Journal of Physics: Conference Series **617** (2015) 012017 doi:10.1088/1742-6596/617/1/012017

The impact of instilled carbide nanoparticles on rat lungs: an *in vivo* perspective on acute intratracheal instillation

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Abstract. In order to study a scenario of acute high concentration exposure via the pulmonary pathway of silicon carbide and titanium carbide nanoparticles, female Wistar rats were administered by intratracheal instillation doses of 0.5 and 5 mg/rat of each nanomaterial. Inflammatory parameters were studied: protein concentration, lactate dehydrogenase activity, total cell count and differentiated cell count (macrophages, neutrophils, oesonophils, lymphocytes). The genotoxicity potential was assessed by the formation of micronuclei from pneumocytes type II. It was found that silicon carbide nanoparticles induce an inflammatory response and a dose dependent genotoxicity, although the genotoxicity levels are comparably lower to the inflammatory response.

1. Introduction

The principal route of exposure to nanomaterials (NMs) is the pulmonary pathway [1]. Carbide nanomaterials, like Silicon Carbide (SiC) and Titanium Carbide (TiC) are widely used in several industries requiring high performance products [2, 3], yet they have not been widely studied in comparison of other material families such as their oxide versions, silica and titania. Given the exposure hazard of such materials at the nanoscale could be due to wear, breakdown or decommissioning, our research group has dedicated several efforts to map the impact of exposure on rats to such NMs in terms: toxicity[4] and biopersistence [5] by whole body exposure (WBE), biopersistence from acute intratracheal instillation [6], oral administration in acute and subacute exposures [7, 8], and *in vitro* [9]. An unexplored subject has been the toxicological impact of SiC and TiC nanoparticles (NPs) via intratracheal instillation at doses simulating an acute high concentration

exposure scenario. This study presents addresses such question using rats, focusing on the inflammatory parameters from broncheoalveolar lavages (BAL) and the genotoxicity potential of SiC and TiC NPs.

2. Materials and methods

2.1. Nanoparticles

SiC and TiC NPs were provided by Sirris (www.sirris.be). They were heated at 200 °C during 2h to eliminate possible endotoxin traces. Their hydrodynamic diamters are 31 and 25 nm for SiC and TiC NPs, respectively. Their full physicochemical characterization has been reported elsewhere [6].

2.2. Animals

Wistar female rats, 200-220 g, were used in this study.

2.3. Exposure protocol

The NPs were prepared in suspensions with a physiologically sterile solution (0.9% NaCl) with 1 % Tween 20 and sonicated. Suspensions were prepared in two doses: 0.5 (low dose) and 5 (high dose) mg/rat. The exposure was acute, delivering the suspensions, 300 µL/rat, to the rat lung via intratracheal instillation. During this procedure rats were anesthetized, exposing surgically the trachea. After the exposure procedure the incision was closed with a suture point.

2.4. Genotoxicity studies

The genotoxicity potential of SiC and TiC NPs was studied *in vivo* in the rats pneumocytes type II. The inflammatory response was assessed 3 days after instillation by sacrificing the rats and measuring different parameters in a broncheoalveolar lavage (BAL) with a saline solution: total protein concentration, enzymatic activity of lactate dehydrogenase (LDH), and the total and differential number of cells. Pneumocytes type II were recovered after several BAL to eliminate inflammatory cells and a lung perfusion to eliminate the erythrocytes. Lung enzymatic digestion was carried out by an elastase solution flowing through the lung trachea for 30 minutes at 37 °C. Then the lung was cut in small sections and the cell suspension was filtered and then incubated in Petri boxes covered with rat IgG for 1 hour at 37 °C. The cells possessing the Fc receptor (macrophages) will adhere while the pneumocytes type II will rest in suspension and were recovered for culture. After two days, the culture chambers are washed and fixed with methanol. The plates are colored with orange acridine and counted with a fluorescent microscope.

The negative control was NaCl and the positive control was WC-Co (94:6 mass ratio, 5 mg) which generates micronuclei [10].

BAL was centrifuged, the supernatant was used to quantify the total proteins and LDH activity, and the cellular sediment was resuspended in NaCl for the cellular count. Protein concentration was measured by spectrometry after complexation with pyrogallol red-molybdate. LDH activity was measured by spectrometry after reduction of the NAD^+ catalysed by the enzyme. Total cell counting was done with a Neubauer plate and differential cells were counted from colored cytospins with Diff-Quick [11].

