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Extended Abstract

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Diesel Exhaust Particles and Human Health: An Insight into their (Geno)Toxicity *In Vitro*

Combustion derived nanoparticles (NPs) have formed the basis for much of what is known regarding the potential for NPs to exhibit potential adverse effects towards human health. Although such a plethora of information exists, essential questions remain concerning exactly how combustion derived NPs may drive the reported human health effects.

In order to gain an insight into such concerns a number of sophisticated *in vitro* approaches have been employed in order to study how combustion derived NPs may elicit immuno-regulatory and (geno)toxic effects.

As a basis for such research, and since the lung is the primary organ following human exposure to combustion derived NPs, a sophisticated 3D co-culture model the epithelial airway barrier was used. The specific *in vitro* system used contained the 16HBE14o- bronchial epithelial cell-line to mimic the key epithelial cell layer, as well as the two most important cells of the human immune system (macrophages and dendritic cells)[1].

Initial investigation using suspension culture experiments, in which standardized diesel exhaust particles (DEPs) (SRM #2975) from the National Institute for Science and Technology (NIST) were employed, showed a significant (p<0.05) dose-dependent (0.005 to 0.04mg/mL) pro-inflammatory cytokine (tumor necrosis factor(TNF)-α) and chemokine (interleukin(IL)-8) effect over a 24hr period, independent of any cytotoxicity (as measured via lactate dehydrogenase release). The pro-inflammatory response noted was further correlated to a significant (p<0.05) decrease in the intracellular thiol glutathione, indicative of an increase in cellular oxidative stress compared to baseline levels. Subsequent research into the potential for NIST DEPs to cause genotoxicity has also shown these DEPs to have a profound effect upon cell proliferation, with a significant (p<0.05) increase observed after 4 and 24hrs exposure at concentrations up to 0.02mg/mL as determined by the BrdU assay. Ongoing investigation into how the DEPs may directly affect cellular DNA also indicates that such combustion derived NPs can potentially cause oxidative DNA damage after 4hrs exposure.

To further understand the potential mutagenicity caused by the NIST DEPs, the bacterial based Ames test was performed[2]. It was observed that the DEPs caused a significant (p<0.05) mutagenicity, however never directly interacted with the bacterial strain (*S. Thyphymurium*) itself. Such a result suggests that the DEPs themselves do not drive the observed

mutagenicity, but, in fact, additional factors such as polyaromatic hydrocarbons (PAHs) could be a key factor in the observed adverse effects. To determine this further, ongoing research using similar endpoints, however with air-liquid exposed co-culture samples as used for determining the NIST DEPs effects *in vitro* is being conducted.

The indirect mutagenicity seen for the DEPs in the Ames test was not however, observed for a plethora of physico-chemically different engineered NPs, or another 'combustion' NP type, cerium dioxide (CeO_2). Since CeO_2 is now commonly used as a fuel additive to increase engine efficiency, the potential for the release of CeO_2 NPs, through the combustion process, into the environment, thus creating an exposure scenario towards humans, is inevitable.

Therefore, to study such effects, the afore mentioned sophisticated 3D in vitro model of the human epithelial airway barrier, 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.[3]. 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). For exposure to DEPs, anestablished exposure system[4] was used in which cell cultures can be 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 the exposure experiments, 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. Before brought into contact with the cells, exhaust samples were diluted 1:10 with absolute clean air, heated to 37°C, and brought to a relative humidity of 85% and a CO_2 concentration of 5%. The volume flow was set to 2l/min.

For exposure to DEPs, the cells were kept in the exposure system for 2 hours or 6 hours, reflecting a low and a high dose. For co-exposures to DEPs+CeO_2 NPs, the cell cultures were sprayed with an aerosolized solution of CeO_2 NPs an aerosolized solution of CeO_2 NPs was nebulized over the cell culture surface after the exposure to diesel exhaust. Cell cultures that had been exposed to low doses of DEPs were sprayed with exposed to a 20µg/ml solution, the ones that had been exposed to high doses of DEPs with 60µg/ml solution. Upon CeO_2 NP exposure, the cultures were incubated for 6 hours at 37°C, 85% relative humidity and 5% CO_2. To be able to differentiate between effects of DEPs, CeO_2 NPs and DEPs+CeO_2 NPs, one set of cell cultures was exposed to DEPs only, one set to CeO_2 NPs only and one set to DEPs+CeO_2 NPs. To further be able to differentiate between effects of DEPs and DEPs independent effects of the conditions in the exposure system, additional sets were exposed to absolute pure air (37°C, 85% relative humidity, 5% CO_2) instead of DEPs. After incubation, the cells and the cell culture media were collected for the measurement of biological responses.

