

## Interaction of Diesel Exhaust Particles with Human, Rat and Mouse Erythrocytes *in Vitro*

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### Key Words

Particulate air pollution • Diesel exhaust particles • Erythrocytes • Oxidative stress

### Abstract

Inhaled ultrafine (nano) particles can translocate into the bloodstream and interact with circulatory cells causing systemic and cardiovascular events. To gain more insight into this potential mechanism, we studied the interaction of diesel exhaust particles (DEP) with human, rat and mouse erythrocytes *in vitro*. Incubation of erythrocytes with DEP (1, 10 or 100 µg/ml) for 30 min caused the highest hemolytic effect (up to 38%) in rats, compared to small but significant hemolysis in mice (up to 2.5%) and humans (up to 0.7%). Transmission electron microscopy of erythrocytes revealed the presence of variable degrees of ultrafine (nano)-sized aggregates of DEP either internalized and/or adsorbed onto the erythrocytes in the three species. A significant amount of DEP was found in rat and mouse (but not human) erythrocytes. Lipid erythrocyte susceptibility to *in vitro* peroxidation measured by malondialdehyde showed a significant and dose-dependent increase in erythrocytes of rats, but not humans or mice. Unlike in human erythrocytes,

total antioxidant status (TAS) and superoxide dismutase (SOD) activity in rats were significantly and dose- dependently decreased. In mouse erythrocytes, DEP caused a decreased in SOD (at 10 µg/ml) and TAS (at 100 µg/ml) activities. In conclusion, DEP caused species-dependent erythrocyte hemolysis and oxidative stress, and were either taken up and/or adsorbed onto the red blood cells. Rat (and to a lesser degree mouse) erythrocytes were susceptible to DEP. Human erythrocytes showed the highest resistance to the observed effects. These species difference should be noted when using rats and mice blood as models for humans.

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### Introduction

Diesel exhaust particles (DEP), the main component of PM<sub>2.5</sub> (particulate matter with a diameter < 2.5 µm) and ultrafine (nano) particles (UFP, diameter ≤ 0.1 µm) in urban areas, are a major contributor to inhaled particulate matter pollution. Experimental exposure studies in healthy and asthmatic individuals have demonstrated oxidative stress, airway inflammation and worsening of

asthma [1], as well as acute cardiovascular events [2]. Indeed, acute exposure to particulate matter (PM) has been associated with risk of myocardial infarction, susceptibility to myocardial ischemia, endothelial dysfunction, arterial stiffness, decreased fibrinolytic capacity, increased platelet activation and increased *ex-vivo* thrombus formation [2]. In experimental animals, DEP have been reported to cause lung inflammation, impair lung function, cause systemic inflammation, thrombosis and aggravate acute renal failure [3-6].

The mechanism by which inhaled DEP exerts cardiovascular effects remains unclear. A number of hypotheses have been presented to explain the mechanisms responsible for the adverse effects of particulate air pollution. The autonomic nervous system may be a target for the adverse effects of air pollution [2]. Inhalation of PM<sub>2.5</sub> may also cause oxidative stress in the airways and induce an inflammatory response with the potential to upregulate systemic inflammatory processes [7]. Moreover, particles can cross barriers into the circulation and may directly interact with circulatory cells (such as erythrocytes or platelets) and vascular tissue to cause effects at various sites of the body [8-11]. Therefore, investigation of the direct toxicity of DEP on erythrocytes is needed, and will help to understand the mechanisms underlying the cardiovascular effects of PM.

Recently, it has been demonstrated that traffic-related air pollutants are associated with decreased erythrocyte antioxidant enzyme activity [12]. A decrease in erythrocytes in association with exposure to PM<sub>10</sub> or UFP in humans or rats has been reported [13-15]. However, the effects of DEP on human, rat and mouse erythrocytes *in vitro* have, as far as we are aware, not been reported hitherto.

The aim of this *in vitro* study was three-fold: (1) to evaluate the hemolytic activity of three graded doses of DEP on human erythrocytes, and to compare it to that in the commonly used animal models (rat and mouse); (2) to assess whether DEP are taken up and/or adsorbed by erythrocytes; (3) to evaluate erythrocyte antioxidant status following exposure to DEP, including malondialdehyde (MDA) levels and total antioxidant status (TAS) and superoxide dismutase (SOD) activity in the three species tested.

