An optimized system to investigate the toxicity of diesel exhaust in lung cells *in vitro*

Sandro Steiner[1], L. Müller[2], P. Comte[3], J. Czerwinski[3], M. Kasper[4], A.C.R. Mayer[5], P.Gehr[6], M.J.D. Clift[1] and B. Rothen-Rutishauser[1]

[1]Department for Clinical Research, Bern University Hospital, Switzerland.
[2]University of North Carolina at Chapel Hill, USA.
[3]Bern University of Applied Sciences, Switzerland.
[4]Matter Aerosol AG, Nanoparticle Measurement, Switzerland.
[5]Technik Thermische Maschinen (TTM), Switzerland.
[6]Institute of Anatomy, University of Bern, Switzerland

In light of increasingly stringent exhaust emission legislation and the growing market for more efficient, cleaner and more sustainable cars, there is great interest in the development of new fuels, lubrication oils, fuel additives and exhaust after-treatment systems for diesel cars. Since any changes to these parameters will result in a change to the chemical composition of the emissions, thereby potentially altering their toxicity, human exposure to the emissions of vehicles using these new technologies requires consideration[1-4].

The aim of the present study was to investigate the effects of diesel exhaust on cells of the human epithelial barrier *in vitro*. A sophisticated 3D *in vitro* model of the human epithelial airway barrier,[5] composed of (i) a confluent epithelial cell layer of 16HBE14o$^-$ cells (ii), human whole blood monocyte derived macrophages (MDM) and (iii) human whole blood monocyte dendritic cells (MDDC) was used. To further reflect the physiological conditions *in vivo* this triple cell co-culture system was cultured at the air-liquid interface, as previously described in Blank *et al*. (2007). The use of this *in vitro* system is advantageous over alternative *in vitro* systems, such as monocultures, since it combines both the physical barrier of epithelial cells and two important immune cells (MDM and MDDC).

Using a well-established exhaust exposure system[6], the triple cell co-cultures were exposed to freshly produced diesel exhaust under controlled conditions. As a test vehicle, an Opel Astra X20DTL without exhaust after treatment system was used. The vehicle was running on a dynamometer at constant velocity of 35km/h (engine speed 2180rpm) with low sulfur diesel (Greenergy SA, Steinhausen, Switzerland) and normal lubrication oil (Motorex). The force at the wheel was set to 66N. In parallel to each exposure, the exhaust was analyzed by measuring the elemental carbon mass with a photoelectric aerosol sensor (PAS), the total particles surface with a diffusion charging particle sensor (DC), the number of particles and the distribution of particles between 10-400nm with an electrostatic classifier with differential

mobility analyser (DMA) in combination with a condensation particle counter. Prior to cell exposure, exhaust samples were diluted 1:10 with clean air that was heated to 37℃ and at a relative humidity of 85% and a CO_2 concentration of 5%. The volume flow was set to 2l/min. Cells were exposed to diesel exhaust for 2 or 6 hours, reflecting a low and a high dose exposure respectively. To be able to differentiate between effects of diesel exhaust and diesel exhaust independent effects a reference set of cell cultures was exposed to clean air only (37℃, 85% relative humidity, 5% CO_2). Following the exposure period, all cells and cell supernatants were collected for subsequent biochemical analysis.

To estimate cytotoxic effects of the exposures, the release of lactate dehydrogenase (LDH) into the culture medium was quantified. LDH is a cytosolic enzyme, which when released into the extracellular media is a predictor for the permeability of the cellular membrane, indicative of a cytotoxic episode. Additionally, fluorescent microscopy of cell cultures labelled for nuclear DNA content and the actin cytoskeleton was performed to assess any morphological changes to the epithelial cell layer following exposure to diesel exhaust. The ability for diesel exhaust to cause oxidative stress *in vitro* was assessed via quantification of total reduced glutathione (GSH), a cellular thiol involved in numerous cellular processes, such as the scavenging of reactive oxygen species and the protection of cellular components. In addition, the expression of superoxide-dismutase 1 (SOD1) and heme-oxygenase 1 (HO1) by real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR) was asssessed. (Pro)-Inflammatory responses were estimated by the quantification of the release of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α and chemokine interleukin (IL)-8 into the culture medium by enzyme linked immunosorbent assay (ELISA) and by measurement of their expression levels via real-time RT-PCR. The ability for all exposures to cause apoptosis was assessed by measuring the expression levels of caspase7 and FAS by real-time RT-PCR.