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 response. 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 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 assessed. (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 DEPs, CeO_2 NPs and DEPs+CeO_2 NPs elicit no significant cytotoxicity, independently of the dose. The same was found by microscopy, which did not reveal any changes in cellular morphology or in the density of the epithelial layer. Quantification of total reduced GSH showed that DEPs decreases the amount of antioxidant molecules in the cell to less than 5% of the controls. This effect was statistically significant (p<0.05) and independent of CeO_2 NPs or the dose. Despite this drastic drop in the cellular antioxidant capacity, the expression level of SOD1 did not show significant changes. HO1 expression increased significantly (p<0.05) upon exposure to low doses of DEPs. This effect was abolished by CeO_2 NPs, whereas low doses of CeO_2 NPs alone did not have an effect. High doses of DEPs and CeO_2 NPs both resulted in a significant (p<0.05) increase of HO1 expression and had a synergistic effect upon co-exposure. This synergistic effect of the two particle types was statistically significant (p<0.05). A significant change in expression levels of TNF-α and IL-8 was not detected, independently of the dose and the exposure type. Also, the quantification of the release of the cytokines showed no significant effects. However, a clear dose dependent tendency of elevated TNF-α release upon exposure to DEPs was observable, an effect which was independent of CeO_2 NPs. Significant apoptotic responses were not detectable independently of the dose and the exposure type. However, for FAS, a tendency towards lower expression was observable independently the exposure type but dependent on the dose. Caspase7 showed a tendency towards lower expression upon high dose exposure to CeO_2 NPs and DEPs+CeO_2 NPs, but upon exposure to high doses of DEPs alone.

In summary, the observation that even the highest doses of DEPs and CeO_2 NPs used did not affect cell viability or morphology indicates that the measured responses can be considered as the result of an intact cellular regulatory network. DEPs were found to exert a highly oxidative effect on human respiratory epithelial cells in vitro, an effect which is not influenced by the presence of CeO_2 NPs. The resulting transcriptional activation of oxidative stress-responsive genes however, is influenced by CeO_2 NPs in dose dependent and gene specific manners and synergistic effects of the two particles are observable. An induction of a pro-inflammatory response is observable upon exposure to DEPs but this induction is not statistically significant. The extent of induction is dependent on exposure duration and independent on the presence of CeO_2 NPs. Significant apoptotic responses are not induced. There is however a trend towards lower expression of pro-apoptotic genes upon exposure to DEPs and CeO_2 NPs for FAS and tendency towards lower expression of caspase7 upon exposure to high doses of CeO_2 NPs observable. In conclusion, CeO_2 NPs do not influence the primary toxicity of DEPs, but they cause changes in the cellular responses and might thereby influence secondary toxicity[5].

In conclusion, by undertaking an *in vitro* outlook as described above, it is clear that by incorporating sophisticated exposure and biological systems the ability to systematically determine how combustion derived NPs may affect human health, particularly the lung, can be efficiently achieved with a clear and realistic insight into the risk posed by combustion derived NPs.

References

1. Rothen-Rutishauser *et al*. (2005) *AJRCMB*. **32**: 281-289; **2.** Clift *et al*. Revised Manuscript Submitted; **3.** Blank *et al*. (2007) *AJRCMB*. **36**: 669-677; **4.** Muller *et al*. (2010) *Environ Sci Technol*. **44**: 2632-2638; **5.** Steiner *et al*. Submitted Manuscript.

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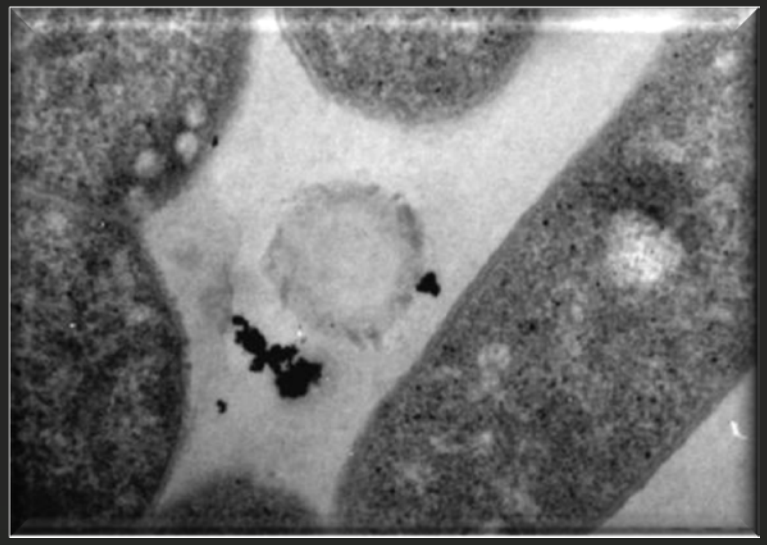

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Introduction

In 2010, 51% of the European passenger cars contained diesel engines.

Diesel engines emit a high amount of compound

Diesel exhaust particles are of particular concern!

nitrogen oxides, polyaromatic hydrocarbons and diesel exhaust particles.

Westerholm and Egeback (1994) *EHP* **102**; 13

Increased number of diesel engine cars equates to an increased environmental burden of these 'volatile' compounds.

Steiner *et al.* **Submitted**

Diesel Exhaust Particles (DEPs)

A complex mixture of particulate materials, *i.e.* carbon and a range of other metals at various sizes (<100nm-10µm).