3. Results and discussion

3.1. BAL analysis

The protein concentration reflects the permeability of the broncheo-alveolar barrier. It is observed that for the high dose of SiC there is an elevated, even if not significant, concentration of proteins when compared to those of TiC. LDH activity, a marker of cytotoxicity, is found the highest for the high dose of SiC, noting that it is statistically significant with a value higher than even the positive control. The LDH activity of the low dose of SiC and the high dose of TiC is close to the positive control. The

total cell number follow the same trend as the LDC activity: high dose of SiC has the highest statistically significant number of cells, higher than the positive control; with both SiC low dose and TiC high dose having similar levels close to the positive control.

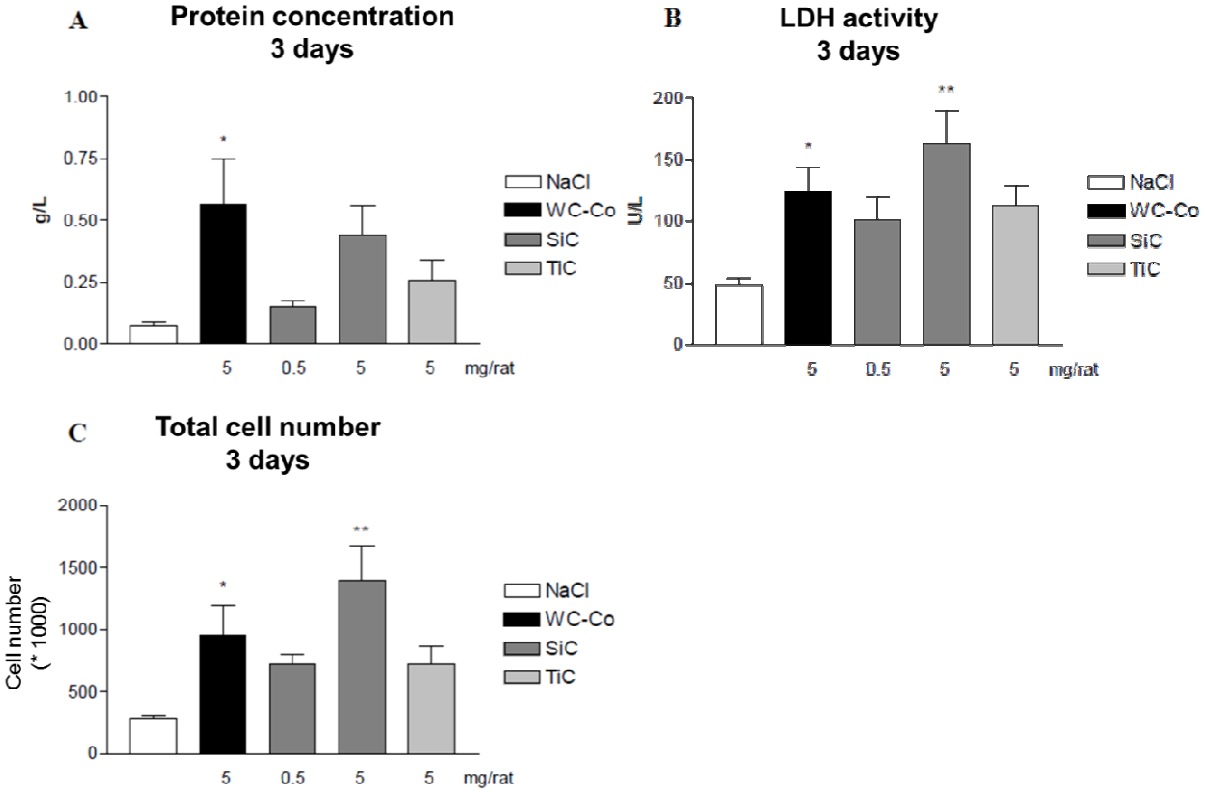

Figure 1. Inflammatory parameters in BAL after exposure. A) Total protein count. B) LDH activity. C) Cell total number. Nar represent the mean ± SEM (n = 4-6). Statistical significance, between negative control (NaCl) and exposed sample, evaluated by Student-Newman-Keuls test with * for $p<0.5$ and ** for $p<0.01$.