## Materials and Methods

### Particles

We used diesel exhaust particles (DEP; SRM 2975) from the National Institute of Standards and Technology (NIST,

Gaithersburg, MD, USA). DEP were suspended in sterile normal saline (NaCl 0.9 %) containing Tween 80 (0.01 %). To minimize aggregation, particle suspensions were always sonicated (Clifton Ultrasonic Bath, Clifton, New Jersey, USA) for 15 min and vortexed before their dilution or incubation with erythrocytes. Control erythrocytes received normal saline containing Tween 80 (0.01 %). These particles were previously analysed by transmission electron microscopy, and showed the presence of a substantial amount of ultrafine (nano) sized particle aggregates [16].

### Blood Collection

This project was reviewed and approved by our Institutional Review Board, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

We obtained erythrocytes from fresh human venous blood, drawn in EDTA from male healthy volunteers (aged between 30 and 55 years old) after informed oral consent, under the full institutional ethical approval.

Male Wistar rats (220-250, Taconic Farms Inc., Germantown, NY, USA), and TO mice (30-35 g, HsdOla:TO, Harlan, UK) were housed in light (12-h light:12-h dark cycle) and temperature-controlled (22 ± 1 °C) rooms. They had free access to commercial laboratory chow and were provided tap water *ad libitum*. For blood collection, rats and mice were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg), and then blood was drawn from the inferior vena cava in EDTA (4 %).

### Hemolysis experiments

The hemolysis experiment has been performed according to a previously described method [17]. Human, rat or mouse blood was mixed separately by gentle inversion of the tube and centrifuged at 1,200 × g for 10 min. The plasma supernatant was discarded and the erythrocytes were washed 4 times by suspending them in saline (0.9%) before centrifugation at 1,200 × g for 10 min. The final suspension consisted of 5% by volume RBC in saline. DEP suspensions (1, 10 and 100 µg/ml) were added, in duplicate, to a flat-bottomed 96-well plate. Negative control wells consisted of normal saline (NaCl 0.9 %) containing Tween 80 (0.01 %), and positive control wells consisted of 150 µL 0.1% Triton-X 100. To each well, 75 µL erythrocyte suspensions was added and mixed gently by pipetting. The plates were incubated for 30 min at room temperature, shaking gently on an orbital plate shaker. After incubation, plates were centrifuged for 5 min and 75 µL carefully removed from each well and transferred to a clean plate. The amount of hemoglobin released into the supernatant was determined spectrophotometrically at a wavelength of 540 nm. The percent hemolysis was calculated using the equation of a straight line,  $y = mx + c$ , where % hemolysis ( $x$ ) = [optical density ( $y$ ) – negative control optical density ( $c$ )]/[positive control optical density – negative control optical density]/100] ( $m$ ).

### Erythrocytes analysis by electron microscopy

To assess whether DEP are taken up and/or adsorbed by erythrocytes, human, rat and mouse erythrocytes were

incubated for 30 min with either saline containing tween 80 (0.01%) or DEP as described above. The samples were then collected and fixed in Karnovsky's fixative. Fixed cells were later rinsed in 0.1 M phosphate buffer, dehydrated in a graded series of ethanol, postfixed in 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer for 1 h, and embedded in Araldite. Ultrathin sections were obtained, stained with uranyl acetate and lead citrate, and examined in a JEOL (JEM 1230) electron microscope.

#### Measurement of antioxidants

One hundred microliters of the erythrocyte suspension ( $1 \times 10^6$  cells/ml) was incubated with DEP at final concentrations of 1, 10 and 100  $\mu\text{g/ml}$ , in a 96-well plate. The erythrocytes treated with normal saline (NaCl 0.9 %) containing Tween 80 (0.01 %) were taken as control. The plate was then positioned in an MS 3 digital microtiter shaker (IKA WERKE GmbH & CO, Stauffer, Germany) and rotated at 300 rpm for 30 min at room temperature. At the end of the incubation period, culture fluid was carefully removed for measurement of antioxidants using commercially available kits. Erythrocytes MDA levels were measured using a thiobarbituric acid-reactive substances method [18] with a commercially available kit (ZeptoMetrix, Buffalo, New York, USA). SOD (Cayman Chemicals, Ann Arbor, MI, USA) and TAS activities (Cayman Chemicals, Ann Arbor, MI, USA) were analyzed spectrophotometrically according to methods described by the manufacturers.

#### Statistics

Data are expressed as means  $\pm$  SD. Comparisons between groups were performed by one way analysis of variance (ANOVA), followed by Dunnett's multiple range tests. P values  $<0.05$  are considered significant.