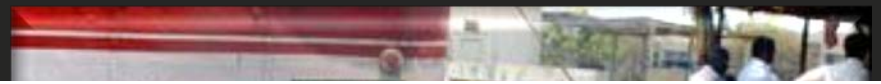
Wilson *et al*. (2002) *TAAP* **184**; 172

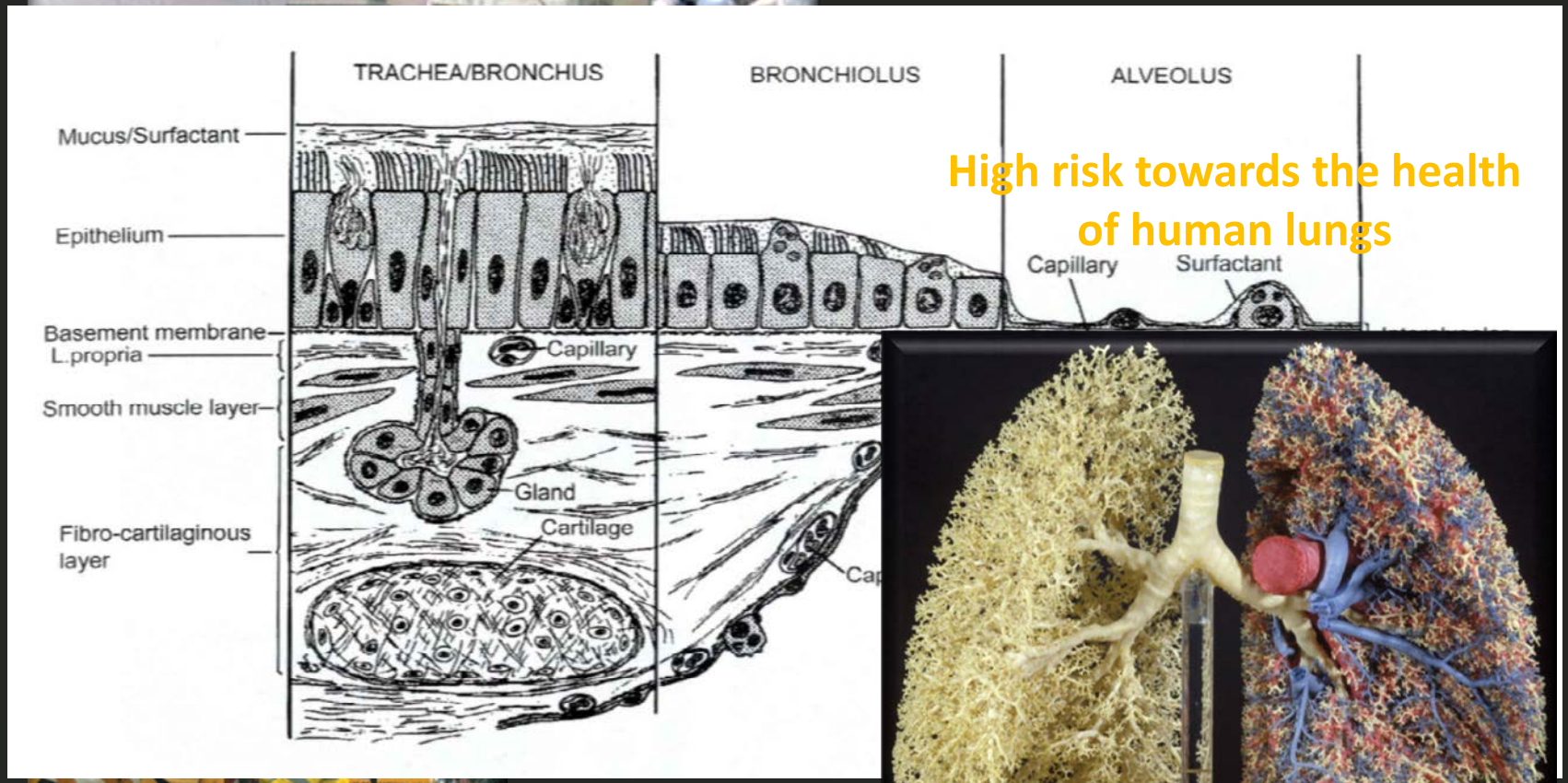
www.tundraheadquarters.com

www.africanhealthmagazine.com

www.wikipedia.org

Human Exposure to DEPs





High risk towards the health of human lungs

Ochs and Weibel (2008); In Fishman´s Pulmonary Diseases and Disorders, New York

Institute of Anatomy, University of Bern

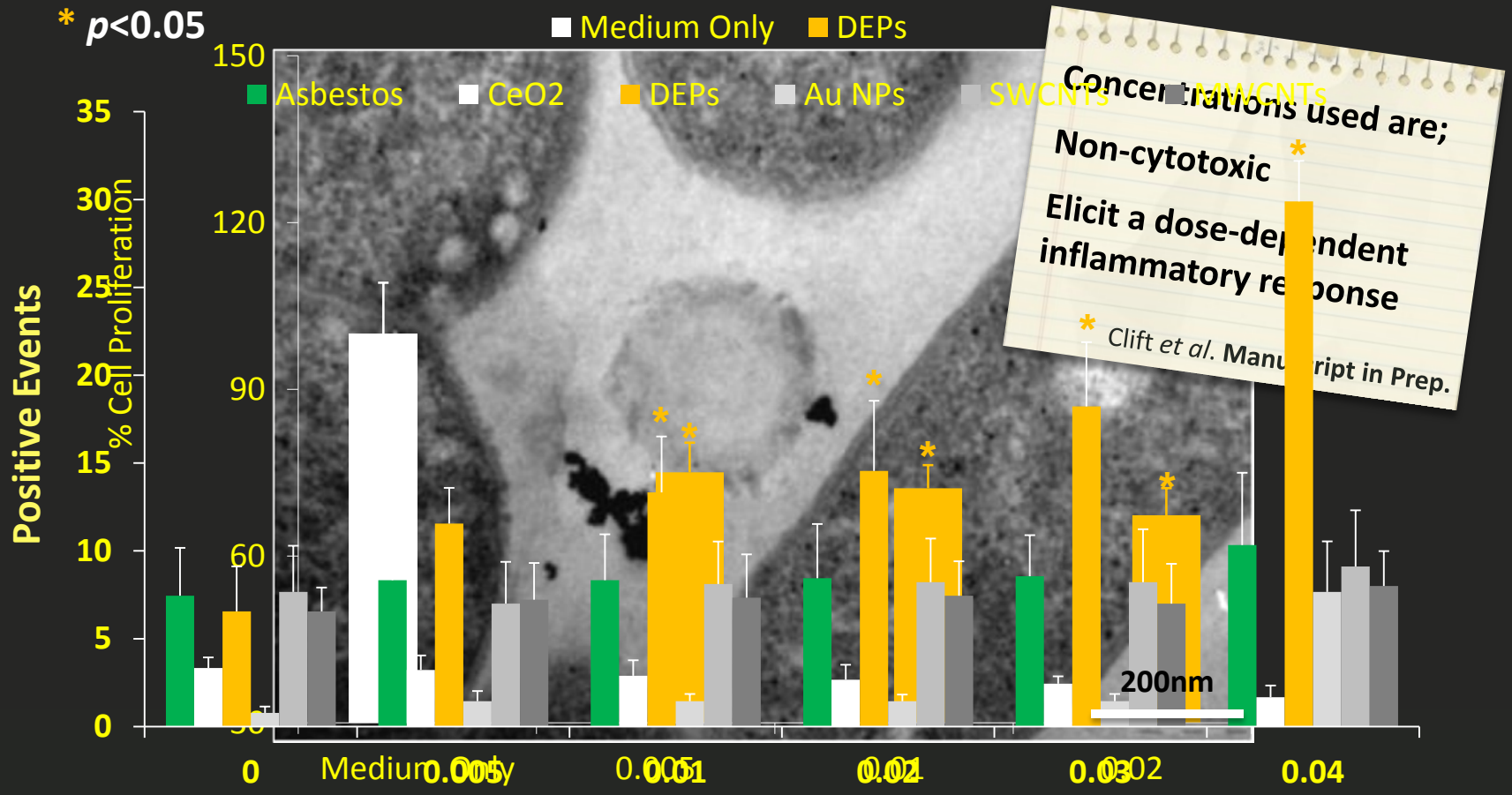
www.makebiofuel.co.uk

DEPs and human health

Decades of Research!

DEP Effects *In Vitro*

n=3 * $p<0.05$



NIST SRM #2975

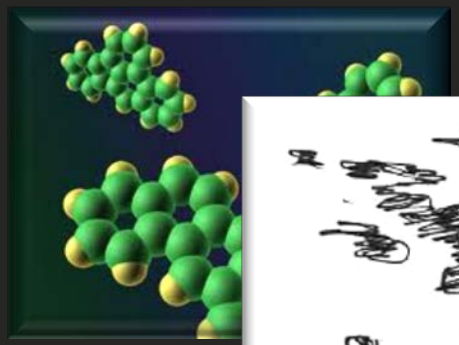
24hrs exposure at 40µg/ml

Clift *et al.* Manuscript in Prep.

BrdU Assay 24hrs (submerged) exposure of human monocyte derived macrophage cells

Ames Test TA98 Strain of *S. Typhimurium*

What fraction of DEPs is of Concern?



