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The genotoxic effects in the leukocytes of workers handling nanocomposite materials

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Abstract

The extensive development of nanotechnologies and nanomaterials poses a number of questions to toxicologists about the potential health risks of exposure to nanoparticles (NP). In this study, we analysed DNA damage in the leukocytes of 20 workers who were long-term exposed (18 ± 10 years) to NP in their working environment. Blood samples were collected in September 2016, before and after a shift, to assess (i) the chronic effects of NP on DNA (pre-shift samples) and (ii) the acute effects of exposure during the shift (the difference between pre- and post-shift samples). The samples from matched controls were taken in parallel with workers before the shift. Leukocytes were isolated from heparinised blood on a Ficoll gradient. The enzyme-modified comet assay (DNA formamido-pyrimidine-glycosylase and endonuclease III) demonstrated a considerable increase of both single- and double-strand breaks in DNA (DNA-SB) and oxidised bases when compared with the controls (2.4× and 2×, respectively). Acute exposure induced a further increase of DNA-SB. The welding and smelting of nanocomposites represented a higher genotoxic risk than milling and grinding of nanocomposite surfaces. Obesity appeared to be a factor contributing to an increased risk of oxidative damage to DNA. The data also indicated a higher susceptibility of males vs. females to NP exposure. The study was repeated in September 2017. The results exhibited similar trend, but the levels of DNA damage in the exposed subjects were lower compared to previous year. This was probably associated with lower exposure to NP in consequence of changes in nanomaterial composition and working operations. The further study involving also monitoring of personal exposures to NP is necessary to identify (i) the main aerosol components responsible for genotoxic effects in workers handling nanocomposites and (ii) the primary cause of gender differences in response to NP action.

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Introduction

Like any new technology, nanotechnology represents a major benefit that, however, may be associated with serious environmental and health risks (1). Specific properties of nanoparticles (NP) (e.g. enhanced reactive surface area, the ability to cross cell and tissue barriers, resistance to biodegradation) may enhance their cytotoxic potential compared to the parent bulk material (2). It is generally accepted that the toxicity of a nanomaterial is determined namely by its physico-chemical parameters such as particle size, shape, surface charge and chemistry, composition and stability (3). Harmful biological effects of NP are usually associated with overproduction of reactive oxygen species (ROS) and oxidative stress. This may subsequently lead to oxidative damage of biological macromolecules, cellular dysfunction and cell death (4–6). The primary genotoxic effects of NP may be induced by direct interaction of NP with genetic material or, indirectly, as a consequence of NP interaction with proteins involved in replication, transcription and repair processes, NP-induced disturbance of cell cycle check point functions, ROS arising from NP surface, or toxic ions released from soluble NPs. Secondary genotoxicity is attributed to ROS production triggered by NP in inflammatory cells (4,7,8). Recently, capability of NP to induce epigenetic changes in mammalian cells (i.e. changes in DNA methylation, histone post-translational modifications and noncoding RNAs) has also been described (9,10).

Despite the growing accumulation of experimental data on adverse biological effects of NP, our knowledge about the risks of NP for human population are still insufficient due to enormous heterogeneity of NP in use concerning their size, shape, chemical composition, surface functionalisation, potential route of exposure etc (11). In the first papers on this topic, decreased antioxidant enzyme activity (superoxide dismutase and glutathion peroxidase), increased expression of cardiovascular markers (fibrinogen and intercellular adhesion molecule), increased levels of oxidative damage to DNA and changes in global DNA methylation were reported in workers occupationally exposed to selected metal oxide NP (12–14). In another study, photocopier operators chronically exposed to printer emitted NP exhibited chronic upper airway inflammation and systemic oxidative stress (15). A cross-sectional study in a nano-TiO_2 manufacturing plant described an association between the occupational exposure to TiO_2 and the markers of lung damage, cardiovascular disease, oxidative stress and inflammation (16). The workers occupationally exposed to TiO_2 and iron oxide NP exhibited elevated markers of oxidative damage to DNA, proteins and lipids in exhaled breath condensate (EBC) (17–19) as well as the signs of inflammation (20).