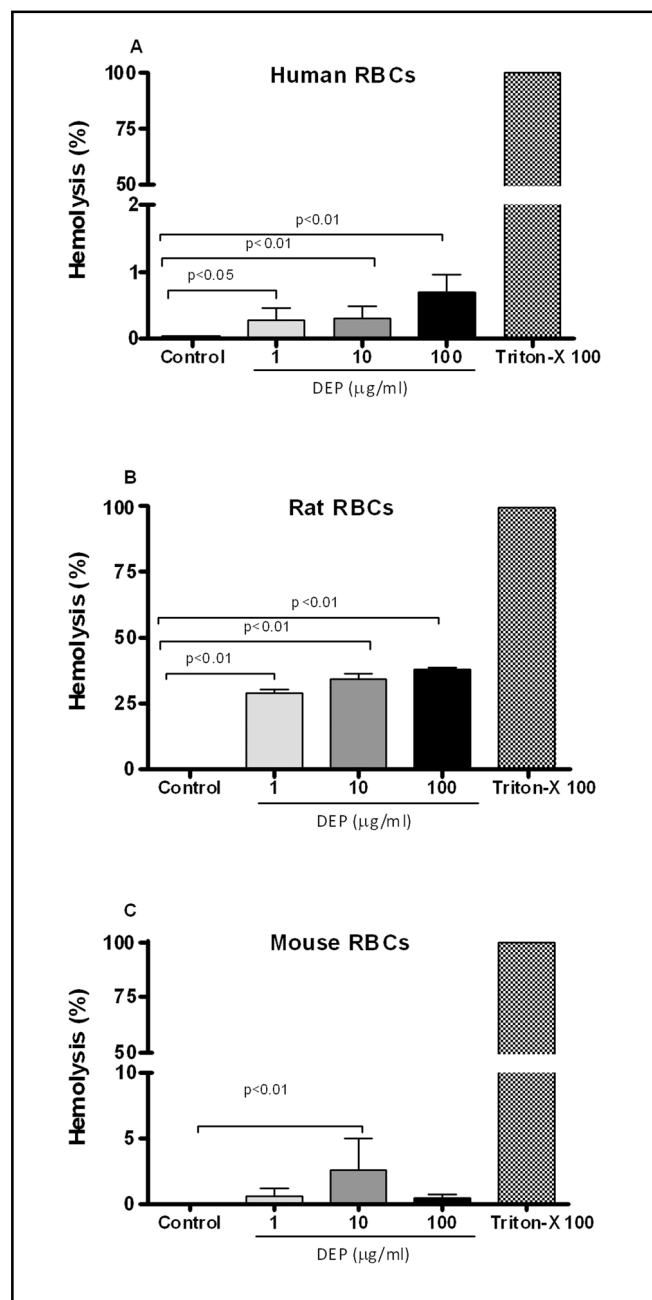
## Results

#### Effect of DEP on erythrocytes hemolysis

In human erythrocytes, DEP caused slight (less than 1%) but significant and dose dependent hemolytic effects (Fig. 1A). The highest hemolytic activity caused by DEP was observed in rat erythrocytes in which significant and dose- dependent effects were observed. The hemolytic effect ranged between 29 to 38 % (Fig. 1B). Compared to rat erythrocytes, an overall small hemolytic activity was observed in mouse erythrocytes. In the latter, a significant increase in hemolysis was only observed at the dose of 10  $\mu\text{g/ml}$ . At this dose, the degree of hemolysis observed (2.5 %) was higher than that observed in human erythrocytes.

