DNA STRAND BREAKS AND OXIDATIVE DAMAGE IN LYMPHOCYTES OF WORKERS HANDLING NANOCOMPOSITE MATERIALS

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Abstract

Many experimental studies have demonstrated the ability of nanoparticles to induce DNA damage and oxidative stress in a number of organs, probably due to the formation of reactive oxygen species. Our knowledge, however, is still limited, especially as regards the persistence of genotoxic effects after long-term exposure to nanoparticles and their health risks. We decided, therefore, to analyze DNA damage in lymphocytes of workers long-term exposed to nanoparticles due to processing of nanocomposite materials.

Exposed group involved 20 employees examined pre-shift and post-shift, samples from 20 controls were taken in the morning. Lymphocytes were isolated from heparinized blood on a ficoll gradient and frozen until further processing. An alkaline comet assay with enzymes of excision DNA repair (Fpg and Endo III) was used to detect DNA damage.

Within the exposed group, the values of total DNA damage in post-shift samples slightly exceed those in pre-shift ones, but the difference was not statistically significant (% tail DNA 4.47 ± 1.43 vs 3.95 ± 1.23, respectively). Compared to controls, however, the workers showed significantly higher levels of genotoxic damage, both in terms of DNA breakage (% tail DNA 3.08 ± 1.04 vs 1.63 ± 0.50, respectively; $p<0.0001$), and oxidized bases (% tail DNA 0.88 ± 0.41 vs. 0.57 ± 0.25, respectively; $p <0.01$). Our preliminary results thus indicate that long term occupational exposure to nanoparticles is associated with an increased risk of DNA damage.

Keywords: Genotoxicity, human, comet assay, nanoparticles, occupational exposure

1. INTRODUCTION

The current period is characterized by an extensive development of nanotechnologies and nanomaterials, and toxicologists thus face a number of questions about the possible health risks of long-term exposure to nanoparticles (NP). The harmful biological effects of NP are usually associated with induction of reactive oxygen species (ROS). Overproduction of ROS may induce oxidative stress and subsequent failure of physiological cell functions resulting in DNA damage, disturbance of cell signaling, protein and lipid alterations, cytotoxicity, apoptosis and carcinogenesis [1-2]. Such effects have been described in a number of *in vitro* and animal *in vivo* studies [3-5]. Nevertheless, it is difficult to extrapolate simply these results to judge the effects in human and so far only few studies reported the human response to NP [6-8]. Namely the workers handling nanomaterials represent a population potentially at risk of chronic effects due to day-to-day exposure at the workplace.

Alkaline version of single-cell gel electrophoresis (comet assay) with an analog of mammalian OGG1-formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III) allows the detection of DNA

strand breaks, alkali-labile sites, transient gaps arising during base excision repair, as well as a broad spectrum of oxidized purines and pyrimidines [9]. Recently, this approach has been approved as a reliable test for genotoxicity of nanomaterials [10-11].

In our study we therefore used comet assay with the aim to assess DNA damage in lymphocytes of workers long-term exposed to nanoparticles during manufacturing and processing nanocomposite materials.

2. METHODS

2.1. Study groups

The exposed group involved 20 nanocomposite-synthesizing and processing workers (13 men and 7 women), the control group consisted of 20 volunteers (13 men and 7 women) from the same town, not employed in this plant nor occupationally exposed to dust or other health risks. All participants of the study completed questionnaire on personal and occupational history, medical treatments, dietary habits, smoking habits, and alcohol intake. History of tuberculosis, myocarditis, congenital heart disease, lung cancer, and recent fever and/or common cold symptoms were the criteria for exclusion from the study.

The workers used personal protective equipment for welding (welding helmets, leather gloves and leather aprons) and smelting (gloves). No respiratory protection was used during any of the procedures.

2.2. Exposure

The workers were exposed to nanoparticles (NP) during three different operations: welding on metal surfaces, smelting of mixtures containing nanoadditives, and machining of the finished nanocomposite. Iron, manganese and silicon represented the highest proportion of elements in the workplace aerosols generated during synthesis and post-processing of nanocomposites and associated operations. Detailed information about the chemical and physical characterization of nanoparticles and aerosols such as the concentration, size distribution and elemental composition has been already reported [12].