Recently, Pelclova with co-workers focussed on the impact of long-term exposure to NP on research workers manufacturing nanocomposite materials. Their first complex study provided detailed characterisation of the workplace processes and aerosol measurements including assessment of particle size distribution and elemental composition. In addition, the markers of oxidative stress in the EBC were analysed. The results showed significant association between working in nanocomposite synthesis and EBC biomarkers (21). A related study revealed deep airway inflammation and respiratory disorders in the group of exposed workers compared to controls (22). Subsequently, the cytogenetic analysis was performed using micronucleus test. Concerning the total frequency of micronuclei (including both chromosomal breaks and losses), this approach demonstrated no effect of chronic exposure to NP; however, some changes in DNA damage pattern in favour of chromosomal breaks were observed (23). In parallel, the material from the exposed workers was subjected to analysis of genotoxic effects using the single cell gel electrophoresis (the comet assay) and the present study summarises the obtained results.

Alkaline version of comet assay supplemented with enzymes of excision repair allows to detect the single- and double-strand breaks in DNA (DNA-SB), transient gaps arising as intermediates during base excision repair, alkali-labile sites, apoptotic DNA fragmentation and a broad spectrum of oxidised purines and pyrimidines (24–29). The method was also recommended for the testing of nanomaterial genotoxicity (30,31). In our laboratory, we have successfully applied the comet assay for analyses of ambient air pollution impact on genetic material of exposed individuals (32,33) as well as in testing the genotoxic properties of different types of manufactured metal NP under *in vitro* and *in vivo* conditions (34–36).

Materials and methods

Exposure

The workers were exposed to NP during three different operations: welding of metal surfaces, smelting of mixtures containing nanoadditives and machining of the finished nanocomposites. In 2016, the work with nanocomposites took place in two workshops—welding and smelting were performed in workshop 1, while machining of surfaces including milling and grinding in workshop 2. In 2017, the work was concentrated in workshop 3 and included machining of surfaces containing epoxide resins with nano-SiO_2 filler, and geopolymers with metakaolin, ash or basalt. Detailed description of the working procedures has already been described (21).

The chemical composition of aerosol in the workplace was determined using a Berner low-pressure impactor—BLPI 25/0,018/2 (HAUKE GmbH., Austria), separating aerosol particles into 10 size fractions in the size range of 25 nm–13.6 µm. The deposits on impactor foils were analysed using gravimetry (M5P balance, Sartorius GmbH., Austria), ion chromatography (IC) in a Dionex 5000 (Dionex Co, USA) and scanning electron microscope (SEM) (Tescan Indusem, Czech Republic) that was equipped by energy-dispersive X-ray spectroscopy (EDS) (XFlash detector 5010, Bruker, Germany) to analyse the elemental content in size-segregated aerosol deposits. The relative mass shares of analysed elements resulting from SEM/EDS were converted to concentrations in $\mu g/m^3$ using concentrations of sulphates from IC and the relative share of sulphur in a sample from EDS, based on the presumption that all sulphur is soluble and present as sulphates.

Particle size distribution during each operation (welding, smelting, grinding and milling) and before the operation (background) was measured by two online aerosol spectrometers—a scanning mobility particle sizer (SMPS 3936L, TSI Inc., USA) and aerodynamic particle sizer (APS 3321, TSI Inc.). These two aerosol spectrometers are capable of measuring the number concentration and its size distribution in the total size range from 6 nm to 20 µm.

All these instruments were located approximately 1.5 m from the particle emission source. Before each working operation, 15 min of measurements was taken as background values.

Study groups

The samples were collected in September 2016 and 2017. In 2016, the exposed group consisted of 20 nanocomposite-synthesising and processing workers [15 males (m) and 5 females (f)]. Eleven workers (9 m/2 f) were employed in workshop 1, the remaining ones in workshop 2 (6 m/3 f). The control group included 21 volunteers (15 m/6

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f) from the same locality, not exposed to dust or other health risks. In 2017, the exposed as well as the control group involved 20 subjects (13 m/7 f). Of the study subjects investigated in 2016, 14 (9 m/5 f) exposed workers and 11 controls (6 m/5 f) were examined again in 2017.

All participants of the study completed a 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. All participants signed an informed consent form and had the opportunity to withdraw from participation at any time during the study, according to the Helsinki II declaration. The ethical committee of the General University Hospital in Prague and First Medical Faculty, Charles University approved the study.

