MICRONUCLEI LEVELS IN NANOCOMPOSITES PRODUCTION WORKERS: INTERPRETATION OF RESULTS FROM TWO YEARS OF MONITORING

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Abstract

Micronucleus assay, which has been used for almost sixty years, is one of the basic methodological approaches of genetic toxicology. This is also evident from more than 10,500 references found in PubMed database. These studies concern the evaluation of cytotoxicity and genotoxicity of numerous chemicals and in the last decade also nanomaterials (NM) and nanoparticles (NP). Surprisingly, no relevant human studies focused on MN formation following the exposure to NP have been reported.

In our study we analyzed 4x (in September 2016 and 2017; pre-shift and post-shift each year) samples in a group of workers, working for almost 18 years in nanocomposites research, and matched controls. Detail aerosol exposure monitoring of particulate matter (PM) including nano-sized fractions was completed during working shift in a sampling day. The micronucleus assay using Pan-Centromeric Chromosome Paint was applied to recognize, beside the frequency of total micronuclei (MN) in binucleated cells (BNC), also other types of chromosomal damage (losses and breaks), including the centromere positive (CEN+) and centromere negative (CEN-) micronuclei.

The monitoring data showed differences in the risk of exposure to NP related to individual working processes, as well as differences in chemical composition of nano-fraction. Cytogenetic results demonstrated consistently (both years): (i) possible adaptation to long-term exposure of NP (related to total frequency of MN), (ii) short-term (2.5 h) exposure could be a reason for the aberration increase, particularly a chromosomal losses (aneugenic effect).

Keywords: Genotoxicity, human, micronucleus assay, nanoparticles, pan-centromeric FISH

1. INTRODUCTION

Assessment of genotoxicity of nanomaterials (NM) and nanoparticles (NP) is one of the current objectives of genetic toxicology due to the increase of their use during the last decade in many areas of human life. One of the important and frequently used methodological tools of researchers is micronucleus assay which is evident from more than 10,500 references found in PubMed database in the middle of 2018. These *in vitro* and *in vivo* studies concern mainly the evaluation of cytotoxicity and genotoxicity of numerous chemicals, which are discussed in context of deleterious effects for humans. Only 300 of them (majority *in vitro*) concern

the toxicity of NM and/or NP. Related to *in vivo* testing of genotoxicity of NP by micronucleus test, about 20 studies in mice (Swiss mice, C57BL/6J mice) or rat models (Wistar rats, Sprague-Dawley rats) are currently found in PubMed databases, but did not show consistent results due to their broad spectrum and various characteristics.

Surprisingly, still no relevant human studies focused on MN formation following the exposure to NP have been reported [1]. Alternatively, it is possible to recognize four studies [2-5] analyzing various cytogenetic markers in welders where the exposure to particulate matter in nano-size range also occurs [6]. A study published already in 1983 analyzed long-term occupational exposure (mean: 19 years) reported no significant differences between the exposed and controls, even though three various cytogenetic methods were used: conventional cytogenetic analysis (CCA), analysis of total MN and sister chromatid exchanges (SCE) [2]. A Mexican study that focused on genetic damage in exfoliated oral mucosa cells also did not observe differences between cases and controls [3] opposite to a comparison of chromosomal alterations induction, including MN, between nasal and buccal cells of welders in another study where a higher sensitivity of the epithelial cells from respiratory system was shown [4]. Additional identification of centromeres and genetics polymorphisms in DNA repair and detoxification genes was performed in another study where the authors reported a significantly higher level of chromosome/genome damage in welders suggesting that the combined analysis of genetic polymorphisms and centromeres in MN may improve the sensitivity of the micronucleus assay in detecting genotoxic effects [5].

Due to this gap in knowledge and potential negative health effects of NP, we analyzed 4x (in September 2016 and 2017; pre-shift and post-shift each year) a group of persons, long-term (years) working in nanocomposites research, and matched controls. Detail aerosol exposure monitoring of particulate matter (PM) including nano-sized fractions was completed during working shift in a sampling day. The micronucleus assay using Pan-Centromeric Chromosome Paint was applied to recognize, beside the frequency of total micronuclei (MN) in binucleated cells (BNC), also origin of chromosomal damage (losses and breaks), including the centromere positive (CEN+) and centromere negative (CEN-) micronuclei.

