GENOTOXICITY OF NANOMATERIALS IN BEAS-2B CELLS ANALYZED BY THE *IN VITRO* MICRONUCLEUS ASSAY

RÖSSNEROVÁ Andrea[1], ČERVENÁ Tereza[1, 2], BRZICOVÁ Táňa[1, 3], VRBOVÁ Kristýna[1], SIKOROVÁ Jitka[1, 2], TOPINKA Jan[1], RÖSSNER Pavel, Jr.[1]

[1]*Institute of Experimental Medicine, Czech Academy of Sciences, Prague, Czech Republic, EU*

[2]*Charles University in Prague, Czech Republic, EU*

[3]*VSB-Technical University of Ostrava, Czech Republic, EU*

Abstract

The tremendous increase of the use of nanomaterials (NMs) has been witnessed during the last decade in many areas of human life including the chemical industry, cosmetics, biomedicine or food technology. The variety of NMs, their unique properties, almost ubiquitous presence and the size range of 1-100 nm raised the interest of toxicologists. The evaluation of the frequency of micronuclei (MN) as a result of the genotoxic events is a broadly utilized and well-established approach in *in vitro* studies for testing the risk of chemical exposure. Nevertheless, properties of the NMs give rise to the questions concerning the optimal methodological variants of the MN assay.

In our study, five types of well-characterized NMs (TiO_2: NM-101 and NM-103; SiO_2: NM-200; Ag: NM-300K and NM-302) of specific size, shape, or e.g. dimensions of aggregates were involved in the genotoxicity testing using four variants of protocols differing in the time of NM exposure, application of cytochalasin-B combined with simultaneous and delayed co-treatment with nanoparticles (NPs). Bronchial epithelial cells (BEAS-2B) were used in this study to fulfil these tasks. Presence of NPs was controlled by transmission electron microscopy (TEM).

Obtained results showed the different genotoxic potential of the various TiO_2 and Ag NMs (NM-101<NM-103 and NM-300K>NM-302, respectively). Comparison of all testing strategies revealed, that the level of DNA damage can differ based on the time of exposure and the methodological approach. In general, using cytochalasin-B led most frequently to the increase of the genotoxic potential of the tested NMs.

Keywords: BEAS-2B cells, genotoxicity, micronucleus assay, nanomaterials

1. INTRODUCTION

The nanomaterials (NMs) have been defined by EU recommendation number 2011/696/EU as “a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm” [1]. The tremendous increase of their application has been witnessed during the last decade in many areas of human life including chemical industry, cosmetics, biomedicine or food technology. Also, the number of registered NM increased rapidly up to almost 4 000 types according to current Nanowerk Nanomaterial Database™ (http://www.nanowerk.com/). Due to possible exposure of humans via a large number of consumer products and the evidence showing that exposure to nanoparticles (NPs) has numerous toxic effects, the interest of toxicologists in these materials has increased in the last few years.

Investigation of the total micronuclei (MN) by cytokinesis-block micronucleus assay (CBMN assay) in binucleated cells (BNC) is a traditional method [2] successfully used for evaluation of the effect of exposure to many chemicals. *In vitro* studies utilizing a broad spectrum of cell lines and evaluating the frequency of MN

are relatively common. On the other hand, this field of research faces many challenges and questions concerning an above-mentioned number of various NMs and variety of their properties like e.g. size, shape or level of aggregation. Also choosing the optimal methodological variant of the micronucleus assay (with or without cytochalasin B) and timing of the exposure experiment is a major task of the genotoxicity testing.

Up to now, more than 50 research papers concerning genotoxicity of NPs investigated by some of the variants of the *in vitro* micronucleus assay have been published according to PubMed database during last decade. Most of them concern some of the size fraction or crystalline structure (anatase or rutile) of titanium dioxide which is one of the most common NPs used in industry. Currently available results obtained by some of the variants of the micronucleus assay after treatment of various cells with mixtures of anatase and rutile NPs differing in their size, shows a wide range of effects, from negative to positive ones. Specifically, no effect of exposure was observed for larger 100% anatase TiO_2 particles >200 nm in BEAS-2B cells opposite to smaller 10 and 200 nm particles which induced significant increase in frequency of MN [3]; no consistent results also observed for different mixtures of both crystalline forms in comparison of various types of cells [4,5]; only higher/non-real dose of tested NPs (1000 mg/ml) was associated with genotoxic/transforming effects in another study [6]. Similarly, as for TiO_2 NPs, the effect of size was also observed for SiO_2 NPs where smaller crystalline forms (7.21 nm) caused a significant increase of MN in opposite to larger amorphous forms up to 104 nm [7,8]. Ag NPs (size range 6-20 nm) induced MN in two different cell lines [9] and induced cytotoxicity and genotoxicity in size- and coating-dependent manner [10]. The effect of the size of NPs on cytotoxicity and/or genotoxicity was presented also by other authors [11-13] while others found that the effect of the size was not always crucial [14].