www.burningissues.org

Polyaromatic H

anthonygonzalez.com

www.hostgator.com

www.3Ddartspace.com

Transition Metals

Metal Nanoparticles (NPs) and DEPs

Up to 17 metals can be present within DEPs…… 75% are <100nm in diameter…..

Lim *et al*. (2009) *J. Environ. Monit.* **11**; 1614

Additional metals (elements) are being added to diesel fuel in order to promote engine performance….

Steiner *et al*. **Submitted**

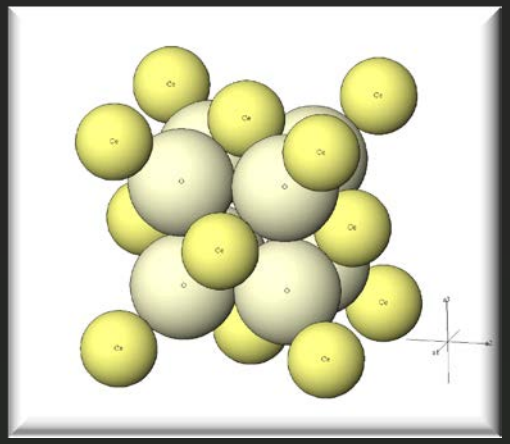
….*i.e.* as Fuel Borne Catalysts (FBCs).

Steiner *et al*. **Submitted**

…..these include: Iron, Lithium, Vanadium, Lead and even Cadmium.

Lim *et al*. (2009) *J. Environ. Monit.* **11**; 1614

Cerium Dioxide (CeO_2)



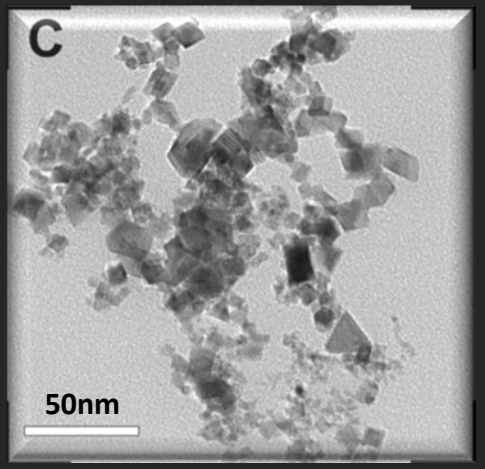
www.aist.go.jp

www.tradekorea.com

What are the consequences of adding such elements?