2. METHODS

2.1. Study groups

A total of 41 and 40 participants were involved in a collection of blood samples in September 2016 and 2017, respectively. The sample set included a group of twenty occupationally exposed nanocomposite processing researchers, working long-term (years) in nanocomposite research, sampled twice each year (pre-shift and post-shift, including processes such as welding mild steel S355J2 and smelting in workshop 1; and machining including the milling and grinding of epoxide resin with nanoSiO_2, and geopolymer nanocomposites in workshop 2) and 21/20 control volunteers living in the same location. Both genders, with a prevalence of men were involved in each subgroup. The researchers spent 2.5 hours during working operations. For simplicity, we refer to these samples as pre-shift and post-shift, even though the remainder of their total 8-h shift was spent in their offices. The exposed subjects did not use personal respiratory protection. More details on basic characteristics are presented in the results and discussion section.

2.2. Aerosol exposure monitoring

The online monitoring during the shift for individual processes and prior to it (background), included two standard aerosol spectrometers Scanning Mobility Particle Sizer (SMPS) (TSI SMPS 3936L, USA) and Aerodynamic Particle Sizer (APS) (TSI APS 3321, USA) which were used to obtain more details in the nanoscale range from 6 nm up. The offline Berner Low Pressure Impactor (BLPI) (HAUKE Gmbh., Austria) was used to sample aerosol particles onto 10 stages up to 13.6 µm including two nanoscales (25-56 nm and 56-100 nm). These samples were consecutively analyzed by gravimetry, ion chromatography [7] and Scanning

Electron Microscope (SEM) (Tescan Indusem, Czech Republic) equipped by Energy-Dispersive X-Ray Spectroscopy (EDS) (XFlash detector 5010, Bruker, Germany) to analyze the elemental composition of size-resolved aerosol fractions.

2.3. Micronucleus assay with Pan-centromeric FISH

***Blood samples*:** Blood cell cultures were incubated at 37°C for 72 h. Cytochalasin B was added to a final concentration of 5 µg/ml after 44 h [8]. Cultures were harvested by centrifugation, treated with hypotonic solution of KCl and fixed with methanol/acetic acid.

***Centromeric FISH technique*:** The protocol performed with FITC labelled Human Pan Centromeric probes was adapted from the manufacturer protocol (Cambio, Cambridge, UK). Stained slides were counterstained with DAPI mixed with Vectashield mounting medium.

***Microscopic analysis*:** One thousand binucleated cells (BNC) were analyzed for each subject under the fluorescence microscope (Axioskop - Zeiss) equipped with filters for DAPI (blue signal) and FITC (green signal). BNC with micronuclei (MN), centromere positive (CEN+) and centromere negative (CEN-), were recorded and analyzed by use of the ISIS software version 5.0 (MetaSystems). See an example including explanation in **Figure 1**.

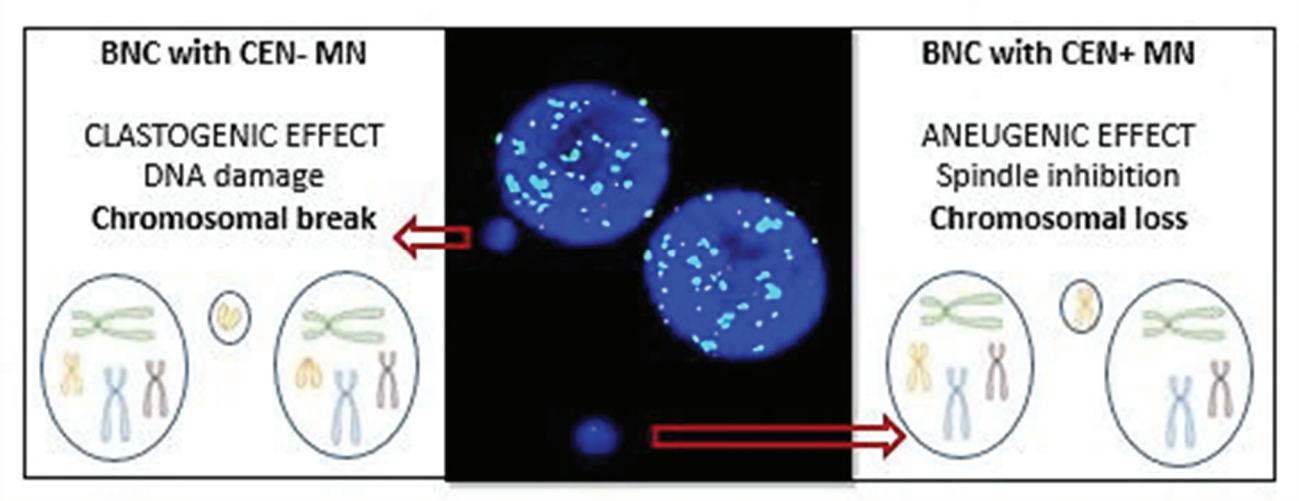

Figure 1 Example of BNC with two MN (both: CEN- and CEN+) with the explanation of their origin (chromosomal break on the left or chromosomal loss on the right)