#### Erythrocytes analysis by electron microscopy

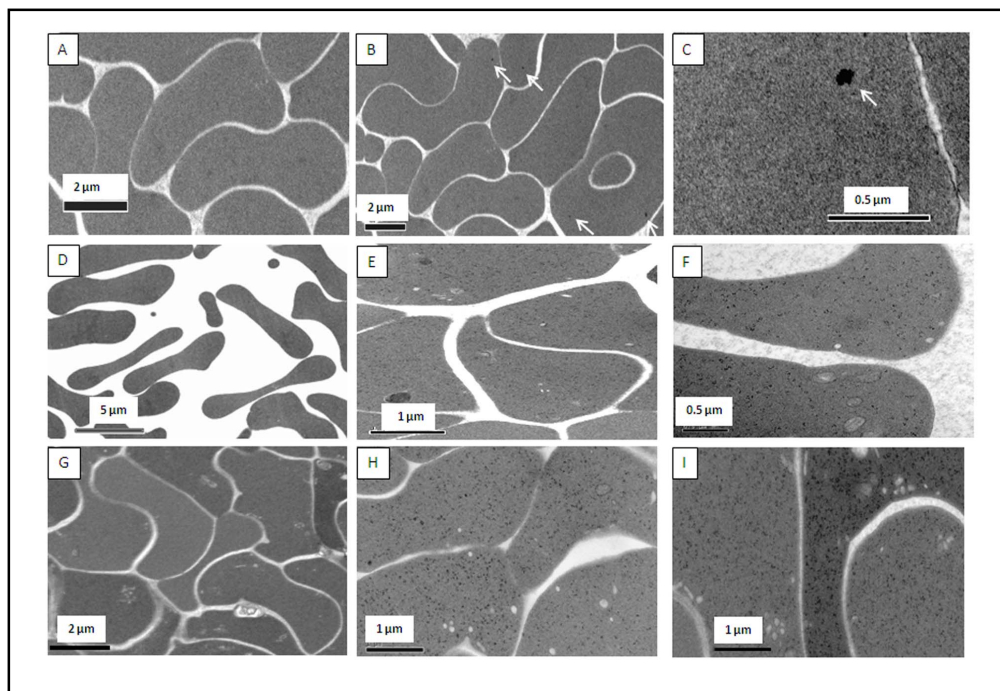
Figure 2 illustrates the TEM analysis in control human (A), rat (D) and mouse (G) erythrocytes exposed to saline containing Tween 80 (0.01%). The incubation of erythrocytes with DEP (1  $\mu\text{g/ml}$ ) for 30 min reveals the



**Fig. 1.** Hemolytic action of diesel exhaust particles (DEP) in the incubation medium of erythrocytes isolated from the blood of humans (A), rats (B) and mice (C). The results are expressed in % of positive control (0.1 % Triton-X 100). Data are mean  $\pm$  SD ( $n=6$  in each group). Statistical analysis by one-way ANOVA followed by by Dunnett's multiple range tests.

presence of only few particles in and/or adsorbed onto human erythrocytes (Fig. 2B-C). However, significant amounts of DEP were seen in and/or adsorbed onto rat (Fig. 2E-F) erythrocytes. Similarly, a large amount of DEP was taken up and/or adsorbed onto mouse erythrocyte

**Fig. 2.** Transmission electron micrographs (TEM) of human (A, B and C), rat (D, E and F) and mouse (G, H and I) erythrocytes after *in vitro* incubation with saline containing 0.01% of Tween 80 (A, D, G) or DEP [1  $\mu\text{g}/\text{ml}$  (B-C, E-F and H-I)]. The presence of nanosized aggregates of DEP which are internalized and/or adsorbed onto erythrocytes was species-dependent, with only few DEP in human erythrocytes (arrows) and large amount of DEP in rat and mouse erythrocytes.



after the incubation period of 30 min (Fig. 2H-I). The majority of DEP found in the erythrocytes were of ultrafine (nano)-size. No differences were observed between DEP 1  $\mu\text{g}/\text{ml}$  and the other studied doses in their accumulation in erythrocytes (data not shown).

#### *Effect of DEP on MDA concentrations and TAS and SOD activities*

Figure 3 illustrates the effect of DEP on the concentrations of MDA in human, rat and mouse erythrocytes suspensions. MDA was used to assess the susceptibility of erythrocytes lipid to *in vitro* peroxidation in human, rat and mouse erythrocyte. The incubation of human (Fig. 3A) or mouse (Fig. 3C) erythrocytes with DEP showed the absence of statistical difference in MDA concentrations at 1 ( $P>0.05$ ), 10 ( $P>0.05$ ) and 100 ( $P>0.05$ )  $\mu\text{g}/\text{ml}$  DEP compared with the control group. However, in rat erythrocytes, a significant and dose-dependent increase of MDA levels was observed (Fig. 3B).