Blood collection and processing

The workers provided blood samples before (pre) and after (post) shift. Although the shifts lasted 8 h, the workers performed operations associated with exposure to NP only part of this time and spent the rest of the shift in the office. The control samples were drawn only once, at the time of pre-shift samples collection. The pre-shift samples were collected to study the long-term (chronic) effects resulting from previous exposures during the whole working history, a comparison of the pre-shift and post-shift samples served to assess the acute effect of exposure during the shift.

Leukocytes were isolated from the whole heparinised blood by density gradient centrifugation over Ficoll-Paque PLUS (Sigma–Aldrich, Germany) and washed with phosphate buffered saline. Then, the cells were diluted with freezing medium as described earlier (32) and stored at −80°C until the further processing.

DNA damage assessment

DNA damage was analysed using an alkaline version of the enzyme-modified comet assay (24,26,32,37). 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%. Two slides per sample were prepared—each with two gels. The slides were submerged for 1 h in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 0.16 M dimethyl sulfoxide, 0.016 mM Triton X-100; all Sigma–Aldrich) at pH 10 and subsequently washed with phosphate buffered saline (3× for 5 min). After that, one slide (i.e. two gels) per sample was treated with a 1:1 mixture of formamido-pyrimidine-glycosylase (FPG) and endonuclease III (ENDO III). Each gel was exposed to 45 µl of enzyme mixture (final concentration of both enzymes 2.5 µg/ml; Sigma–Aldrich) for 30 min at 37°C. In parallel, two remaining gels per sample were treated with the same volume of buffer used for the dilution of enzymes (0.1 M KCl, 4 mM EDTA, 2.5 mM HEPES, 2% bovine serum albumin; all Sigma–Aldrich). Subsequently, the slides were equilibrated for 40 min in alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH 13) to allow the DNA to unwind. Electrophoresis was performed in fresh alkaline buffer (30 min, 1 V/cm, 300 mA) at 4°C. Finally, the slides were neutralised in 0.4 M Tris (pH 7.5), stained with 0.005% ethidium bromide (Sigma–Aldrich) for 7 min, washed with distilled water (7 min), fixed in methanol (15 min), dried at room temperature and stored at 4°C.

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. Each sample was characterised by two parameters: (i) median value of total DNA damage and (ii) median value of DNA-SB. The level of DNA oxidation was assessed as the difference between these medians.

To verify reproducibility of the comet assay results obtained from different electrophoresis runs, the levels of DNA-SB in a reference standard (the sample of reference leukocytes tested in each electrophoresis run) were measured in parallel with experimental samples.

Statistical analysis

Basic descriptive statistics [mean, standard deviation and range (minimum – maximum)] were calculated using Microsoft Excel 2013. An advanced statistical analysis was performed using the IBM SPSS Software version 22.0 (Chicago, IL). The Kolmogorov–Smirnov test was used to test normality of the data distribution. For the independent groups (exposed vs. controls; 2016 vs. 2017), *t*-test (normally distributed variables) or the nonparametric Mann–Whitney *U* test (non-normally distributed variables) were used for the comparison of the studied parameters (total DNA damage, DNA-SB, oxidised bases). For the paired values (pre-shift vs. post-shift within the given year; pre-shift 2016 vs. pre-shift 2017 in repeatedly analysed subjects), paired sample *t*-test or the Wilcoxon signed-rank test, depending on the data distribution, was used. Spearman correlation analysis was used to test the relationship between body mass index (BMI) and oxidative damage to DNA. We further applied bivariate and multivariate logistic regression analysis to assess the impact of the studied parameters (age, gender, BMI and exposure) on the levels of the total DNA damage, DNA-SB and oxidised bases. Logistic regression was performed separately: (i) for controls and pre-shift data to evaluate the effect of long-term (chronic) exposure and (ii) for controls and post-shift data to evaluate the effect of short-term (acute) exposure on the background of chronic exposure. For logistic regression, all dependent variables were transformed into a two-level scale using medians. These results were expressed as odd ratios with a 95% confidence interval and significance level (*P* value).

