis due to a cytotoxic effect. The results indicate that vaping of „Menthol“ or „Strawberry-Menthol“ 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 - July 2, 2015

Prof. Dr. Peter C. Dartsch
Diplom-Biochemiker

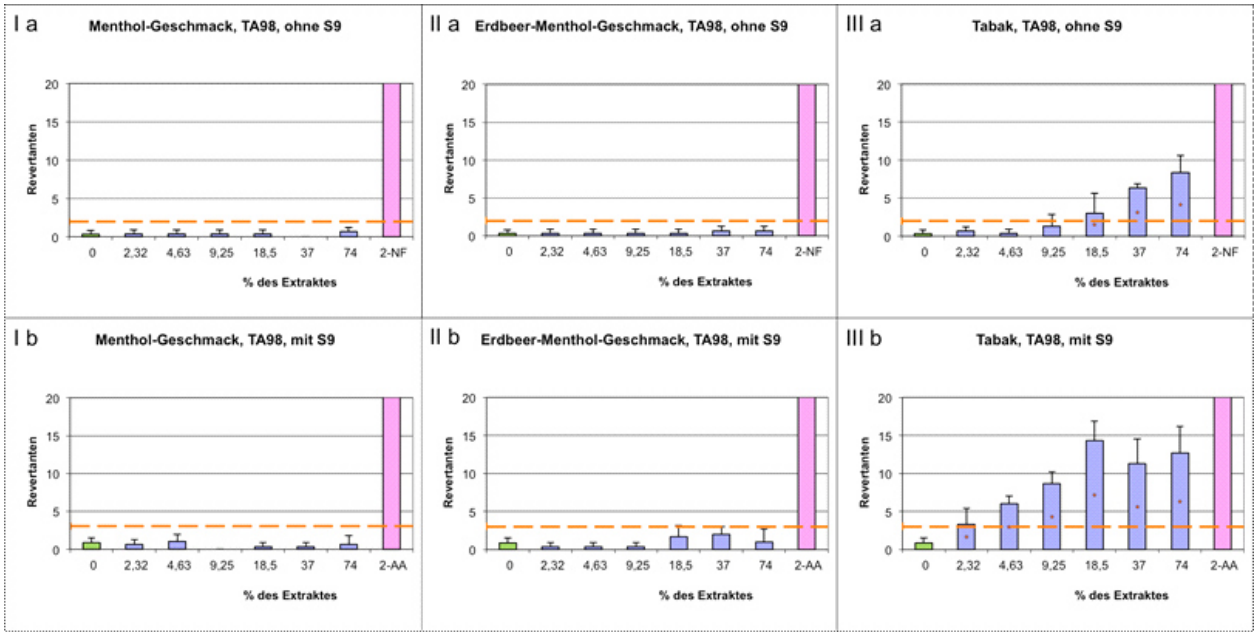

Fig. 2: Results of Ames MPF Aqua reverse mutation assay with strain TA98. I: Menthol, II Strawberry-Menthol, III Tobacco cigarette. a: without S9 metabolisation, b: with S9 metabolisation. Positive controls: 2-nitrofluorene (NF) and 2-aminoanthracene (2-AA).

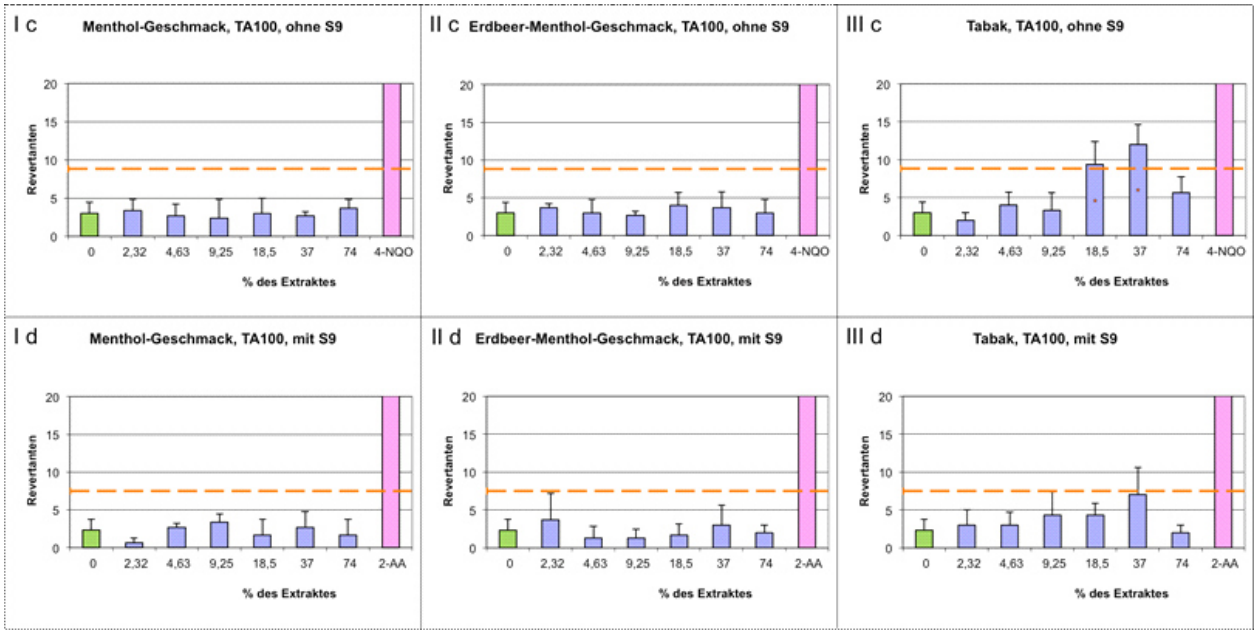

Fig. 3: Results of Ames MPF Aqua reverse mutation assay with strain TA100. I: Menthol, II Strawberry-Menthol, III Tobacco cigarette. a: without S9 metabolisation, b: with S9 metabolisation. Positive controls: 4-nitroqionoline-N-oxide (4-NQO) and 2-aminoanthracene (2-AA).