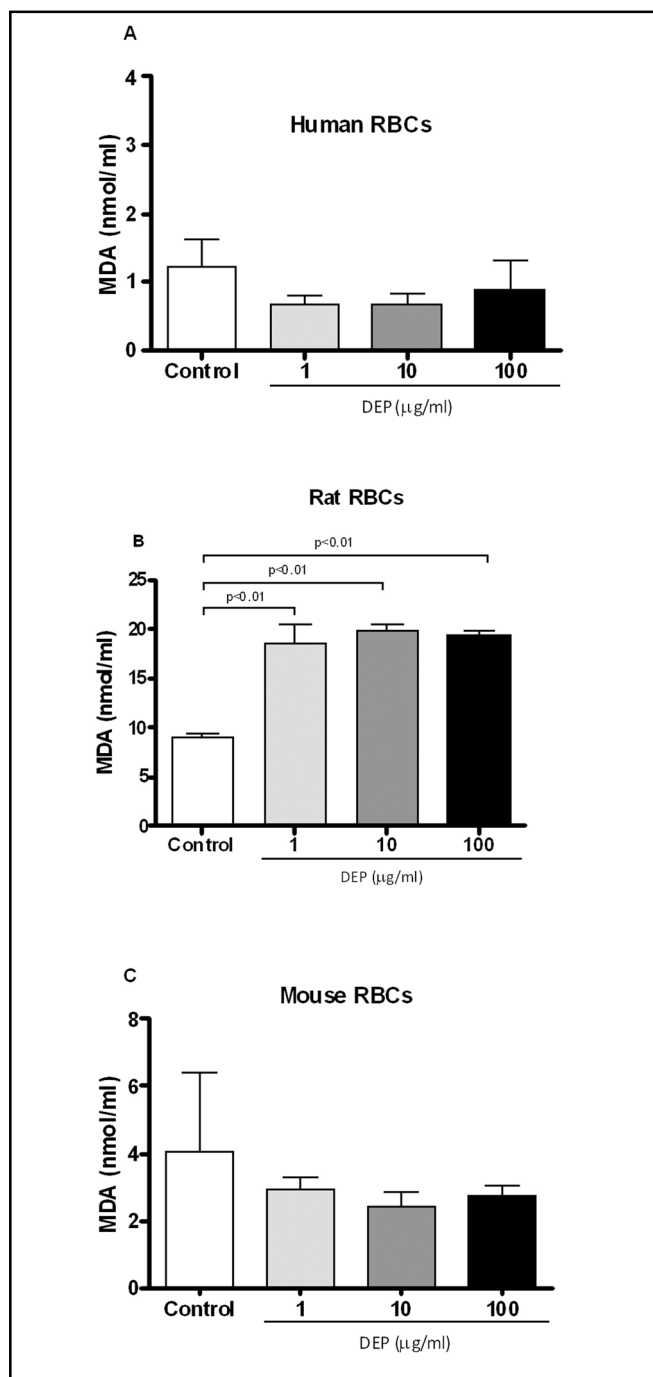
Figure 4 shows the effect of DEP on TAS activity. Compared to control, the TAS activity in human erythrocytes was not significantly affected by the different doses of DEP used (Fig. 4A). However, in rat erythrocytes, a significant and dose-dependent decrease in TAS activity was observed at 1 ( $P<0.05$ ), 10 ( $P<0.01$ ) and 100 ( $P<0.01$ )  $\mu\text{g}/\text{ml}$  DEP following a 30 min incubation period (Fig. 4B). In mouse erythrocytes, only the dose of 100  $\mu\text{g}/\text{ml}$  DEP caused a significant ( $P<0.05$ ) decrease of TAS activity compared to the control group (Fig. 4C).

Figure 5 shows the SOD activity after incubation of human, rat and mouse erythrocyte suspensions with DEP. Similarly to TAS, the SOD activity in human erythrocytes was not affected by the different doses of DEP (Fig. 5A). Conversely, a dose-dependent and significant decrease of SOD activity was seen following the incubation of rat erythrocytes with 1 ( $P<0.01$ ), 10 ( $P<0.01$ ) and 100 (0.01)  $\mu\text{g}/\text{ml}$  DEP compared to the control group. In mouse erythrocytes, a significant decrease of SOD activity was observed at 10  $\mu\text{g}/\text{ml}$  DEP ( $P<0.05$ ) compared to the control group (Fig. 5C). The two other doses of DEP did not affect the SOD activity.