2.4. Statistical analysis

Basic descriptive statistics [mean, standard deviation (SD), median and range (minimum and maximum)], were calculated using Microsoft Excel 2013. T-test, Mann-Whitney *U* test, paired sample t-test or the Wilcoxon signed-rank test were used for the comparison of the studied parameters (total MN/1000 BNC, CEN+ MN/1000 BNC and CEN- MN/1000 BNC).

3. RESULTS AND DISCUSSION

Main characteristics of the studied groups working in nanocomposite research analyzed repeatedly in September 2016 and 2017 twice per day (pre-shift and post-shift) each year, and matched controls are summarized in **Table 1**. There was no significant difference between the exposed and control groups for any of the selected characteristics and year even though the studied groups were not 100 % identical between years (an overlap of 14 and 11 participants in exposed and controls between years, respectively.

Table 1 Characteristics of the studied groups (format: N or mean±SD (min-max))

	2016		2017	
Characteristics	Exposed	Controls	Exposed	Controls
Number (N)	20	21	20	20
Males/Females (N)	15/5	15/6	13/7	13/7
Age (years)	41.8±11.4 (29-63)	38.7±9.1 (20-55)	38.6±10.7 (23-63)	39.8±7.1 (27-55)
BMI (kg/m^2)	28±6.2 (18-42.1)	25±4.7 (18-37.2)	24.6±4.4 (17.5-34.3)	26.2±4.4 (20.1-36.6)
Smoking (N)	1	4	1	3

Two types of exposure data were available for the group of nanocomposite researchers: (i) related to time of exposure (years of exposure, common working day exposure and exposure in monitoring day), (ii) particular data from aerosol exposure monitoring including nano-fraction as described in detail previously [9]. Comparison of these data revealed differences in years of exposure between groups monitored in year 2016 and 2017 (17.8±10 and 7.3±4.8 years, respectively). Also, individual working processes were accompanied by differences in nano-fraction exposure and their chemical composition.

