valeo

The e-Liquid laboratory

Study 10/2014

Acute toxic effect of e-Liquids on human lung cells

dartschSCIENTIFIC
INSTITUT FÜR ZELLBIOLOGISCHE TESTSYSTEME

Dartsch Scientific GmbH · Oskar-von-Miller-Str. 10 · D-86956 Schongau

Firma
valeo laboratories GmbH
Lise-Meitner-Straße 21

D-25524 Itzehoe

Oskar-von-Miller-Straße 10
D-86956 Schongau, Germany

Fon Diessen: +49 8807 2759-650
Fon Schongau: +49 8861 256-5250
Fax: +49 8861 256-7162
Email: info@dartsch-scientific.com
Web: www.dartsch-scientific.com

November 5, 2014

TEST REPORT

Evaluation of the acute toxicity of tobacco smoke in comparison to the vapour of two basic liquids from valeo laboratories

Investigations using 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 glycerin, 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 the health effects related to the variability of vaporisers, e-liquid ingredients and their quality.
Prompted by this background, the present study with human lung cells was conducted to compare the acute toxicity of tobacco smoke with the vapour of two basic liquids from valeo laboratories GmbH, D-25524 Itzehoe, Germany.

TOBACCO CIGARETTE AND LIQUID BASES

The investigations were done by using a common cigarette brand of medium strength with 10 mg tar, 0.8 mg nicotine und 10 mg carbon monoxide and two basic liquids from valeo laboratories GmbH, D-25524 Itzehoe, Germany. In detail, the following basic liquids were examined: (1) Valeo Nicotine Liquid Sample with 12 mg/ml nicotine, and (2) Valeo BioNic Liquid Nicotine Substitute Medium 1.5 %.

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. For smoking a ciga-

rette, 10 puffs with a duration of 3 to 5 seconds and a pause of 30 seconds between two puffs was presumed (see 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).
The e-cigarette used for the experiments was a common product with a vaporiser of 2.2 Ω and a voltage of 3.7 V. The smoke of the cigarette and the vapour of the e-cigarette were aspirated by a pump and piped into 20 ml of HEPES-buffered cell culture medium. The resulting primary extracts had a neutral pH value of 7.4 ± 0.3. The extract was brownish for tobacco cigarette smoke and colourless for basic liquid vapour. Both primary extracts were filtrated sterile by pressing them through a sterile porous membrane (porous size 0.45 µm) and were added to the lung cells cultures as described below.

EXPERIMENTAL SETUP

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 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).
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 & 100 µg/ml of streptomycin. All cell culture reagents were from GE Healthcare Life Sciences, Cölbe, Germany. For the experiments, cells were taken from 80 to 90 % confluent mass cultures and were seeded into 96-well plates for enzymatical test (200 µl/well) and 12-well plates for morphological examination (2 ml/well). Seeded cell densities were chosen 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 basic liquid vapour to yield the following concentrations of the primary extracts 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, cells in 12-well plates were observed morphologically for signs of an acute toxic effect such as perinuclear vacuolisation, rounding or detachment of the cells. The culture medium of the 96-well plates was discarded and replaced by 190 µl/well of culture medium and 10 µl/well of WST-1. Multiwell plates were incubated for another 30 minutes at 37 °C in the incubator and the optical density of each well was examined by a difference measurement at $\Delta OD = 450 - 690$ nm using a double-wavelength elisa reader (BioTEK

dartschSCIENTIFIC
INSTITUT FÜR ZELLBIOLOGISCHE TESTSYSTEME

Elx 808). WST-1 (Roche Diagnostics, Mannheim, Germany) is a tetrazolium salt which is used in a colorimetric assay for the quantification of cellular proliferation, viability, and cytotoxicity. The stable tetrazolium salt (red colour) is cleaved to a water-soluble formazan (yellow colour) by a complex cellular bioreduction process which is largely dependent on the glycolytic production of NAD(P)H in viable cells. In particular, WST-1 is cleaved to formazan by the succinate tetrazolium reductase system which belongs to the respiratory chain of mitochondria, and is only active in metabolically intact cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. Experiments were done in triplicate.