## **Discussion**

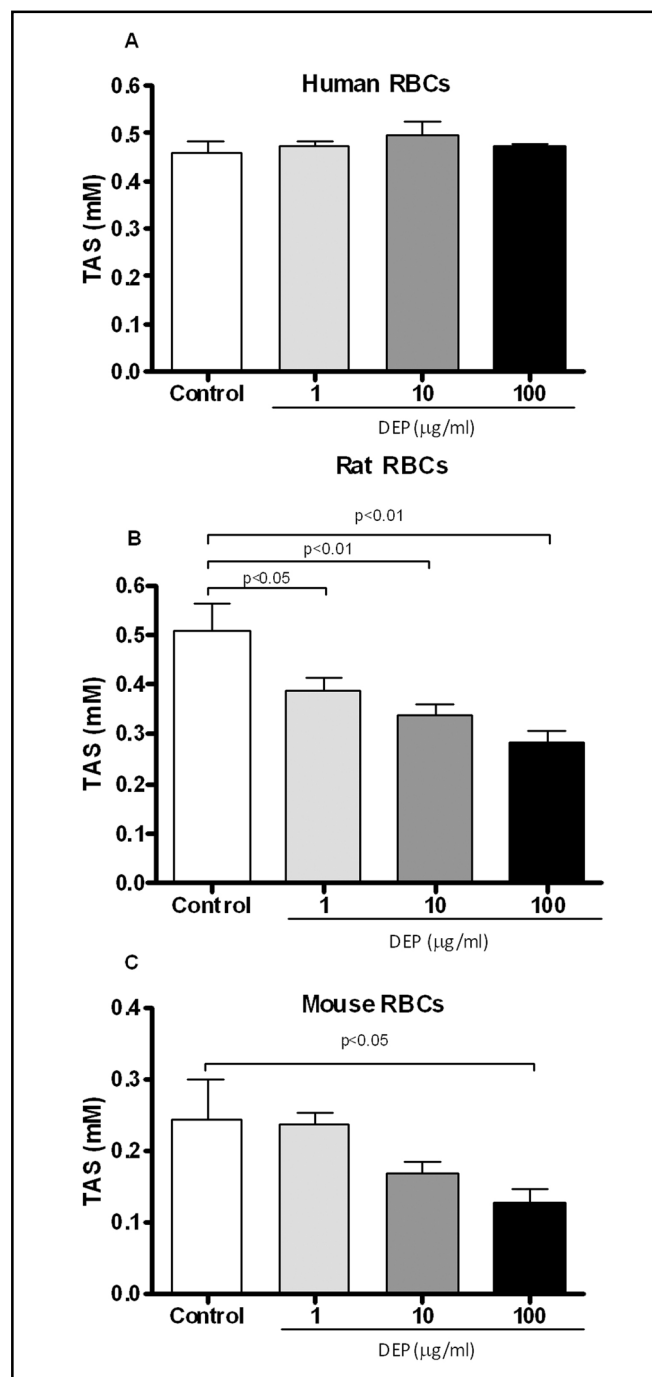
In this work, DEP induced species-dependent erythrocyte hemolysis and oxidative stress. Moreover, DEP were found to be internalized and/or adsorbed onto the red blood cells. Rat erythrocytes were found to be more prone to DEP toxicity than those of mice. The human erythrocytes showed the highest resistance to these effects.

Epidemiologic studies showed an association between elevated concentrations of ambient particulate matter and increased cardiovascular morbidity and mortality [2]. Exposure to UFPs, of which DEP are a significant contributor, in urban and industrial areas have increased dramatically because of anthropogenic sources such as internal combustion engines, power plants,



**Fig. 3.** Effect of diesel exhaust particles (DEP) on malondialdehyde (MDA) levels measured in the incubation medium of erythrocytes isolated from the blood of humans (A), rats (B) and mice (C). The results are expressed in % of positive control (0.1 % Triton-X 100). Data are mean  $\pm$  SD (n=6 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

incinerators and many other sources of thermo-degradation [11]. Once inhaled, UFP penetrate deeply into the respiratory tract, are able to inhibit phagocytosis,