Despite significant achievements in the testing of genotoxicity of NPs, there are many methodology variants and designs of MN assay which can be potentially used and which can affect the final results. In this study we utilized previously obtained published data and concentrated on the additional gaps in knowledge in optimizing the micronuclei assay for testing of the broad range of NPs. Five types of well-characterized NMs (TiO_2 NM-101 and NM-103, SiO_2 NM-200, Ag NM-300K and NM-302) differing by their properties, including size, shape, or e.g. dimension of aggregates were involved in the testing of four variants of protocols characterized by time of exposure and application of cytochalasin-B combined with simultaneous or delay co-treatment with NPs.

2. METHODS

2.1. Nanomaterials

Five types of NMs differing by their characteristics were involved in genotoxicity experiments: (i) TiO_2 [titanium dioxide - anatase form, primary particle size 5 nm, NM-101 (Joint Research Centre)]; (ii) TiO_2 [titanium dioxide - rutile form, primary particle size 20-100 nm, NM-103 (Joint Research Centre)]; (iii) SiO_2 [synthetic amorphous silicon dioxide produced by precipitation, particle size 10-20 nm, NM200 (Joint Research Centre)]; (iv) Ag [silver with prevalently round shape, particle size < 20 nm, NM-300K (Joint Research Centre)] and; (v) Ag [silver with rods shape, particle size 100-200 nm width, 5-10 μm length, NM-302 (Fraunhofer)].The preparation of NMs for genotoxicity testing was performed according to instructions of the generic NANOGENOTOX dispersion protocol (www.nanogenotox.eu).

2.2. Cell cultivation and treatment

Adherent human bronchial epithelial cells BEAS-2B obtained from American Type Culture Collection (ATCC®) (CRL9609™) were used to evaluate the genotoxic effect of selected NMs. The protocol recommended by ATCC® for cultivation of the BEAS-2B cells was used. Briefly, cultivation surfaces were coated with a mixture of 0.01 mg/ml fibronectin (Sigma-Aldrich, MO, USA), 0.03 mg/ml bovine collagen type I (Sigma-Aldrich, MO, USA) and 0.01 mg/ml BSA (Sigma-Aldrich, MO, USA) dissolved in bronchial epithelial basal medium (BEBM™) (Lonza, Switzerland) and kept at 37°C overnight. Before seeding the cells, all the coating media were removed.

Serum-free cultivation conditions (BEGM™ kit CC3170) (Lonza, Switzerland) were used. The cells confluence did not exceed 70%. Three concentrations (see details in results) of each tested NM were selected for testing in this study.

2.3. Micronucleus assay

Genotoxicity of selected NMs was determined using the micronucleus assay in both mononucleated (MONO) and binucleated cells (BNC) [15,16]. Four methodological variants: two without cytochalasin-B and two with cytochalasin-B were tested in this study (see **Figure 1 A-D**). NPs were applied for 28 h and 48 h before the end of cultivation. The 8-well Lab-Tek™ Chamber Slide System where the cells were grown and treated was used as described previously [17]. Concentration of cytochalasin-B (Sigma-Aldrich, MO, USA) for blocking of cytokinesis was 1 µg/ml. At the end of cultivation, the cells were treated with a hypotonic solution of KCl (0.075M, Sigma-Aldrich, MO, USA) and fixed with a mixture of methanol (Merck Millipore, MA, USA) and acetic acid (Penta, Czech Republic) (3:1). Finally, the slides were dried and stained by 5% Giemsa (Merck Millipore, MA, USA).

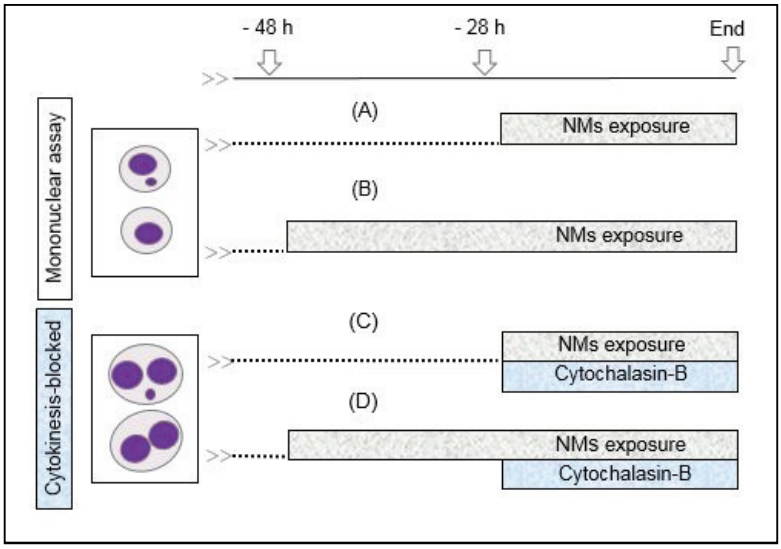

Figure 1 Overview of the design of micronucleus assay tested in the study: Mononuclear assay with 28 h (A) and 48 h (B) NMs exposure; CBMN assay with 28 h simultaneous co-treatment (C) and 48 h delayed co-treatment (D) of NMs with cytochalasin-B.