The quantification of extracellular LDH showed that diesel exhaust elicits no significant cytotoxicity, independently of the dose applied. Similarly, no changes to the cell morphology was observed. Quantification of GSH showed that diesel exhaust significantly decreases (p<0.05) the antioxidant capacity of the triple cell co-culture independent of the dose. Despite this drastic drop in the cellular antioxidant capacity, the expression level of SOD1 did not show significant changes. HO1 expression however, increased significantly (p<0.05) upon exposure to low and high doses. Significant changes (p<0.05) in the expression levels and the release of pro-inflammatory cytokines as well as chemokines were not detectable. For TNF-α however, a clear dose dependent tendency towards higher release upon exposure was observable. Independently of the dose, no significant apoptotic responses were detectable. However, for FAS, a dose dependent tendency towards lower expression was detected.

In summary, the observation that even the highest dose used did not affect cell viability or morphology indicates that the measured responses are the result of an intact cellular regulatory network. Diesel exhaust exposed cells reveal a decrease in their pools of oxidative stress-defensive proteins (GSH). This resulted in transcriptional activation of oxidative stress-responsive genes such as HO-1, a response which was found to be dose independent. Additionally a weak pro-inflammatory response was triggered in a dose dependent manner.

In conclusion, exposure to diesel exhaust causes no acute cytotoxic effects, but significant cellular responses are triggered, which might subsequently result in secondary cytotoxicity.

The presented data will serve as a reference for future experiments, in which triple cell co-culture models will be exposed using the same exposure system, however with varying technical settings, such as the use of a particle filter, bio-fuel, low and zero SAPS lubrication oils, an iron based fuel additive and the variation of the nitrogen dioxide concentration in the exhaust. The results of this study will gain insights into how different technical parameters at diesel engines affect the lung toxicity of their exhaust. This will help to decide which technologies are best suited for future use with respect to adverse health effects.

Acknowledgments

We thank the Bern University of Applied Sciences, the Institute of Aerosol and Sensor Technology, the University of Applied Sciences Northwestern Switzerland and the University of Rouen for technical assistance and the Swiss Federal Office for the Environment, Erdölvereinigung EV and VSS lubes for the founding.

References

1 Knothe, G., Sharp, C.A., Ryan, T.W. (2006) Energy and Fuels, 20, 403-408.

2 Sjögren, M., Li, H., Banner, C., Rafter, J., Rannug, U. (1996) Chemical Research in Toxicology, 9, 197-207.

3 Westerholm, R., Egebäck, K-E. (1994) Environmental Health Perspectives, 102, 13-23

4 Vaaraslahti, K., Ristimäki, J., Virtanen, A., Keskinen, J., Giechaskiel, G., Sola, A. (2006) Environmental Science and Technology 40. 4776-4781.

5 Rothen-Rutishauser, B., Kiama S.G., Gehr, P. (2005) American Journal of Respiratory Cell and Molecular Biology, 32, 281-289.

6 Müller, L., Comte, P., Czerwinski, J., Kasper, M., Mayer, A.C.R., Gehr, P., Burtscher, H., Morin, J-P., Athanasions, K., Rothen-Rutishauser, B. (2010) Environmental Science and Technology, 44, 2632-2638.

INSELSPITAL
UNIVERSITÄTSSPITAL BERN
HOPITAL UNIVERSITAIRE DE BERNE
BERN UNIVERSITY HOSPITAL

UNIVERSITÄT BERN

Berner Fachhochschule
Haute école spécialisée bernoise

Toxicity of Diesel Exhaust in Human Lung Cells *in vitro*

Sandro Steiner[1], Loretta Müller[2], Pierre Comte[3], Jan Czerwinski[3], Peter Gehr[4], Markus Kasper[5], Andreas C.R. Mayer[6], Martin J.D. Cliff[1] and Barbara Rothen-Rutishauser[1]
1. Bern University Hospital, Department for Clinical Research, Switzerland; 2. University of North Carolina at Chapel Hill, USA; 3. AFHB, Bern University of Applied Sciences, Biel-Bienne, Switzerland; 4. University of Bern, Institute for Anatomy, Switzerland; 5. Matter Engineering AG, Nanoparticle Measurement, Wohlen, Switzerland; 6. Technik Thermische Maschinen (TTM), Zurich, Switzerland; E-mail: sandro.steiner@bfh.ch