Co-Exposure of DEPs and CeO_2 NPs *In Vitro*

Muller *et al*. (2010) *Environ Sci Tech*. **44**; 2632
Muller *et al*. (2011) *InSciences J. Nanotech*. **1**; 30



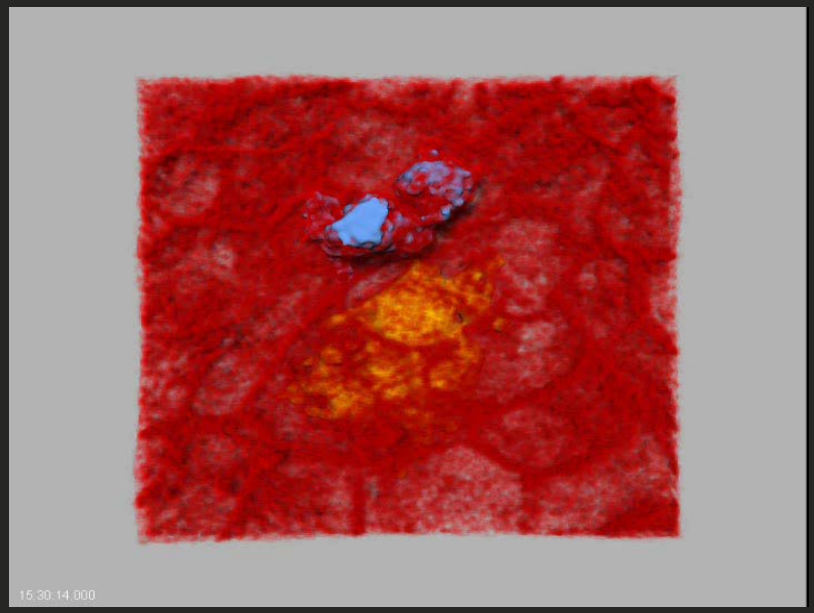
CeO_2

Raemy *et al*. (2010) *Eur J Pharm Biopharm* **77**; 368

Steiner *et al*. **Submitted**

Co-Exposure of DEPs and CeO_2 NPs *In Vitro*

3D *in vitro* model of the epithelial airway barrier



Fabian Blank, DKF, Bern

Human blood isolated macrophage cells

16HBE14o- epithelial cell-line

Human blood isolated dendritic cells

All exposures were performed at the Air Liquid Interface (ALI).