2.3. Blood collection and processing

Workers provided blood samples before and after aerosol exposure in the workshop during September 2017. For simplicity, these examinations are referred to as pre-shift and post-shift, despite that the workers spent the remainder of their total 8-hour shift in the office. The controls were withdrawn only once at approximately the same time of day as workers. The pre-shift samples were taken to study the subacute/chronic effects resulting from previous exposures, comparison of the pre-shift and post-shift samples was performed to assess the acute effect of exposure during the shift.

Lymphocytes were isolated from the whole heparinized blood by density gradient centrifugation over Ficoll-Paque PLUS (Sigma, Germany) and washed with phosphate buffered saline (PBS). Then, the cells were diluted with freezing medium as described earlier [13] and stored at -80 °C until the further processing.

2.4. DNA damage assessment

DNA damage was analyzed using an alkaline version of the comet assay [14-15]. The cells were quickly thawed in a 37 °C water bath, and the viability of cells was estimated by trypan blue exclusion. The number of trypan blue-positive cells did not exceed 15 % per sample. Four slides were prepared per sample (for details see [13]). Two slides per sample were treated with 45 μl of formamido-pyrimidine-glycosylase (FPG) and endonuclease III (ENDO III) in a 1:1 mixture (final concentration of both enzymes 2.5 μl/ml; Sigma-Aldrich, Germany) for 1 h at 37°C. In parallel, two slides were treated with the same volume of buffer used for the dilution of enzymes (0.1M KCl, 4mM EDTA, 2.5mM HEPES, 2 % BSA, all Sigma-Aldrich, USA). Subsequently, the slides were equilibrated for 40 min in alkaline buffer (0.3M NaOH, 1mM EDTA, pH 13) to allow the DNA to

unwind. Electrophoresis was performed in fresh alkaline buffer (30 min, 1.2 V/cm, 300 mA). Finally, the slides were neutralized in 0.4M Tris (pH 7.5), stained with 0.005 % ethidium bromide (Sigma-Aldrich, Germany) for 7 min, washed with distilled water (7 min), fixed in methanol (15 min), dried at room temperature and stored.

Before analysis, the slides were rehydrated in distilled water, and images were captured with a CCD-13008 camera (VDS, Vosskuhler, Germany) attached to a BX51 fluorescence microscope (Olympus, Japan). The extent of DNA migration was quantified using Lucia Comet Assay 7.00 software (Laboratory Imaging, Prague, Czech Republic), and the results were expressed as the percentage of DNA in the tail (Tail DNA %). Both total DNA damage (with enzymes) and DNA strand breaks (DNA-SB; without enzymes) were measured in 100 randomly selected cells per sample. Finally, each sample was characterized by two medians from the measured data, i.e., by medians calculated from (a) values of total DNA damage and (b) values of DNA-SB, and the level of oxidative DNA damage was assessed as the difference between these medians.

2.5. Statistical analysis

An unpaired two-tailed Student's t-test was used to analyze the significance of differences between the groups.

3. RESULTS AND DISCUSSION

The basic information about the groups is summarized in **Table 1**. We found no significant differences in age, gender, body mass index, and alcohol consumption between the analyzed groups (all $p > 0.05$). On the other hand, 20 % of exposed subjects used vitamins, while no one in the control group. This probably reflects the outcomes of raising awareness of workers on potential health risks of nanomaterials.

Table 1 Characterization of the exposed and control subjects. SD - standard deviation; BMI - body mass index.

	Exposed Mean ± SD (min - max)	**Controls** Mean ± SD (min - max)
Age (years)	38.6 ± 10.7 (23 - 64)	39.8 ±7.1 (27 - 55)
BMI (kg/m^2)	24.6 ± 4.4 (17.5 - 34.3)	26.2 ± 4.4 (20.1 - 36.6)
Smoking	1 + 2 ex	3
Vitamin intake (C, E)	2 + 2 occasionally	0

On average, the workers were exposed to aerosol for 7 years, with the usual length of stay in the workshop around 2 hours a day (**Table 2**). As concerns the total length of exposure, there were big differences between individual workers. Nevertheless, the workers No 9 and 10 with the longest working age (18 and 21 years, respectively) exhibited the highest levels of DNA damage within the exposed group (% Tail DNA 6.26 and 5.87, respectively), although their average daily exposure was relatively short - 1 and 1.5 hours, respectively.