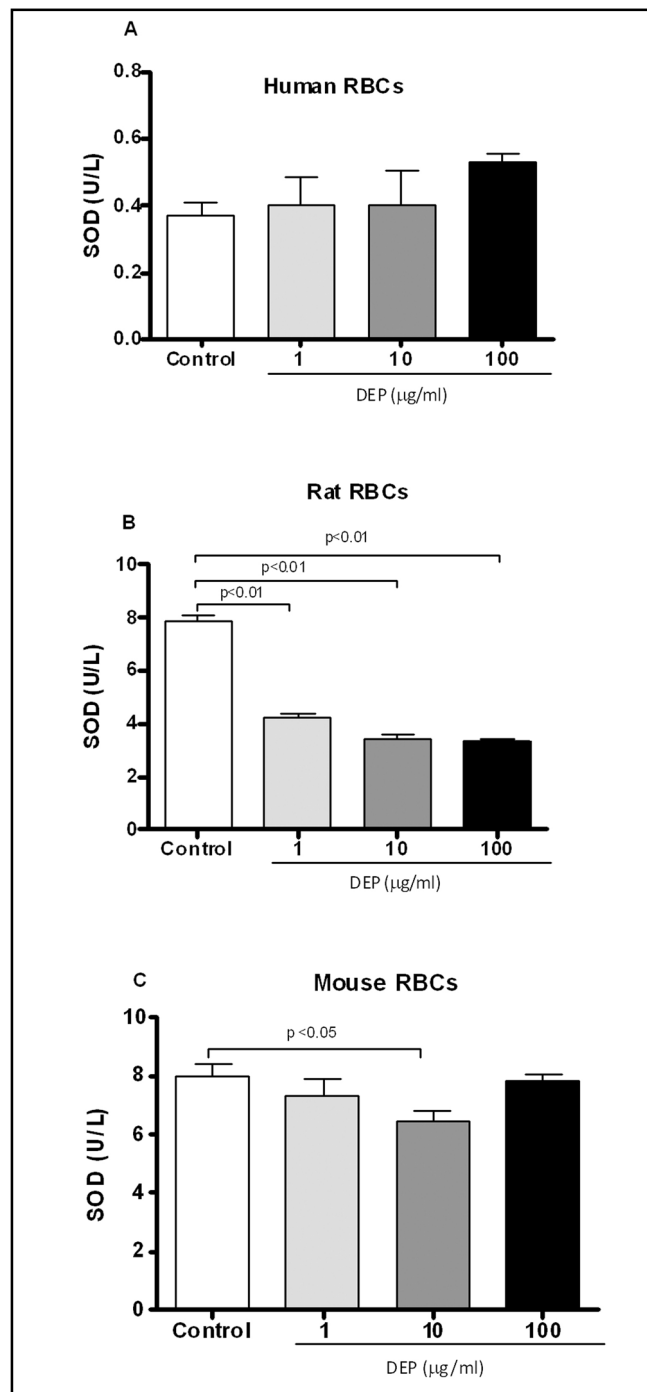
**Fig. 4.** Effect of diesel exhaust particles (DEP) on total antioxidant status (TAS) activity measured in the incubation medium of erythrocytes isolated from the blood of humans (A), rats (B) and mice (C). The results are expressed in % of positive control (0.1 % Triton-X 100). Data are mean  $\pm$  SD (n=6 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

and to stimulate inflammatory responses, damaging epithelial cells and potentially gaining access to the interstitium [11, 19]. Studies from our laboratory and

others showed that UFP can translocate through the respiratory epithelium towards circulation and subsequently exert toxicity to vascular endothelium, interact with circulatory cells such as erythrocytes, platelets and cause alteration of blood coagulation [8-10, 20, 21]. However, as far as we are aware, the effect of DEP on human, rat and mouse erythrocytes has not been reported before.

Our data show that DEP exert a slight but statistically significant hemolytic activity on human (0.7%) and mouse (2.5%) erythrocytes. On the contrary, a marked dose-dependent hemolytic activity was observed in rat erythrocytes. It has been reported that exposure to particulate air pollution causes a decrease in erythrocyte numbers in humans and rats [13-15]. These *in vivo* observations were explained by the systemic inflammation caused by particles, and subsequent adhesion of red blood cells to endothelial cells via adhesion molecules including inter-cellular adhesion molecule 1 [22] and/or the direct toxicity of particles on circulating erythrocytes. Our *in vitro* findings demonstrate that DEP exerts hemolytic activity on human, rat and mouse erythrocytes. A direct hemolytic effect of TiO<sub>2</sub> nanoparticles has been previously reported in rabbit erythrocytes [23]. The difference in amplitude of the hemolytic effects that we observed could be explained by species differences in erythrocyte sensitivity to DEP. Our results corroborate previous findings which demonstrated that erythrocytes from healthy blood donors have been reported to be less susceptible to the hemolytic activity of butoxyacetic acid and alkoxyacetic acids *in vitro* than the rat erythrocytes [24]. Moreover, it has been shown that human erythrocytes showed no hemolysis when incubated in butoxyacetic acid at concentrations up to 2.0 mM, a level which results in greater than 30% hemolysis of rat red cells after a 2 to 4 h incubation [25-27].