RESULTS AND CONCLUSIONS

The morphological alterations of lung cells due to a 24 hour exposure to the primary extract of tobacco smoke were dramatic and resulted in a rounding and detachment of lung cells (not depicted). This morphological results correlated well with the results of the enzymatical tests which are depicted in the table and figure. Even the lowest test concentration of 10 vol% of the primary extract of tobacco smoke caused a decrease in cell vitality by about 50 %; in case of the undiluted primary extract only 2 % of cells were viable.
In contrast, human lung cells which were exposed to the vapour of both basic liquids behaved completely different. At all concentrations tested no signs of an altered cell morphology were observed. This correlated well with the cell vitality data of basic liquid vapour which did not differ signficantly from those of the untreated controls (see table and figure for details). Thus, the test system used here was unable to detect an acute toxic effect for both basic liquids. To what extent any flavouring substances present in the liquids change the results must be clarified from case to case.

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

Schongau – November 5, 2014

CELL-BIOLOGICALLY TESTED PRODUCT
dartsch SCIENTIFIC
2014
PRODUKT ZELLBIOLOGISCH GEPRÜFT

Prof. Dr. Peter C. Dartsch
Diplom-Biochemiker

dartschSCIENTIFIC
INSTITUT FÜR ZELLBIOLOGISCHE TESTSYSTEME

Tabular presentation of the absolute and relative results of each single experiment and the calculated final results for cell vitality in comparison to controls. S.D. = standard deviation.

Tobacco smoke

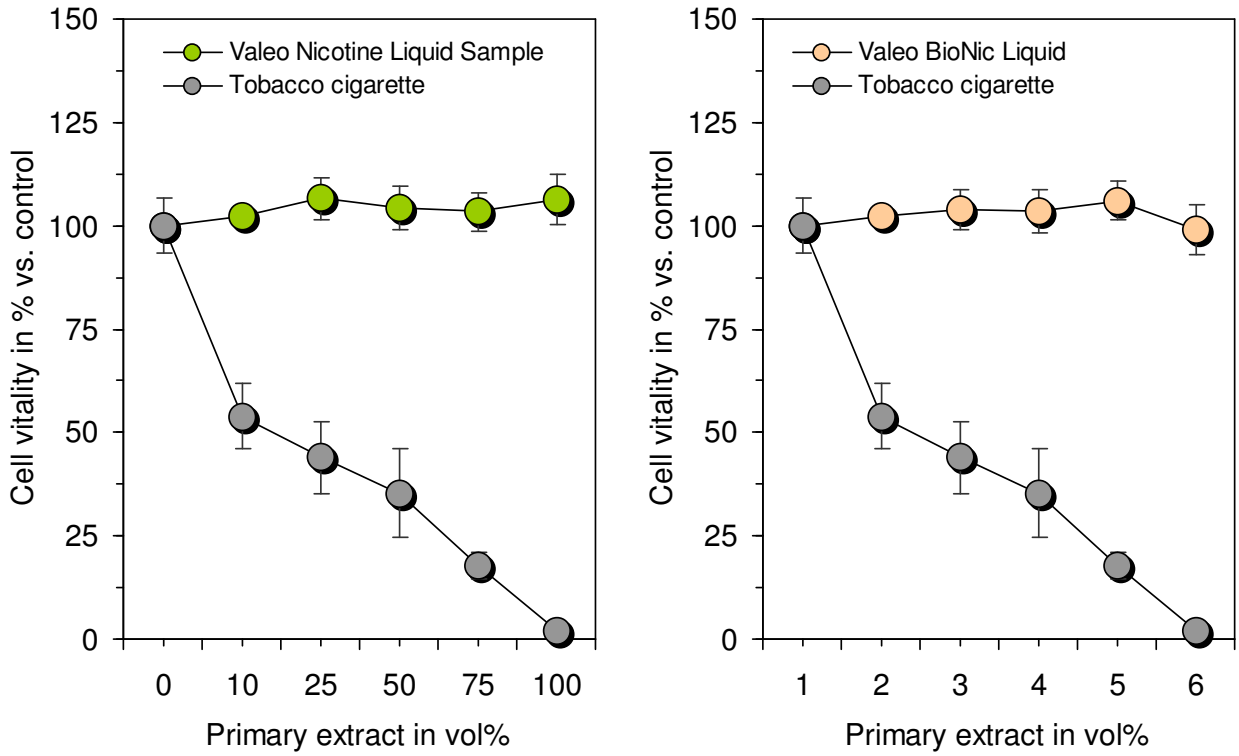
Sample	Optical density (single values)			Mean value	±	S.D.	Rel. cell vitality in % vs. control	±	S.D. in %
Control (= 0 vol%)	734	721	743	733	±	11	100.0	±	1.5
Primary extract 10 vol%	448	335	403	395	±	57	54.0	±	7.8
Primary extract 25 vol%	394	273	299	322	±	64	43.9	±	8.7
Primary extract 50 vol%	287	318	169	258	±	79	35.2	±	10.7
Primary extract 75 vol%	124	109	158	130	±	25	17.8	±	3.4
Primary extract 100 vol% (undiluted)	11	21	9	14	±	6	1.9	±	1.6

