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Automotive airborne brake wear debris nanoparticles and cytokinesis-block micronucleus assay in peripheral blood lymphocytes: A pilot study

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ABSTRACT

Motor vehicle exhaust and non-exhaust processes play a significant role in environmental pollution, as they are a source of the finest particulate matter. Emissions from non-exhaust processes include wear-products of brakes, tires, automotive hardware, road surface, and traffic signs, but still are paid little attention to. Automotive friction composites for brake pads are composite materials which may consist of potentially hazardous materials and there is a lack of information regarding the potential influence of the brake wear debris (BWD) on the environment, especially on human health. Thus, we focused our study on the genotoxicity of the airborne fraction of BWD using a brake pad model representing an average low-metallic formulation available in the EU market. BWD was generated in the laboratory by a full-scale brake dynamometer and characterized by Raman microspectroscopy, scanning electron microscopy, and transmission electron microscopy showing that it contains nano-sized crystalline metal-based particles. Genotoxicity tested in human lymphocytes in different testing conditions showed an increase in frequencies of micronucleated binucleated cells (MNBNCs) exposed for 48 h to BWD nanoparticles (NPs) (with 10% of foetal calf serum in culture medium) compared with lymphocytes exposed to medium alone, statistically significant only at the concentration 3 $\mu g/cm^2$ ($p=0.032$).

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1. Introduction

Emissions from combustion-related processes have been studied thoroughly, reflecting the interest of the general public in this issue. In the European Union the emission limits for light-duty vehicles are set by Euro V and Euro VI legislation (EuP, 2007). On the other hand, emissions from non-combustion processes are still relatively ignored. A huge amount of BWD is generated and released to the environment, but no regulations exist to control the wear debris particles emitted from single materials.

Fine particles play a significant role in adverse health effects; in particular, exhaust emissions are considered to be the major source of the finest particulate matter (PM). However, published studies (Amato et al., 2011; Denby et al., 2013; van der Gon et al., 2013; Pant and Harrison, 2013; Amato et al., 2014) report that exhaust and non-exhaust emission sources (wear of brakes, tires, automotive hardware - *e.g.* engine parts, road surfaces, and traffic signs) contribute almost equally to the finest airborne PM.

According to Gasser et al. (2009), brake wear contributes up to 20% of total traffic emissions, and Sanders et al. (2003) revealed that approximately 50% by weight of the wear debris became airborne within all the vehicles tested in their study. Also automotive friction materials are complex composites typically consisting of more than 10 ingredients (Filip et al., 1997; Eriksson et al., 2002). Some of the raw materials used by manufacturers may be potentially hazardous, *e.g.* copper. More importantly, compared to the original bulk materials, particulates with a different chemistry and morphology are released by friction processes during braking; they are characterized by complex physico-chemical interactions on their surfaces (Filip et al., 2002; Kukutschova et al., 2011).

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Studies of brake wear particles are hindered by a lack of standardized sampling procedures and measurement techniques. Complex information regarding the potential influence of BWD on environmental and human health is also missing. However, recent reviews summarize the latest research in this field (Grigoratos and Martini, 2015; Peikertova and Filip, 2016).

Kukutschova et al. (2009) evaluated the mutagenic potency of initial friction composite and wear particles by two *in vitro* bacterial assays (SOS Chromotest, Ames test). They report an interaction of brake wear particles with DNA after metabolic activation, which indicates the presence of indirect mutagens. van der Gon et al. (2012) found that the presence of brake wear particles causes damage to tight junctions well before any significant cell mortality occurs. It also leads to negative health consequences, possibly through the formation of reactive oxygen species (ROS) which then may lead to inflammation and cardiovascular effects. According to Gasser et al. (2009) the content of metals (especially Fe, Cu, and Mn) in BWD may damage tight junctions, probably through a mechanism involving oxidative stress and proinflammatory response.

The purpose of this pilot study was to investigate genotoxicity of automotive airborne BWD by cytokinesis-block micronucleus (CBMN) assay. The *in vitro* micronucleus assay in human peripheral lymphocytes was performed following the general principles of the OECD Guideline 487 (2014). Only long-term continuous treatment was used because it is the most informative, taking into account that the uptake of the nanomaterials (NMs) by the cell has been shown to be time-dependent (Jin et al., 2007; Doak et al., 2012). For the characterization of the BWD Raman microspectroscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used. Airborne BWD were generated in the laboratory using a brake dynamometer test apparatus with the model brake pad, which corresponds to the low-metallic formulations of automotive brake pads available in the European markets with component materials typical of those used by the majority of brake pad manufacturers worldwide.