Blank *et al.* (2007) *AJRCMB*. **36**; 669

model was

DEPs for

and

All samples were then incubated for 6hrs prior to biochemical analysis

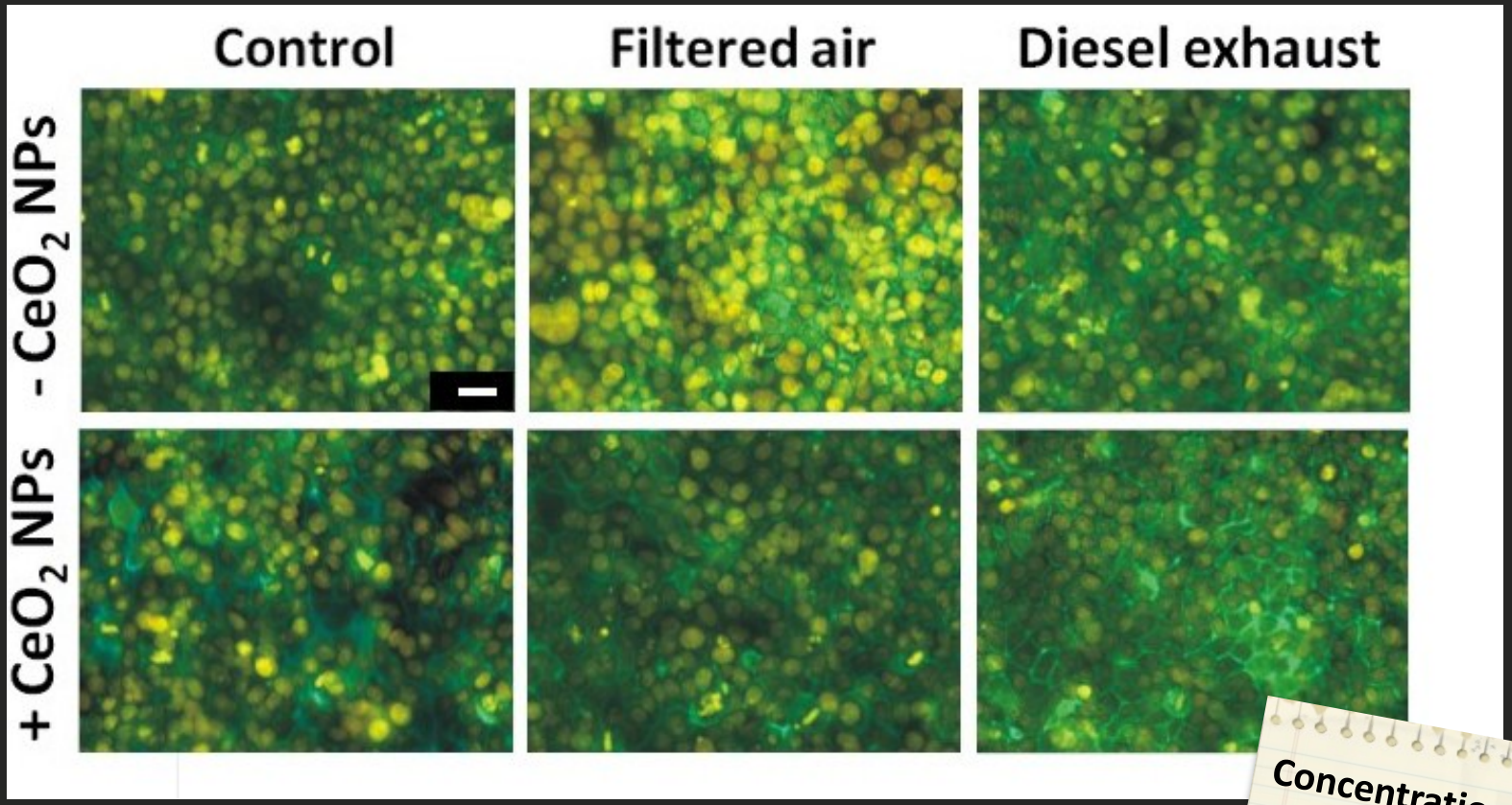
Steiner *et al.* **Submitted**

CeO_2 NPs

then applied at 20µg/ml (low dose) and 60µg/ml (high dose) after the DEP exposure period.

Steiner *et al.* **Submitted**

Effects of DEPs and CeO_2 NPs *In Vitro*



F-Actin 'Cytoskeleton **Nucleic Acids (DNA)** Scale Bar = 25µm

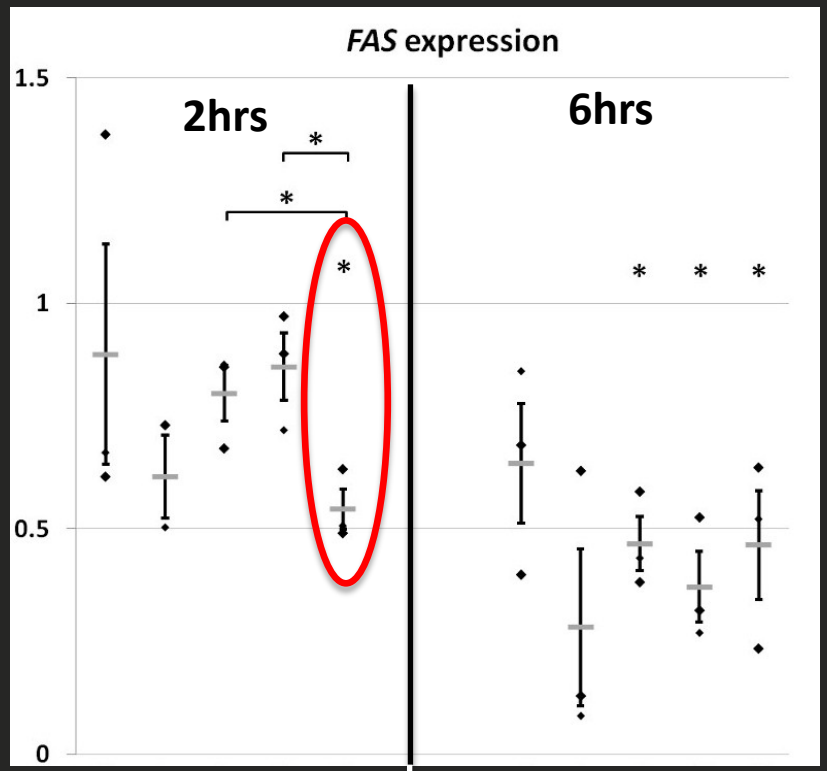
Fluorescent Microscopy of 'High Dose' exposed 3D model

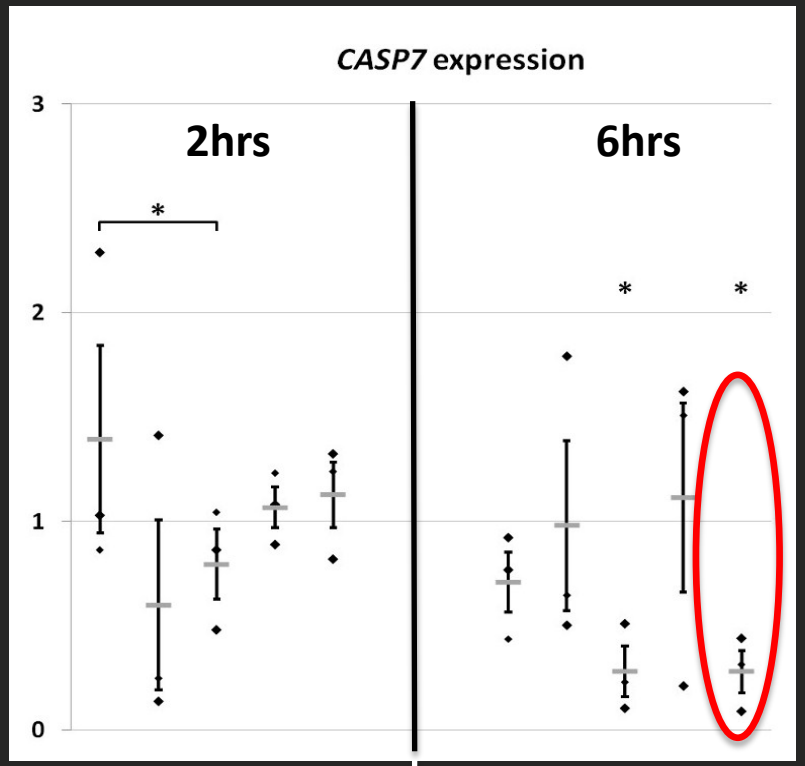
Concentrations used;
All were non-cytotoxic
(as determined by lactate dehydrogenase release)