Valeo Nicotine Liquid Sample with 12 mg/ml nicotine

Sample	Optical density (single values)			Mean value	±	S.D.	Rel. cell vitality in % vs. control	±	S.D. in %
Control (= 0 vol%)	707	761	742	737	±	27	100.0	±	3.7
Primary extract 10 vol%	754	783	720	752	±	32	102.1	±	4.3
Primary extract 25 vol%	769	810	776	785	±	22	106.6	±	3.0
Primary extract 50 vol%	761	800	743	768	±	29	104.3	±	4.0
Primary extract 75 vol%	753	770	761	761	±	9	103.0	±	1.2
Primary extract 100 vol% (undiluted)	788	816	745	783	±	36	106.3	±	4.8

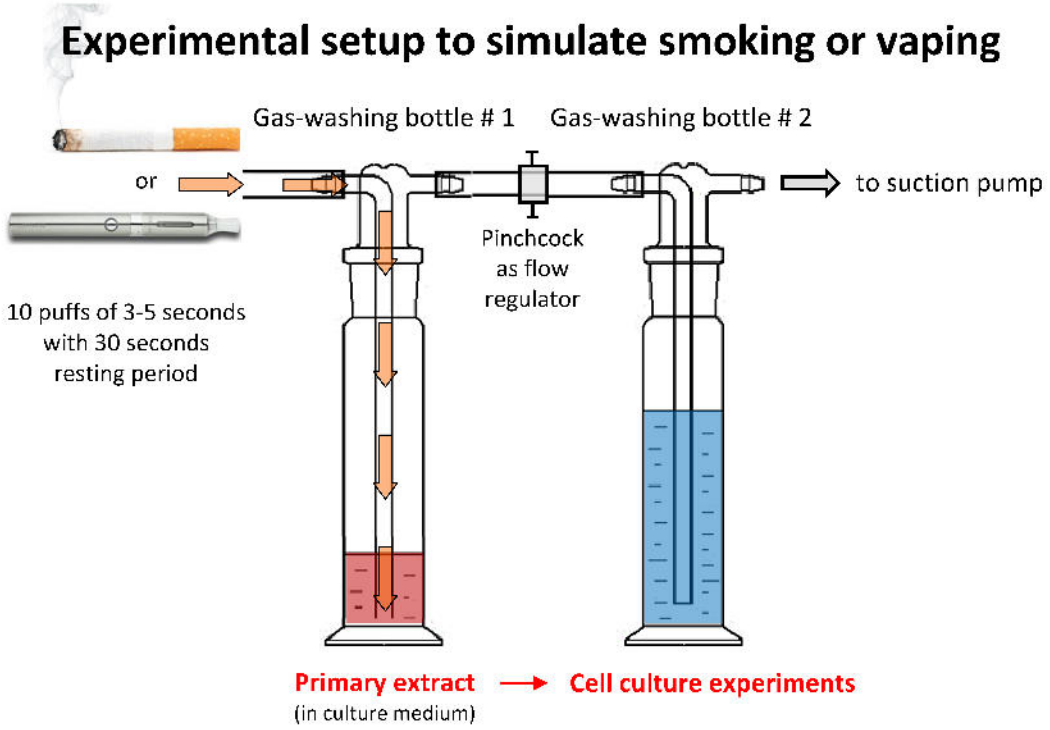
Valeo BioNic Liquid Nicotine Substitute Medium 1.5 %