The cell count for macrophages and neutrophils has a significant increase for the high dose of SiC NPs, and lymphocytes had an increase for the low dose of SiC NPs. Eosinophils had similar cell counts for all doses, although showed no significant difference versus the negative control. Exposure to TiC NP showed higher levels (not significant) of macrophages and neutrophils with respect to the negative control.

3.2. Pneumocyte type II analysis

The number of micronuclei formed from pneumocytes type II for SiC and TiC NPs exposure were not significantly different from those formed on the negative control. In fact there seems to be a similar genotoxicity potential for the high dose of SiC and TiC NP, even if the high dose of SiC generated cytotoxicity and a higher presence of macrophages and neutrophils, while TiC did not generate a neutrophils response. It is also contrasting that a similar level of micronuclei is generated for both nanoparticle doses even if the biopersistence of TiC NPs in the lung is 2 times lower than SiC NPs [6]. This level of micronuclei production is quite lower than those produced by WC-Co. In addition, it is clear that the production of micronuclei is dose dependent as evidenced by the low and high dose of SiC NPs.

As previously reported, pulmonary toxicity of WC-Co is related to the capacity of induce both, reactive oxygen species and transient acute inflammation [12]. Here, after 72 h exposure to WC-Co particles no cellular infiltration was observed in BAL, however micronuclei formation in type II

epithelial cells was present. Meanwhile, SiC induced a pronounced inflammatory response in the lung and no signs of genotoxicity were observed. From this differences, we propose that SiC produces a substantial lung toxicity through the damage of resident cells in the alveoli, as demonstrated by the significant increase of LDH concentration in BAL. When cellular integrity is compromised, several molecules are liberated from cytosol, among them the damage-associated molecular-pattern (DAMP) molecules (such as Ca^{++}, ATP, RNA, DNA, etc.) could trigger inflammation and promote recruitment of innate inflammatory cells such as macrophages and neutrophils towards the alveoli, even in the absence of pathogens [13].