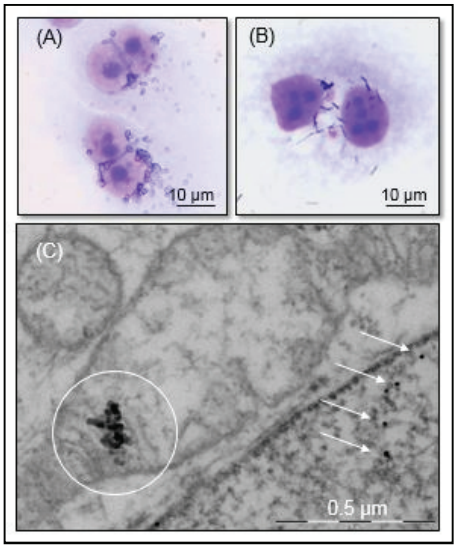

Figure 2 Examples of images from microscopic analyses: Giemsa-stained BNC with the NM-101 (A) and NM-302 (B) on the surface; TEM image showing the NM-300K (C) inside the cell

2.4. Microscopic analysis

Visual scoring using the Olympus BX41 microscope was performed to analyze the MONO or binucleated cells in final magnification 1000x. A total of 3x 1000 MONO or 3x 500 BNC per each tested compound were evaluated. In case of evaluation of the BNC, the cytokinesis-block proliferation index (CBPI) was calculated to control for cell division. Transmission electron microscopy (TEM) was applied with the aim to control the presence of NPs and their agglomerates inside the cells and/or nuclei. See an example of images from both microscopic analyses in **Figure 2**.

3. RESULTS AND DISCUSSION

Five types of well-characterized NMs (TiO_2 NM-101 and NM-103, SiO_2 NM-200, Ag NM-300K and NM-302) of specific size, shape, or e.g. dimension of aggregates were involved in the testing of four variants of protocols

differing by the time of exposure (28 h or 48 h) and application of cytochalasin-B. Simultaneous or delay co-treatment with NMs was applied in case of use of methodological variant with cytochalasin-B. The results, summarized in **Figure 3 A-E,** are expressed as a percentage of aberrant cells (% ABB) in MONO or BNC for assay without or with cytochalasin-B, respectively.