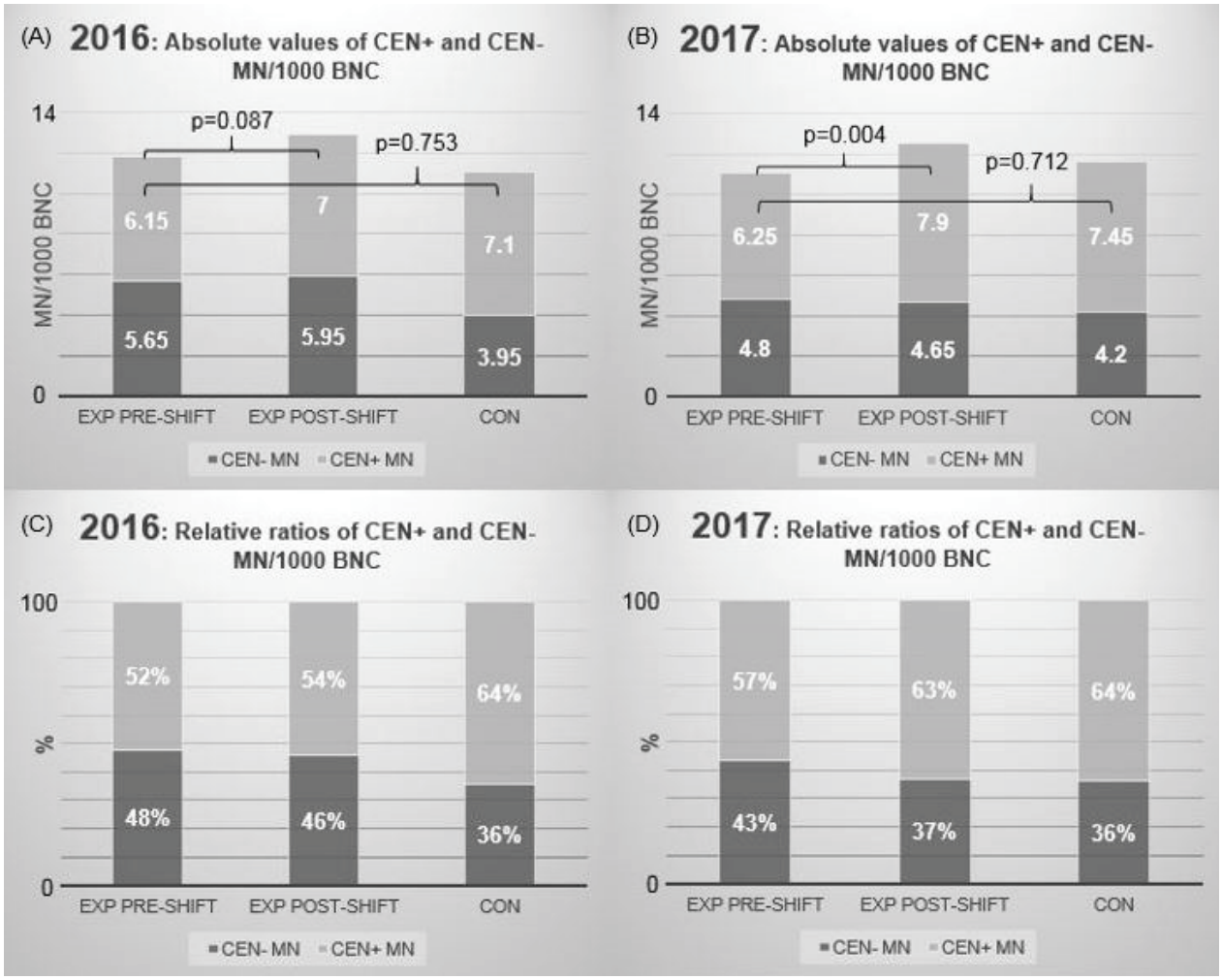

Figure 2 Overview of cytogenetic results (absolute values (A, B) and relative ratios (C, D) of CEN+ and CEN- MN in 1000 BNC) obtained by micronucleus assay using Pan-Centromeric Chromosome Paint in years 2016 and 2017

Summary of most important cytogenetic results (absolute values and relative ratios of CEN+ and CEN- MN in 1000 BNC) obtained by micronucleus assay using Pan-Centromeric Chromosome Paint is shown in **Figure 2**. We already analyzed in detail the sample set followed in year 2016 [10]. These results showed differences in the impact of exposure to NP related to individual working processes as well as differences in chemical composition of nano-fraction. Cytogenetic results demonstrated possible adaptation to long-term (years) exposure to NP (related to total frequency of MN) and generally corresponded with above mentioned studies related to welders [2-5], although this exposure may be responsible for DNA damage pattern changes (increase of chromosomal breaks from 36 % to 48 % - clastogenic effect). However, short-term (daily) exposure could be a reason for the increase of chromosomal losses (aneugenic effect) as well as breaks (clastogenic effect) in the exposed group, depending on the particular type of exposure. New data from year 2017 are generally in agreement with the previous year trend of results (related to both long-term and short-term exposure). Moreover, a trend to increase of DNA damage related to daily exposure (short-term exposure of long-term exposed subjects) has even greater significance ($p=0.004$) in comparison with the previous year ($p = 0.087$). This can be explained by a change in participants of the studies during the year and their individual exposure history differences. The exposure history of a group followed in 2017 was shorter and the effect of adaptation was not so strong.

4. CONCLUSION

The principal aim of this study was to fill the gap in human biomonitoring studies [1] and focus on the effects of exposure to NP on DNA damage in occupationally exposed subjects. We also aimed to verify the versatility of our hypotheses on human adaptation to the environment [11]. Obtained cytogenetic results demonstrated consistently (for both years): (i) possible adaptation to long-term exposure of NP (related to total frequency of MN), (ii) short-term (about 2.5-h per shift) daily exposure could be at least a reason for the aberration increase, particularly chromosomal losses (aneugenic effect), although particular type of exposure as well as exposure history play crucial role in resulting effects. As this type of human study is unique, more work is urgently needed for the understanding of the effect of both long-term and short-term exposure to NP on the level of DNA damage.

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