Table 2 The length of exposure of workers handling nanomaterials. SD - standard deviation.

Length of exposure	**Mean ± SD (min - max)**
Total (years)	7.3 ± 4.8 (2.5 - 21)
Common working day (hours/day)	2.1 ± 1.4 (0.5 - 6)
Current - i.e. on the monitoring day (hours)	3.4 ± 0.9 (2.5 - 6)

The average values of total DNA damage in the exposed group appeared to be slightly higher in post-shift samples than those in samples obtained prior to beginning of the shift, however this difference was not statistically significant (**Figure 1**). In contrast, pre-shift as well as post-shift values of DNA damage in lymphocytes of exposed workers considerably exceeded the levels detected in control subjects.

Figure 1 documents that both DNA-SB and oxidized lesions were significantly increased in exposed group compared to controls. The previous study from the same workplace demonstrated in the exhaled breath condensate (EBC) of exposed subjects an increase of markers of nucleic acid oxidation over the control values already in the pre-shift samples, and no further significant elevation was observed in post-shift EBC samples [12]. This fully corresponds with our present finding.

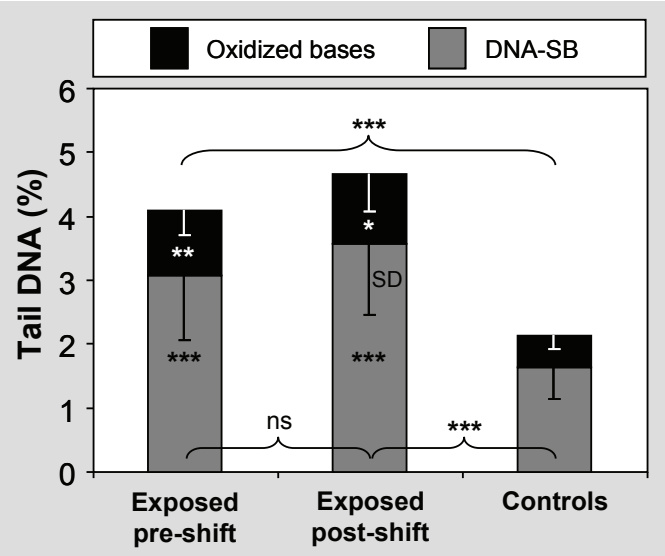

Figure 1 DNA damage in lymphocytes of workers long-term exposed to nanoparticles and control subjects. DNA-SB - DNA strand breaks, SD - standard deviation, *p < 0.05, **p < 0.01, ***p < 0.001.

Depression of antioxidant enzymes (superoxide dismutase and glutathione peroxidase) and increased expression of cardiovascular markers (fibrinogen and intercellular adhesion molecule) were found among workers handling nanomaterials [16]. The same author subsequently suggested that exposure to metal oxide NP may lead to global methylation, DNA oxidative damage, and lipid peroxidation [17]. Chronic upper airway inflammation and systemic oxidative stress were documented in photocopier operators chronically exposed to nanoparticles [18]. A cross-sectional study performed in a nano-TiO_2 manufacturing plant in China described an association between the occupational exposure to nano-TiO_2 and the markers of lung damage, cardiovascular disease, oxidative stress and inflammation [19].

Our results thus support the recent data on harmful biological effects associated with long-term occupational exposure to nanoparticles and illustrate the need for further studies aimed at a detailed analysis of the mechanism of genotoxic effects and their health consequences.

4. CONCLUSION

Long-term inhalation exposure of workers handling nanocomposite material led to considerable increase of DNA damage. The levels of both DNA-SB and oxidized lesions in peripheral blood lymphocytes of exposed subjects significantly exceeded those detected in control persons.

Comet assay provided a useful tool for monitoring potential harmful effects of nanoparticles on DNA.

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