2. Material and methods

2.1. Nanoparticles (NPs) and their characteristics

2.1.1. Friction composite for automotive brake pads

For evaluation of genotoxicity of the airborne BWD the model low-metallic polymer matrix composite brake material whose formulation is presented in Table 1 was chosen. The model brake pad represents a composite with component materials typical of those used by the majority of brake pad manufacturers worldwide. The total mass of one brake lining pad is 113 g after machining and two pads are used in one dynamometer test. Brake linings were manufactured in the Southern Illinois University laboratories by mixing all ingredients in a Littleford W-10 vertical batch mixer (30 min), moulding, and hot pressing (180 °C/45 kN/15 min) followed by post curing in air at 180 °C for 4 h and machining to specifications given by the manufacturers.

Table 1
Formulation of the studied low metallic brake pad composite.

Constituent	Weight [%]	Constituent	Weight [%]
Steel fiber	10	Twaron	2
Synthetic graphite	5	Barite	9
Synthetic graphitic carbon	5	Aluminium oxide	1
Coke	15	Magnesium oxide	3
Iron powder	3	Stibnite	3
Vermiculite	5	Tin	3
Zircon	2	Molybdenite	1
Nitrile rubber	5	Phenolic resin	24
Copper	4		

2.1.2. Friction testing and brake wear debris generation

Link full scale brake dynamometer model M2800 (VŠB-Technical University of Ostrava) with the AK Master test procedure (SAE J 2522 – Dynamometer Global Brake Effectiveness) was used for generating the airborne BWD. The test consists of the following 15 sections which include 404 braking cycles with varying speed (40–180 kph), pressure (0.1–0.8 MPa) and temperature (100–550 °C). During the dynamometer test the airborne and non-airborne fractions of the wear debris were generated and after the test both fractions were collected. However, only the airborne fraction was evaluated for genotoxicity testing.

2.1.3. Analytical Techniques

Raman spectra of airborne BWD were obtained using Smart Raman Microscopy System XploRA™ (HORIBA Jobin Yvon, France). Raman spectra were acquired with 532 nm excitation laser source, and 1200 grooves/mm grating. Conventional TEM analyses were performed in a JEOL 1200EX electron microscope at 120 kV with airborne BWD deposited onto a TEM grid with amorphous carbon film. Scanning electron microscope Philips XL 30 (operating at 0.5–30 keV) equipped with EDS detector was used for determining morphology and elemental composition of the BWD.

2.1.4. NP Dispersion protocol

Stock solutions of BWD were prepared fresh each time. Concentrations were selected according to the FP7 NanoTest project (Dusinska et al., 2015). To prepare 1 ml of stock solution, 1 ml of 10% foetal bovine serum (FBS) in PBS was added to 6.75 mg of BWD NPs in a glass centrifuge tube. The dispersion was sonicated with a HD 3100 SONOPULS sonicator by BANDELINE electronic (Berlin, Germany) for 15 min at 100 W (cycle: 100%). The dispersion was cooled during sonication with an ice/water bath in order to prevent heating of the dispersion. The stock solution was equal to the highest concentration (75 μg/cm^2). Serial dilutions in the dispersion medium were made to obtain the full range (3, 15 and 75 μg/cm^2 equivalent to 5.4; 27 and 135 μg/ml) of BWD NPs suspensions. BWD NP solutions were added to cultures immediately in a volume of 100 μl.

2.2. Cytokinesis-block micronucleus test

Peripheral blood samples from a single donor (non-smoking female, aged 28) with no recent exposures to genotoxicants or ionizing radiation were used. Ethical approval was obtained before the study. Blood was collected by venipuncture from fasted subjects, and aliquoted in heparinised tubes. Cultures were set up in duplicate by adding 0.5 ml of whole blood to 4.5 ml of RPMI medium with L-glutamine and $NaHCO_3$ (Sigma) supplemented with 10% or 2% Foetal Bovine Serum (FBS, Sigma), phytohaemagglutinin (Remel, 0.18 mg/ml,) and antibiotics (penicillin and streptomycin, Gibco) in 9 cm^2 flasks. Two different protocols were used:

Protocol 1: 24 h after seeding (G1/S exposure), cultures were treated with different concentrations of BWD (3, 15 and 75 μg/cm^2) 20 h prior to cytochalasin B addition (Sigma, 6 μg/ml) and the cultures were incubated for additional 28 h (48 h treatment total).

Protocol 2: 44 h after seeding (G1/S/G2/M exposure), cultures were treated with different concentrations of BWD (3, 15 and 75 μg/cm^2), 4 h prior to cytochalasin B addition (Sigma, 6 μg/ml) and the cultures were incubated for additional 24 h (28 h treatment total).

After the incubation period, cells were hypotonically treated in

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