Sample	Optical density (single values)			Mean value	±	S.D.	Rel. cell vitality in % vs. control	±	S.D. in %
Control (= 0 vol%)	692	718	785	732	±	48	100.0	±	6.6
Primary extract 10 vol%	756	762	731	750	±	16	102.5	±	2.2
Primary extract 25 vol%	784	719	778	760	±	36	103.9	±	4.9
Primary extract 50 vol%	799	722	750	757	±	39	103.5	±	5.3
Primary extract 75 vol%	788	737	802	776	±	34	106.0	±	4.7
Primary extract 100 vol% (undiluted)	776	701	695	724	±	45	99.0	±	6.0



Graphical presentation of the acute toxicity of tobacco cigarette smoke in comparison to both tested basic liquid vapours (Valeo Nicotine Liquid Sample with 12 mg/ml nicotine (left) and Valeo BioNic Liquid Nicotine Substitute Medium 1.5 % (right)). The primary extract of tobacco smoke causes a loss in cell vitality of about 50 % at a concentration of only 10 vol%, whereas the undiluted primary extracts of both basic liquids do not cause any alterations in cell vitality in comparison to untreated controls. Thus, tobacco smoke has a much higher acute toxic effect than the tested basic liquids. Data represent mean value ± standard deviation of 3 experiments.

Experimental setup to simulate smoking or vaping



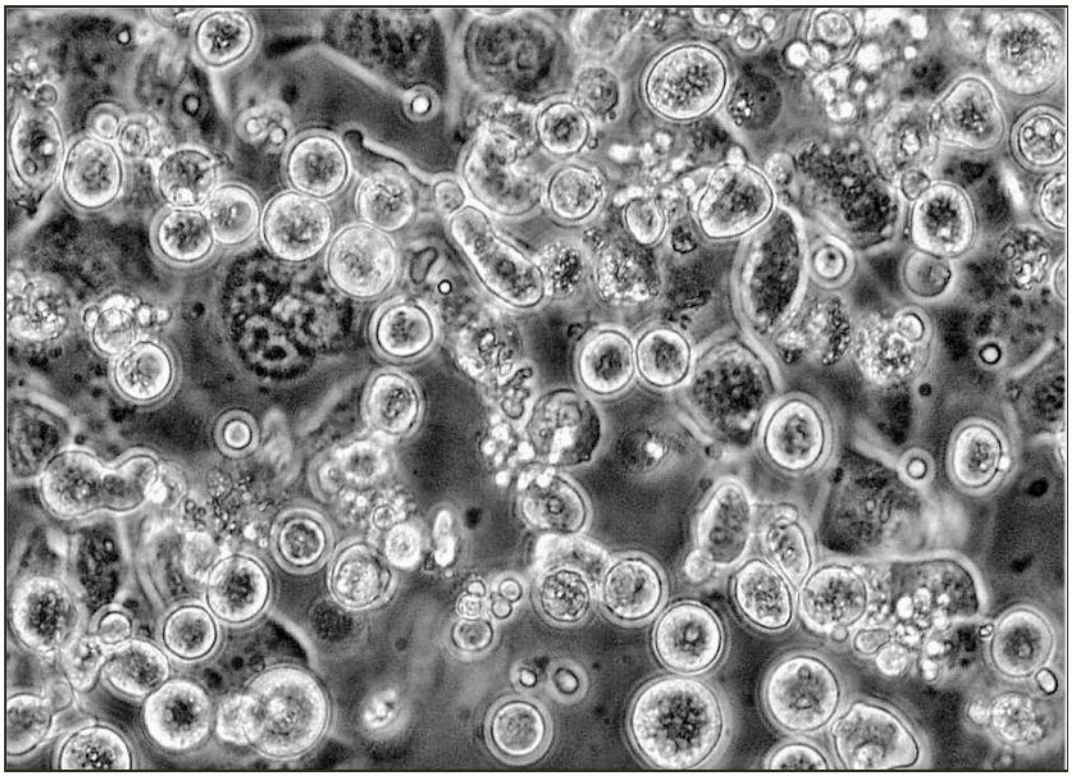
valeo laboratories gmbh

Valeo Laboratories GmbH
Laboratorium: Lise Meitner Str. 21, 25524 Itzehoe
Tel: +49 4821 60492-0 | Fax: +49 4821 60492-29

HRB: AG Pinneberg HRB9730PI
Vertreten durch Frau Ilonka Paul | GF – CEO
USt.ID: DE814156169 | St.Nr.: 18/293/23504
Web: valeo-laboratories.com | valeo-one.de
eMail: info@valeo-one.de

Bankverbindung: Valeo GmbH
KontoNr: 192048874 | BLZ: 21852310
Kreditinstitut: SPK Hennstedt/Wesselburen
SWIFT: NOLADE21WEB
IBAN: DE41218523100192048874

Effect of ***tobacco smoke*** on human lung cells



Effect of ***tobacco smoke*** on cell morphology of cultured human lung cells after 24 hours of exposure to an undiluted primary extract which corresponds to the smoking of one single cigarette. Note the rounding and detachment of the vast majority of cells due to an acute toxic effect.