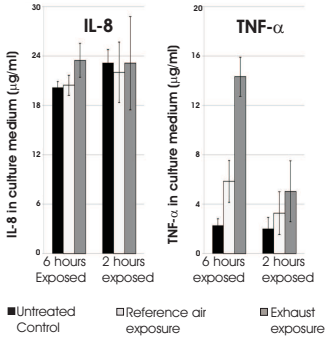
Introduction

Because of more stringent exhaust emission legislations and the growing market demand for more efficient and cleaner vehicles, new exhaust after-treatment systems, fuels, fuel additives and lubrication oils for diesel cars are being developed continuously. The resulting alteration in diesel exhaust composition makes reconsidering diesel exauhst toxicity necessary (1-4).

In the presented study, a cellular model of the human respiratory epithelium (5) was exposed to diesel car exhaust, the cellular responses were measured and will in further experiments serve as a reference to assess the influence of variations in fuel and oil type, fuel additives and exhaust after-treatment on diesel exhaust toxicity

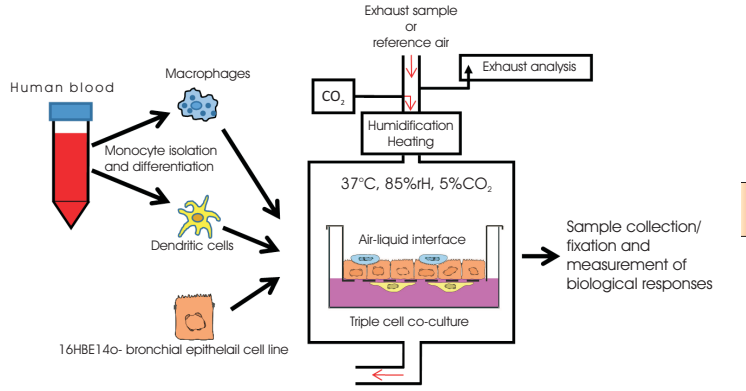
(Pro-)inflammatory response

Estimated by measurement of interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α)

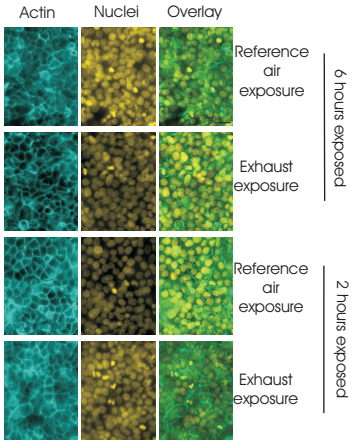


Extracellular concentrations of IL-8 were not changed upon exposure, whereas for TNF-α, a dose dependent increase was observed

Experimental procedure



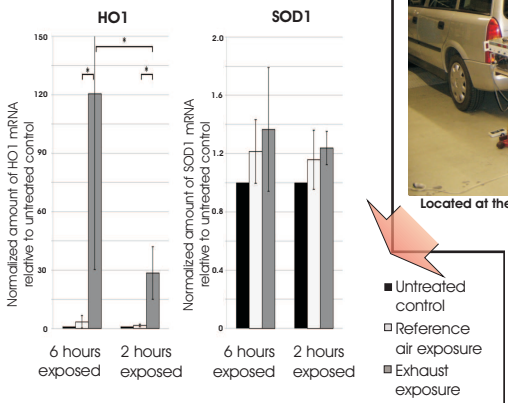
Cellular morphology



No effects on cellular morphology were detected, independently of exposure duration

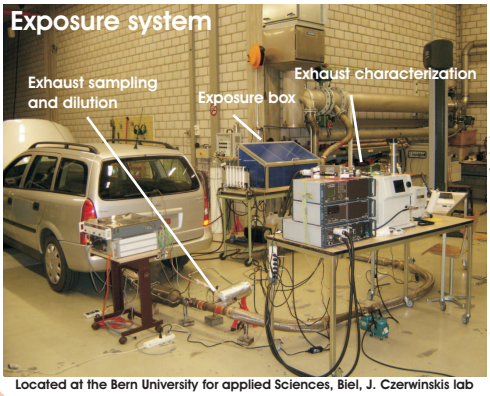
Response to oxidative stress

Estimated by real-time RT-PCR on superoxide dismutase 1 (SOD1) and haem oxygenase 1 (HO1)



A strong and dose dependent induction of HO1 but not of SOD1 expression was detected.