Steiner *et al*. **Submitted**

Effects of DEPs and CeO_2 NPs *In Vitro*

Steiner *et al*. **Submitted**

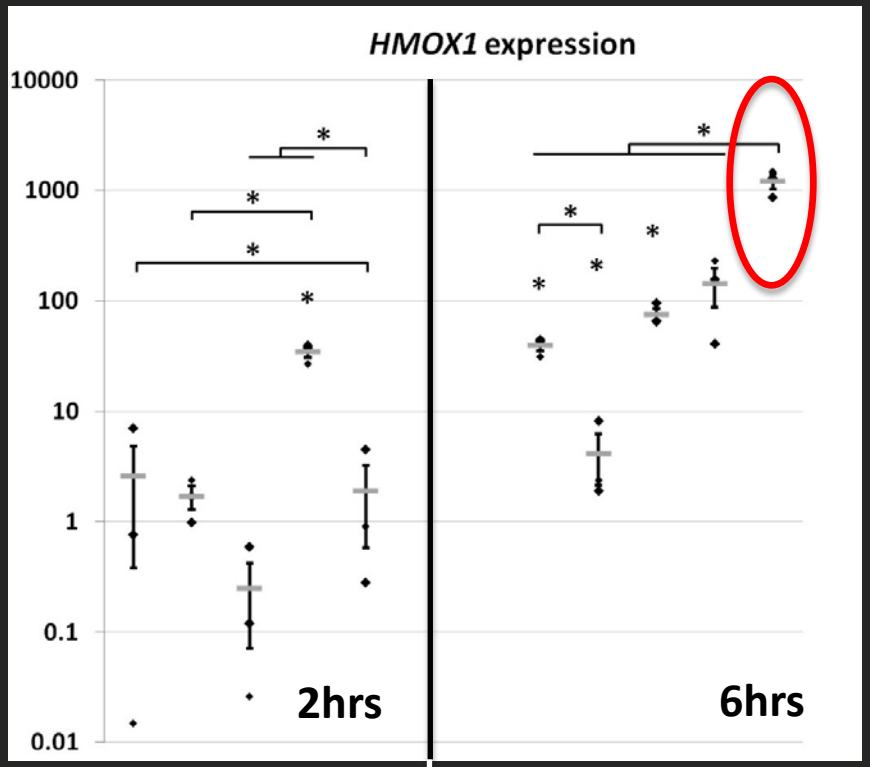




n=3

* $p<0.05$

Effects of DEPs and CeO_2 NPs *In Vitro*



n=3

* $p<0.05$

No effect upon reduced glutathione (antioxidant) status in 3D model following dual exposure.

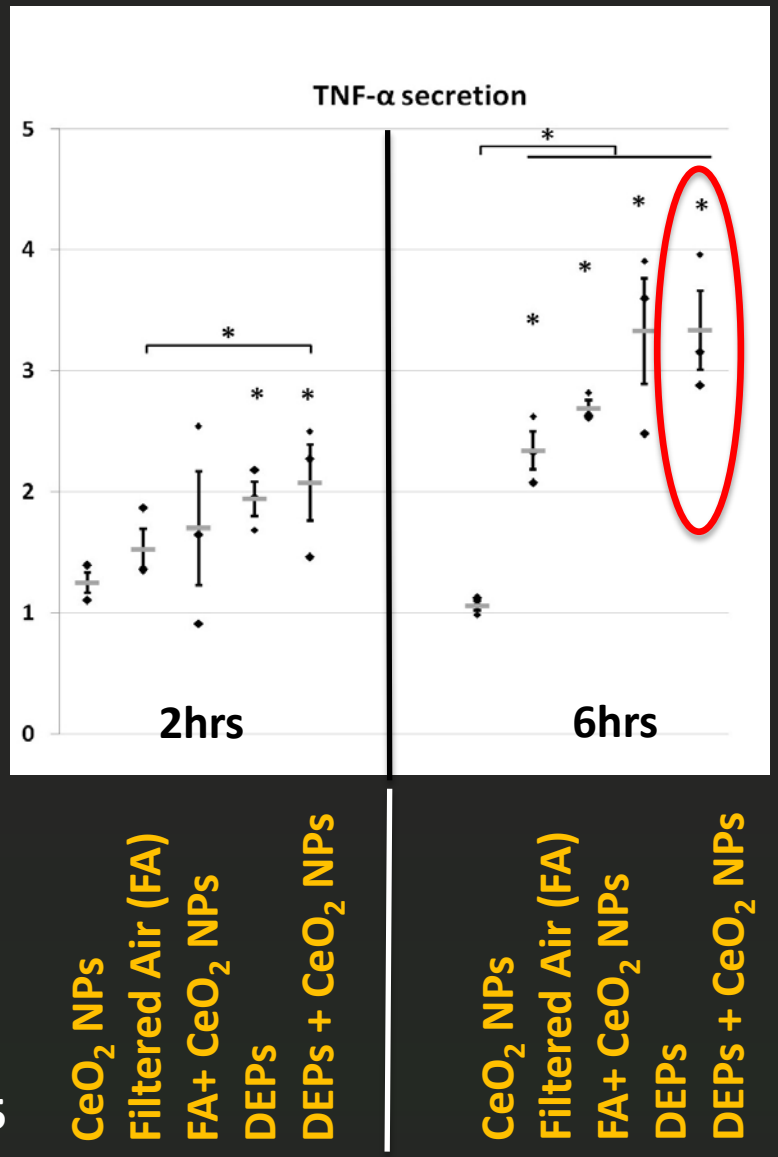
No effect upon alternative gene marker for oxidative stress (superoxide dismutase1 (SOD1)) in 3D model following dual exposure.