Results

Workplace aerosol

Table 1 summarises the results of particulate matter (PM) fraction measurements, while Table 2 presents the data on elemental composition of nanosized fractions. In 2016, smelting (workshop 1) generated the highest relative amount of nanosized particles in the working environment—about 95% of the total PM amount, while welding (workshop 1) or machining (workshop 2) only 40 or 60%, respectively. On the other hand, the NP number concentrations detected during machining were about 7× higher than those detected during welding and smelting. But as can be seen in Table 2, welding generated 20 µg of NP/m^3, while the values detected during smelting and machining were 10× and 100× lower, respectively.

In 2017, nanosized fraction arising during machining accounted for 96% of the total PM in the working environment, while this was only 60% in the previous year. However, the absolute mass

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Table 1. Proportions of PM fractions measured by online monitoring (SMPS and APS) during the shift related to the individual working processes

Year	Processes	Proportion of PM fractions (%)			Total number concentration (#/cm³)
		<100 nm	100 nm–1 µm	1–10 µm	<100 nm
2016	Welding *workshop 1*	40.13	59.85	0.02	4.88×10^4
	Smelting *workshop 1*	94.64	5.35	0.01	4.60×10^4
	Background *workshop 1*	96.76	3.23	0.00	2.01×10^4
	Machining *workshop 2*	61.23	38.76	0.01	3.22×10^5
	Background *workshop 2*	40.89	59.10	0.01	1.16×10^5
2017	Machining *workshop 3*	95.74	4.20	0.05	8.99×10^4
	Background *workshop 3*	53.30	46.69	0.01	4.68×10^4

The background was measured 15 min before the start of individual working process in the workshop.

Table 2. Elemental composition of nanosized fraction (25–100 nm) for individual working processes

Year	Process	Absolute amount of NP (µg/m³) [relative amount of NP (%)]								
		Fe	Si	S	Cl	Mn	Na	Al	K	Total
2016	Welding *workshop 1*	16.73 [82.29]	1.58 [7.77]	0.08 [0.39]	0.03 [0.15]	1.85 [9.10]	0.06 [0.30]	N/A	N/A	20.33 [100.00]
	Smelting *workshop 1*	1.693 [79.52]	0.139 [6.53]	0.019 [0.89]	0.028 [1.32]	0.175 [8.22]	0.042 [1.97]	0.010 [0.47]	0.023 [1.08]	2.129 [100.00]
	Machining *workshop 2*	0.093 [48.95]	0.036 [18.95]	0.017 [8.95]	0.030 [15.79]	N/A	N/A	0.014 [7.37]	N/A	0.190 [100.00]
2017	Machining *workshop 3*	N/A	0.024 [41.01]	0.009 [15.88]	0.003 [5.41]	N/A	0.010 [16.75]	0.012 [20.94]	N/A	0.057 [100.00]

N/A, not applicable.

concentration of NP generated during this operation was 3× lower compared to 2016 (0.19 vs. 0.06 µg/m³). Likewise, the NP number concentration in aerosol decreased 3.6× in 2017 vs. 2016. Analysis of the elemental composition of NP in aerosol also revealed significant differences between the years. In 2016, iron dominated in workplace aerosols, irrespective of the type of working process. Especially during welding and smelting, the iron content in the nanosized fraction of aerosol reached up to 80% of the total amount of detected elements. In contrast, even iron traces were not detected in the aerosol produced during machining in 2017. On the other hand, the amount of silicon more than doubled compared to the previous year (19 vs. 41%).

Subjects—general and exposure characteristics

Exposed workers and control subjects showed no significant differences in general characteristics, such as gender, age, BMI, smoking status or alcohol consumption either within the given year or between the years (Table 3). In 2016, however, the exposed males tended to have higher values of BMI (29 ± 6 kg/m²) compared to those from the control group (26 ± 5 kg/m²). Majority of the obese males were in the subgroup (six subjects, BMI = 32 ± 7 kg/m²) that did not participate in the repeated analysis in 2017. New males who completed the exposed group in 2017 had BMI in the normal range (23 ± 2 kg/m²), similarly to both exposed (24 ± 5 and 23 ± 4 kg/m² in 2016 and 2017, respectively) and control (23 ± 4 kg/m² in both years) females.

Exposure characteristics are summarised in Table 4. Compared to 2016, the total length of the occupational exposure was shorter by about 5.5 years in the group analysed in 2017 as the newly enrolled individuals had substantially shorter working history in the field than those participating in 2016 only (on average 6 vs. 23 years, respectively). In contrast, the average daily exposure was slightly higher in 2017 vs. 2016 (by about half an hour) and on the monitoring day this difference reached almost 40 min. In terms of the total or average daily exposure, the workers engaged in workshop 2 tended to be more exposed than those from workshop 1. Nevertheless, the statistical analysis showed no significant differences between these subgroups (Table 5).