Exposure system



Located at the Bern University for applied Sciences, Biel, J. Czerwinskis lab

Exposure settings:
Low dose (exposure for 2h)
High dose (exposure for 6h)
6h post-incubation

Exhaust exposure (diluted exhaust sample)
Reference exposure (filtered ambient air)

Technical information:

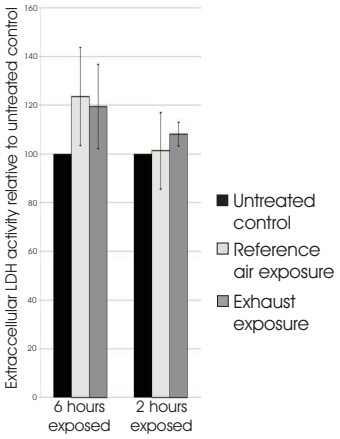
Test vehicle:	Opel Astra
Velocity:	35 km/h
Engine speed:	2180 rpm
Inertia mass:	1360 kg
Force at wheel:	66 N
Exhaust dilution:	1:10

Exhaust characterization:

Particles:	Elemental carbon mass Size-number distribution Total active surface area
Gases:	CO, NO_x, NO, Hydrocarbons

Cytotoxicity

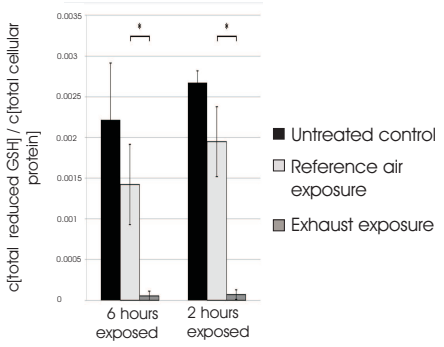
Estimated by quantification of extracellular lactate dehydrogenase (LDH)



No significant cytotoxic effects were detected upon exposure, independently of exposure duration

Cellular antioxidant capacity

Estimated by quantification of total reduced glutathione (GSH)



Independently of the exposure duration, the cellular antioxidant capacity was strongly reduced, reflecting the high oxidative potential of diesel engine exhaust

Materials and Methods

Monocyte isolation was done by CD14 positive selection (CD14 MicroBeads, Miltenyi Biotec). Monocyte differentiation and preparation of the triple cell co-cultures is described in (5), the exposure system in (6). Determination of extracellular LDH concentration: LDH Assay Kit, Roche Applied Science; extracellular GSH: Glutathione Kit, Cayman Chemical; TNF-α and IL-8: Bio-Plex Pro Assay, Bio-Rad; Real-time RT-PCR: RT: Omniscript Reverse Transcriptase (Qiagen), real-time PCR: Fast SYBR Green Mastermix (Applied Biosystems), 7500 Fast Real-Time PCR System (Applied Biosystems)

Conclusion

We have established a system which allows the exposure of a cellular model of the human respiratory epithelium to fresh and whole diesel exhaust. In further experiments, this system will be used for the assessment of the impact of various technical settings at diesel engines on exhaust toxicity

Literature
1) Knothe *et al.*, Energy & Fuels 2006. 2) Sjögren *et al.*, Chem. Res. Toxicol. 1996. 3) Vaaraslahti *et al.*, Environ. Sci. Technol. 2006. 4) Heeb *et al.*, SIAT 2005. 5) Rothen-Rutishauser *et al.*, Am J Resp Cell Mol Biol 2005. 6) Müller *et al.*, Environ. Sci. Technol. 2010

Acknowlegments
We thank the Bern University of Applied Sciences, the Institute of Aerosol and Sensor Technology, the University of Applied Sciences Northwestern Switzerland and the University of Rouen for technical assistance and the Swiss Federal Office for the Environment, Erdölvereinigung EV and VSS lubes for the founding