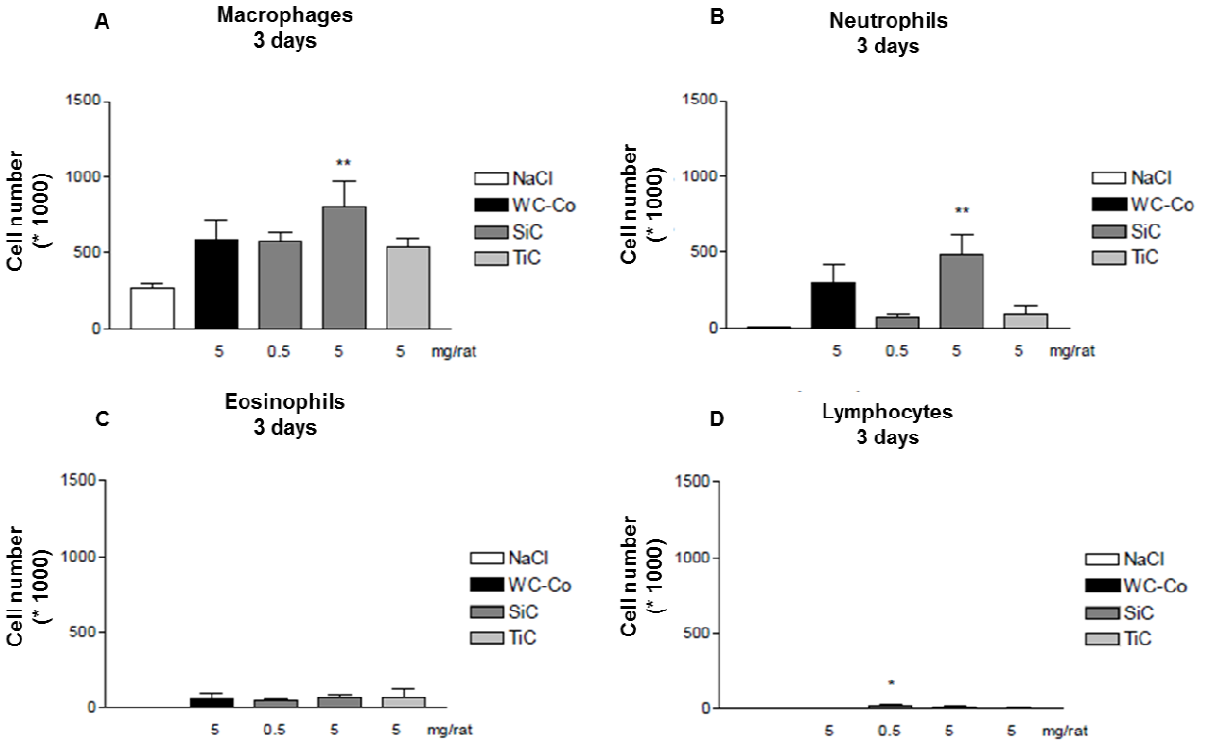

Figure 2. Number of (A) macrophages, (B) neutrophils, (C) eosinophils, and (D) lymphocytes after exposure. Statistical significance, between negative control (NaCl) and exposed sample, evaluated by Student-Newman-Keuls test with * for $p<0.5$ and ** for $p<0.01$.

Mechanistically, production of reactive oxygen species (ROS) is considered as the major player in NP induced toxicity [14]. It is possibly that SiC, but not TiC, induce free radicals that arise from redox reactions between their highly oxidized surface [6] and the biological milieu. For SiC, the aforementioned process is more relevant because their increased surface/volume ratio make it more capable to produce in ROS in higher amounts compared to TiC and WC-Co. Therefore, given the relatively similar hydrodynamic diameter of both SiC and TiC NPs, this is a NP surface-chemical mediated effect.

Depending on the severity, ROS could elicit different cellular responses: at low levels, an increase in proliferation and antioxidant defense is promoted, in mild levels DNA damage and release of inflammation mediators, whereas at high concentration, ROS provoke excessive organelle and DNA damaged leading to cell death [15]. Therefore, one possibility by which SiC did not shown DNA damage to type II cells is due to an overwhelming ROS production and subsequent cell death, since micronuclei formation could only be detected in cells without compromised viability, in which mild DNA damage allowed them progress to cell division.

The impact of SiC NPs was studied recently in a Whole Body Exposure (WBE) model [4], evaluating the impact of a SiC nanoaerosol when the whole respiratory pathway is taken into account. The toxicological analysis was found very similar to this study, where a limited inflammatory response was evidenced by a elevated presence of macrophages and neutrophils 24 h after an increment in the LDH activity.

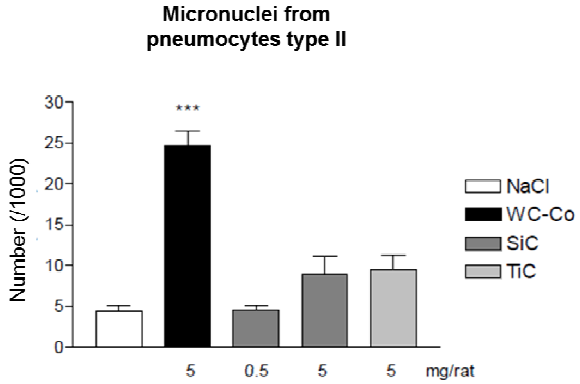

Figure 3. Number of micronuclei from pneumocytes type II after exposure. Statistical significance, between negative control (NaCl) and exposed sample, evaluated by Student-Newman-Keuls test with *** for $p<0.01$.

4. Summary

There is a dose dependent production of micronuclei with both SiC and TiC NPs exposed by intratracheal instillation. This genotoxicity, however seems not related to the prominent inflammatory response and more specially the relation with the neutrophils presence. The high dose, 5 mg, of SiC NPs produced the most important inflammatory response. TiC NPs, while not induced a inflammatory response, produced a similar level of micronuclei like SiC NPs.

Acknowledgements. This work was supported by the Service Public de Wallonie (SPW) – Direction générale opérationnelle – Economie, Emploi et Recherche (DGO6), Départément des Programmes de Recherche (Nanotoxico Project, SPW/FUNDP research convention No. 516252). Thanks to Dr. Georgina B. Vega for her insightful dialogues about the inflammatory response.

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