Because of the observed hemolytic effect of DEP, we performed TEM analysis of erythrocytes to assess if these cells take up and/or adsorb DEP and whether they can be affected by the species. Our data show the presence of large amount of nanosized DEP within rat and mouse erythrocytes. However, only relatively few DEP were observed in human erythrocytes. This finding could probably explain the smallest hemolytic activity observed in human erythrocytes. However, in spite of the presence of a large amount of DEP within and/or adsorbed onto mouse erythrocyte, the degree of hemolysis observed was relatively slight (only 2.5%). It has been reported that inhaled TiO<sub>2</sub> UFP were localized within blood capillaries and erythrocytes [28]. More recently, it has



**Fig. 5.** Effect of diesel exhaust particles (DEP) on superoxide dismutase (SOD) levels measured in the incubation medium of erythrocytes isolated from the blood of humans (A), rats (B) and mice (C). The results are expressed in % of positive control (0.1 % Triton-X 100). Data are mean  $\pm$  SD (n=6 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

been demonstrated that small MCM-41-type mesoporous silica nanoparticles (~100 nm) were found to adsorb to the surface of erythrocytes without disturbing the

membrane or morphology. In contrast, adsorption of large SBA-15-type mesoporous silica nanoparticles (~600 nm) to erythrocytes induced a strong local membrane deformation leading to spiculation of erythrocytes, internalization of the particles, and eventual hemolysis [29]. From our experiments, it could not be ascertained whether DEP are adsorbed and/or internalized by the erythrocytes. Further experiments are needed to clarify this point.

Erythrocytes have several biological mechanisms to protect them from oxidative stress. These include antioxidant enzymes such as SOD. Changes of enzyme activities also contribute to oxidative stress, which attenuate antioxidative defense [30, 31]. In spite of their well-developed antioxidant defense system, erythrocytes can be oxidatively damaged, due to exposure to environmental pollutants. The measurement of MDA is commonly used as a method for the quantification of lipid peroxidation [18]. Here, we measured MDA to assess the susceptibility of human, rat and mouse erythrocyte lipid *in vitro*. In line with the hemolysis findings, rat erythrocytes showed a significant and dose-dependent increase of MDA. This suggests that DEP caused oxidative stress to the erythrocyte, the plasma membrane is often the initial site of damage, and that the resulting peroxidation of membrane lipids causes hemolysis and cross-links between protein and lipid molecules to different extents [32]. Further, the increased fragility leading to the observed hemolysis is suggestive of cytosolic enzymes being inefficient in reacting with reactive oxygen species generated in the membrane. Conversely, the absence of significant increase of MDA in human and mouse erythrocytes corroborates the small hemolytic effects of DEP and illustrates that human and mouse erythrocytes are resistant to peroxidation of membrane lipids. Besides measuring the total antioxidant status as an index of overall oxidative stress [33], we have also measured SOD. Our data show a dose-dependent and significant decrease of TAS and SOD in rat erythrocytes. These findings are in line with MDA and hemolysis results. In mouse erythrocytes, a significant decrease of TAS was found at only 100 µg/ml and SOD at 10 µg/ml. Two

main mechanisms may have played a role in the decrease of these antioxidative enzymes activities. The first possible mechanism involves consumption during the breakdown of free radicals and the high level of H<sub>2</sub>O<sub>2</sub> or the inhibition of the enzyme by these radicals [31], while the second involves the direct inhibition of TAS and SOD by DEP. On the other hand, both TAS and SOD activities were not affected in human erythrocytes confirming its resistance to oxidative stress caused by DEP *in vitro*. The mechanisms responsible for species differences in RBC sensitivity to DEP remain uncertain but may be related to the differences in the structure, biochemistry and physiology of the erythrocyte membrane in the different species. Another possible explanation could be related to the human environmental air exposure prior to blood collection which probably differed markedly from that of the rats and mice. Because of possible exposure of humans to environmental contaminants/pollutants, perhaps there were some adaptive or compensatory ongoing processes in the human erythrocytes that did not occur in the rats and mice. Further studies are warranted to clarify this point.

It can be concluded that DEP caused dose- and species-dependent erythrocyte hemolysis, oxidative stress and are either taken up and/or adsorbed by the red blood cells. Rat erythrocytes were found to be very susceptible to DEP and to a lesser extent than those of mouse. The human erythrocytes showed the highest resistance to these observed effects. It would be of interest, in our future experiments to see if these *in vitro* actions also occur *in vivo* in mice and rats.

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