Steiner *et al*. **Submitted**

Effects of DEPs and CeO_2 NPs *In Vitro*

Significant inflammatory effect in the 3D model after CeO_2 + DEP exposure

Similar inflammogenic effects were observed at the gene level (*TNF* expression)



n=3 * $p<0.05$

Steiner *et al*. **Submitted**

Summary and Conclusion

CeO_2 + DEP exposure does not cause significant primary acute toxicity effects *in vitro*.

CeO_2 + DEP exposure has a significant impact on the secondary toxicity.

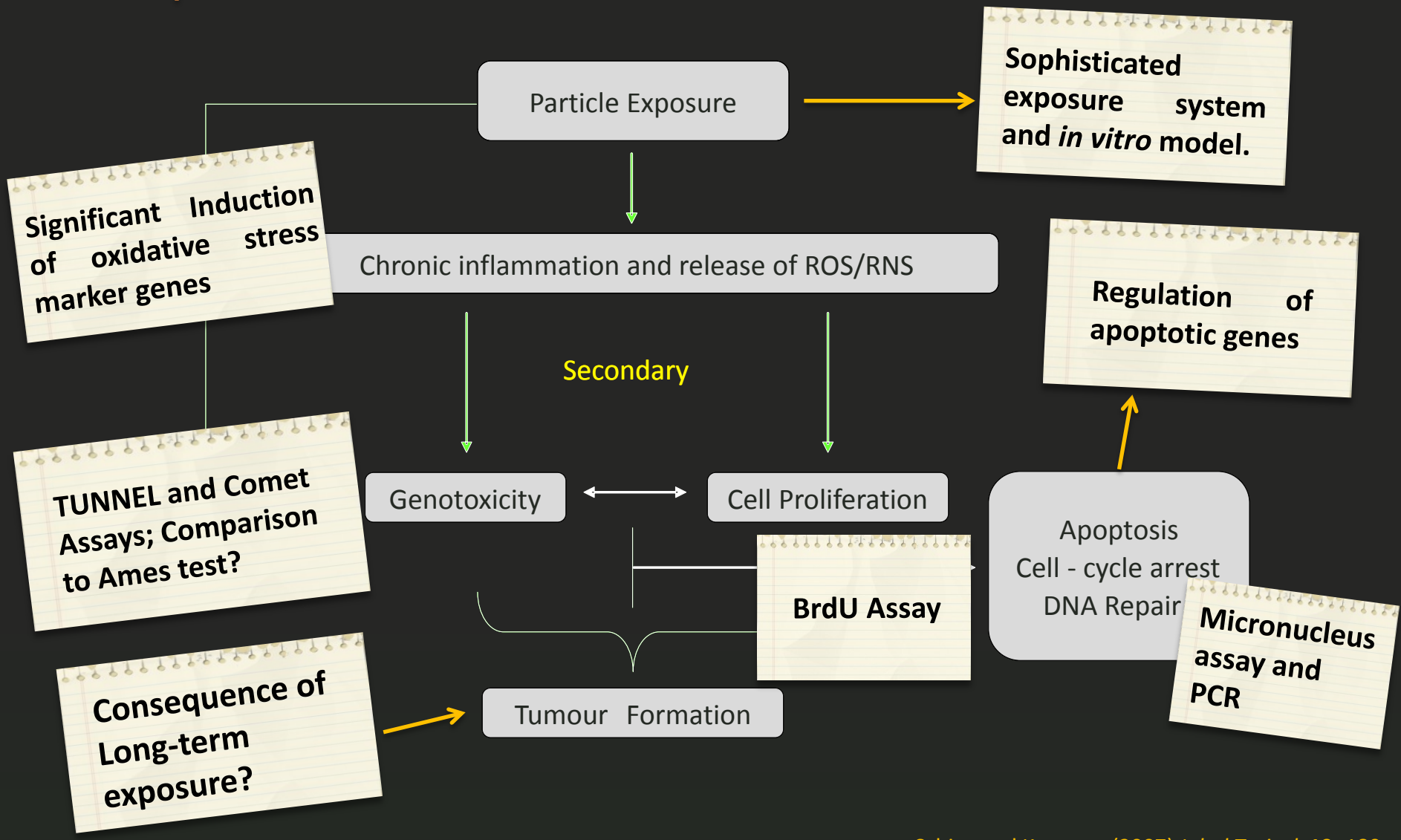
CeO_2 + DEP exposure elicits a significant inflammatory response.

CeO_2 could be an applicable and efficient fuel borne catalyst.

Long-term (genotoxic) effects of this dual exposure?

Steiner *et al*. **Submitted**

Perspective and Outlook



Schins and Knaapen (2007) *Inhal Toxicol.* **19**; 189

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