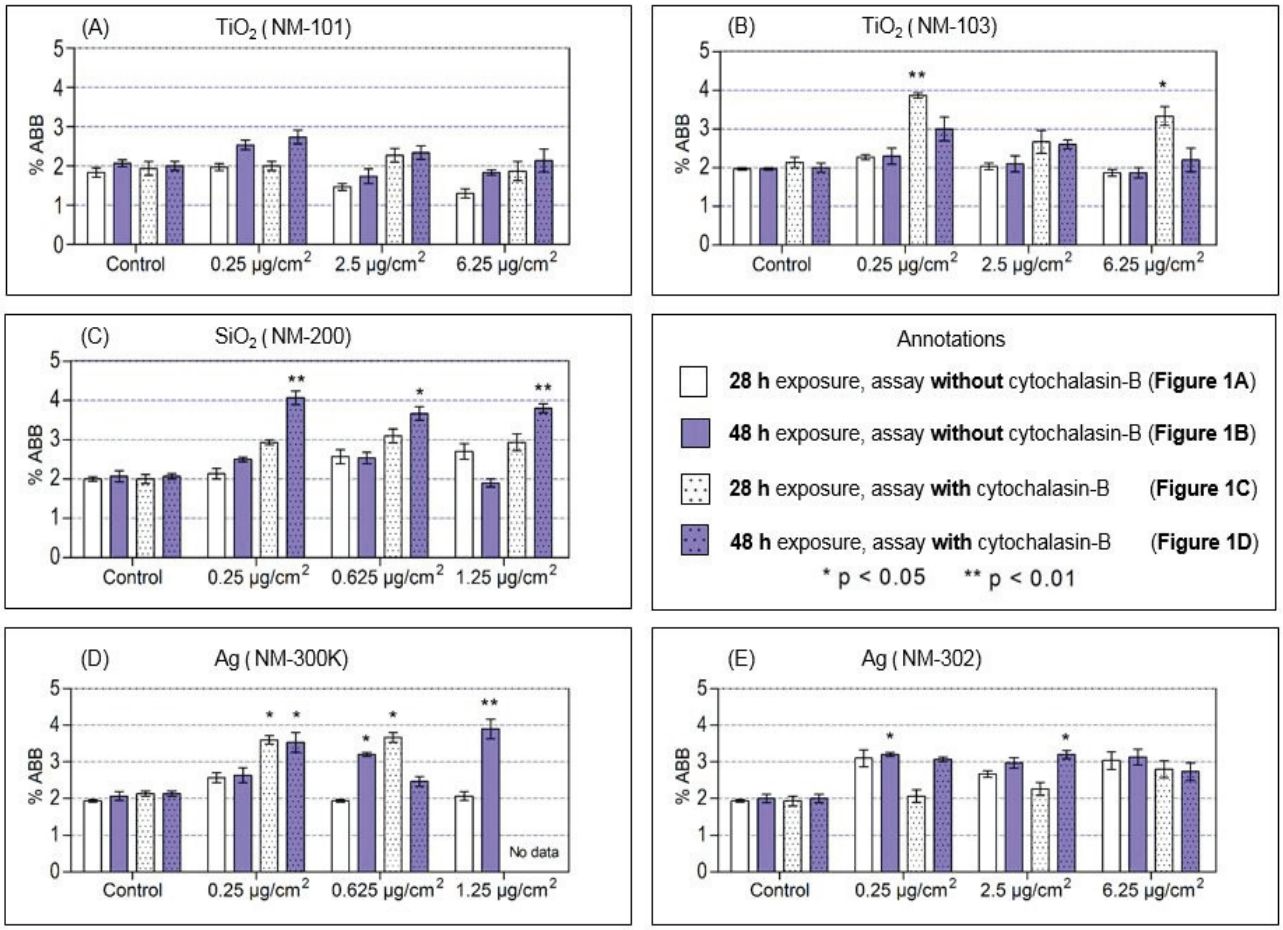

Figure 3 Micronuclei induced after exposure to selected NMs for 28 h and 48 h with and without cytochalasin-B

Such broad testing of NMs, involving NPs of different properties, concentrations, time of exposure and various concepts of the micronucleus assay was inspired mainly by unique physico-chemical properties of NMs responsible for unexpected interactions with experimental components that support the evidence, that they cannot be treated in the same manner as chemical compounds [18]. Beside many confounding factors, experimental design with/without cytochalasin-B seems to be fundamental. Various concepts of MN assays were previously discussed for dextran-coated USPION exposure to MCL-5 cells where a post-treatment with cytochalasin-B induced a significant and dose-dependent increase of MN frequency. The post-treatment as well as delayed co-treatment with cytochalasin-B was also suggested [18,19]. Another discussion, but with opposite worry, concerns the fact, that cytochalasin-B acts as an actin inhibitor which, beside the cytoplasmic cell division, may also inhibit the process of actin-dependent endocytosis as one of the routes for entry of NPs into the cells. This may theoretically result in underestimation of genotoxic potential of NPs [20].

Our results support above mentioned observation, that cytochalasin-B plays a role in increase of the significance of the results as shown for TiO_2: NM-103, SiO_2 NM-200 and Ag NM-300K. In contrast, the testing strategies without cytochalasin-B represent more typical situation related to real exposure of humans. Co-treatment with cytochalasin-B during exposure to NPs can only simulate the possible reaction of the human

during cancer therapy. Concerning other findings, two TiO_2 and Ag NMs which differed substantially by size and crystalline form or shape respectively, are the typical examples of the differences in genotoxic effect caused by the properties of the individual NMs. From our results, we may hypothesized that co-treatment of BEAS-2B cells with NPs and cytochalasin-B is not a responsible for underestimation of genotoxic potential, but just opposite, probably due to alternative pathway/s of the NPs transport through cell membrane.

4. CONCLUSION

In conclusion, NPs and their aggregates of different size were present in cells after the treatment as observed by TEM. Their genotoxic potential differed depending on the individual physicochemical properties of the tested NMs. Even though the DNA damage level measured by micronucleus assays without blocking of cytokinesis was mostly not significant, the simultaneous or delayed co-treatment with cytochalasin-B produced more significant increase of micronuclei. Standardized testing strategy should be thus thoroughly verified in future studies.

ACKNOWLEDGEMENTS

This work was supported by the grant of the Ministry of Education Youth and Sports CR #LO1508. The authors also acknowledge the assistance provided by the Research Infrastructure NanoEnviCz, supported by the Ministry of Education, Youth and Sports of the Czech Republic under Project No. LM2015073. The project was supported by the Operational Program Prague Competitiveness under Project No.CZ.2.16/3.1.00/24507.

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