Micrograph at phase contrast with an Olympus IX50 inverted microscope and Olympus E-10 digital camera with 4 mega-pixels.

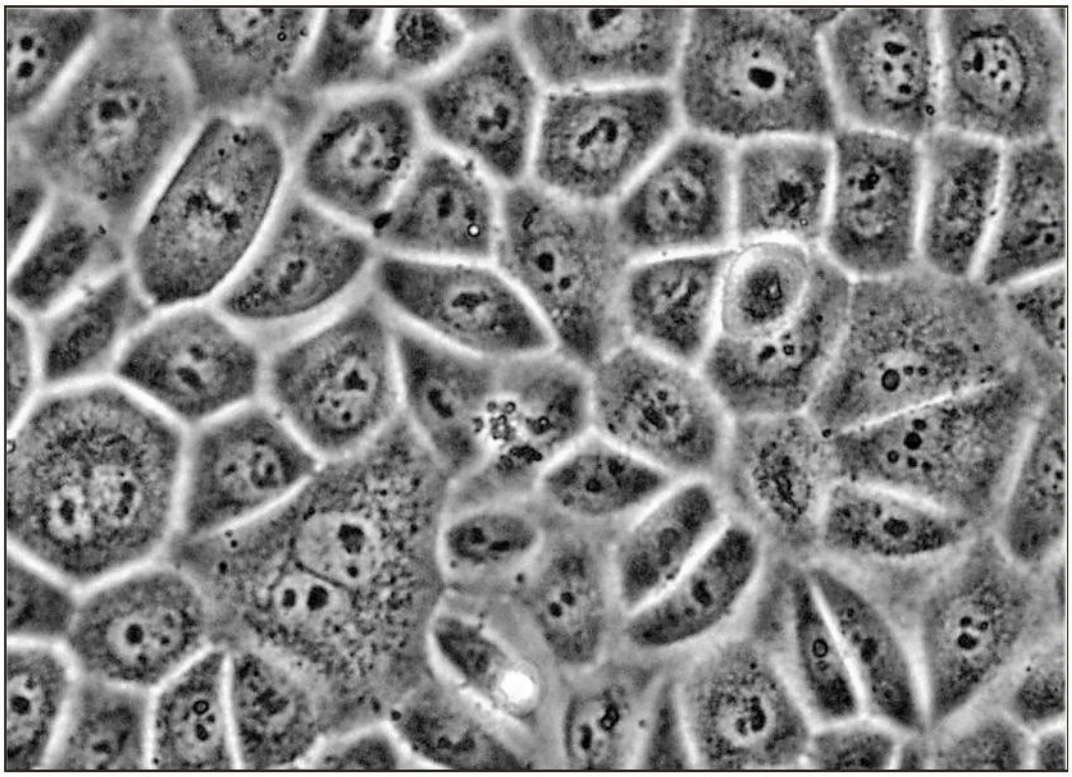
valeo laboratories gmbh

Valeo Laboratories GmbH
Laboratorium: Lise Meitner Str. 21, 25524 Itzehoe
Tel: +49 4821 60492-0 | Fax: +49 4821 60492-29

HRB: AG Pinneberg HRB9730PI
Vertreten durch Frau Ilonka Paul | GF – CEO
USt.ID: DE814156169 | St.Nr.: 18/293/23504
Web: valeo-laboratories.com | valeo-one.de
eMail: info@valeo-one.de

Bankverbindung: Valeo GmbH
KontoNr: 192048874 | BLZ: 21852310
Kreditinstitut: SPK Hennstedt/Wesselburen
SWIFT: NOLADE21WEB
IBAN: DE41218523100192048874

Effect of ***e-liquid vapour*** on human lung cells



Effect of ***base liquid vapour*** on cell morphology of cultured human lung cells after 24 hours of exposure to an undiluted primary extract which corresponds to the vaping of one single cigarette.

Note that cells are not rounded or detached and cell morphology is completely normal with its triangular appearance. No toxic effect.

Micrograph at phase contrast with an Olympus IX50 inverted microscope and Olympus E-10 digital camera with 4 mega-pixels.

valeo laboratories gmbh

Valeo Laboratories GmbH
Laboratorium: Lise Meitner Str. 21, 25524 Itzehoe
Tel: +49 4821 60492-0 | Fax: +49 4821 60492-29

HRB: AG Pinneberg HRB9730PI
Vertreten durch Frau Ilonka Paul | GF – CEO
USt.ID: DE814156169 | St.Nr.: 18/293/23504
Web: valeo-laboratories.com | valeo-one.de
eMail: info@valeo-one.de

Bankverbindung: Valeo GmbH
KontoNr: 192048874 | BLZ: 21852310
Kreditinstitut: SPK Hennstedt/Wesselburen
SWIFT: NOLADE21WEB
IBAN: DE41218523100192048874

INSTITUT FÜR ZELLBIOLOGISCHE TESTSYSTEME
DARTSCH SCIENTIFIC

DARTSCH SCIENTIFIC GMBH

Institute of Cell Biological Test Systems

herewith certifies that the basic liquid named

Valeo BioNic Liquid Nicotine Substitute 1.5 %

manufactured and distributed by

valeo laboratories GmbH, D-25524 Itzehoe, Germany

has been tested for its acute toxic effects by using in vitro tests
with cultured human lung cells (cell line A549).

Results

In the present study the vaping of liquid base according to the smoking of a tobacco cigarette was simulated and the vapour was passed into an aqueous buffered medium yielding the primary extract. For comparison, the same experimental conditions were applied to a usual tobacco cigarette of medium strength. The primary extracts were added to cultivated human lung cells at concentrations ranging from 100 vol% (undiluted primary extract) to 0 vol% (pure medium) for 24 hours. Thereafter, vitality of the cells was examined by morphological and enzymatical tests. The primary extract of the tested basic liquid did not cause any toxic effects at all concentrations. In contrast, the primary extract of tobacco smoke caused a decreased vitality and even death of cells at much lower concentrations.

Schongau: November 5, 2014

Prof. Dr. rer. nat. Peter C. Dartsch
Diplom-Biochemiker

DARTSCH SCIENTIFIC GMBH

Institute of Cell Biological Test Systems

herewith certifies that the basic liquid named

Valeo Nicotine Liquid Sample with 12 mg/ml nicotine

manufactured and distributed by

valeo laboratories GmbH, D-25524 Itzehoe, Germany

has been tested for its acute toxic effects by using in vitro tests with cultured human lung cells (cell line A549).

Results

In the present study the vaping of liquid base according to the smoking of a tobacco cigarette was simulated and the vapour was passed into an aqueous buffered medium yielding the primary extract. For comparison, the same experimental conditions were applied to a usual tobacco cigarette of medium strength. The primary extracts were added to cultivated human lung cells at concentrations ranging from 100 vol% (undiluted primary extract) to 0 vol% (pure medium) for 24 hours. Thereafter, vitality of the cells was examined by morphological and enzymatical tests. The primary extract of the tested basic liquid did not cause any toxic effects at all concentrations. In contrast, the primary extract of tobacco smoke caused a decreased vitality and even death of cells at much lower concentrations.

Schongau: November 5, 2014

Prof. Dr. rer. nat. Peter C. Dartsch
Diplom-Biochemiker

valeo

The e-Liquid laboratory

Production & Experimental Lab
valeo laboratories gmbh
Lise-Meitner-Strasse 21
25524 Itzehoe - Germany

Tel: +49 (0)4821-60492-0
Fax: +49 (0)4821-60492-29

Logistics & Storage
valeo laboratories gmbh
Margarete Steiff Weg 2
25524 Itzehoe - Germany

Tel: +49 (0)4821-40822-0
Fax: +49 (0)4821-40822-22

Webshop B2C DE
www.valeo-one.de

Web B2B EN ES FR DE
www.valeo-laboratories.com

Olaf Meinert | Head of Development - o.meinert@valeo-one.de
Ilonka Paul | Executive management - i.paul@valeo-one.de