DNA damage

The results of comet assay are shown in Table 6. The data from 2016 demonstrated strong effect of long-term exposure to NP (pre-shift samples) on the levels of DNA damage as the values of both DNA-SB and oxidised bases in the exposed group considerably exceeded those detected in the controls (2.4× and 2×, respectively). Acute exposure (post-shift samples) resulted in further increase of total DNA damage. Comparison of pre- and post-shift samples revealed that mainly DNA-SB were responsible for this effect.

The samples collected in 2017 exhibited similar trend (Table 6), although the levels of the total DNA damage were lower compared to the previous year in both exposed and control subjects ($P \leq 0.001$ and $P \leq 0.01$, respectively). Because even the controls investigated in 2016 and 2017 were not entirely identical, the comparison was repeated only with the subgroups of subjects analysed in both consecutive years (Figure 1). Within the exposed group, the results confirmed a significant decrease of DNA-SB and oxidised bases in 2017 compared to the previous year. In contrast, repeatedly analysed control subjects showed no differences between the years.

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Table 3. General characteristics of the study subjects

Characteristics	2016			2017			2016 vs. 2017	
	Exposed	Controls	P^a	Exposed	Controls	P^a	P^b	P^c
N (male/female)	20 (15/5)	21 (15/6)	0.796	20 (13/7)	20 (13/7)	1.000	0.588	0.728
Age (years) Mean ± SD (range)	41.8 ± 11.4 (29–63)	38.7 ± 9.1 (20–55)	0.334	38.6 ± 11.01 (23–64)	39.9 ± 7.3 (27–55)	0.472	0.371	0.348
BMI (kg/m^2) Mean ± SD (range)	28.0 ± 6.2 (18–42)	25.0 ± 4.7 (18–37)	0.100	24.6 ± 4.6 (18–34)	26.1 ± 4.6 (20–37)	0.294	0.060	0.409
Smoking	1	4	0.169	3	3	1.000	0.579	0.828
Alcohol (occasionally)	18	16	0.240	16	18	0.661	0.582	0.437

N, number of subjects; SD, standard deviation.
[a]Exposed vs. controls within a given year.
[b]Exposed in 2016 vs. exposed in 2017.
[c]Controls in 2016 vs. controls in 2017.

Table 4. Exposure characteristics of the study subjects: comparison of two consecutive years

Exposure time	2016	2017
	Mean ± SD (range)	Mean ± SD (range)
Total (years)	17.8 ± 10.0 (5–40)	12.2 ± 9.3 (2–31)
Common daily (min)	101.4 ± 60.0 (30–240)	128.3 ± 87.3 (30–360)
On the monitoring day (min)	156.3 ± 61.9 (60–330)	203.5 ± 54.7 (150–360)

SD, standard deviation.

No differences in the level of genotoxic effects were observed between the subjects exposed in workshop 1 and 2 (Table 7). In both workshops, the long-term exposure to NP increased DNA-SB and oxidised DNA bases above the control levels and the acute exposure (post-shift samples) further enhanced the total DNA damage. However, the increase of DNA-SB above the pre-shift levels was significant only in samples from workshop 1 and the DNA oxidation remained at the pre-shift values regardless the workshop.

Table 8 presents the gender differences in sensitivity to genotoxic effects of NP. In 2016, long-term exposure to NP (pre-shift samples) induced significant increase of DNA-SB and oxidised DNA bases in both males and females. However, only the males exhibited an additional increase of the total DNA damage after the acute exposure to NP (post-shift samples) due to the significant increase of DNA-SB above the pre-shift values. The levels of oxidised DNA bases remained unchanged. In 2017, the results of comet assay in males basically copied the trend observed in 2016, despite the fact that the detected levels of DNA damage were evidently lower. On the other hand, the female samples from 2017 showed—in contrast to the previous year—no significant difference between the pre-shift (or post-shift) and control level of DNA oxidation. The gender differences in response to NP exposure with a view to particular workshop were also analysed. Nevertheless, the results were difficult to interpret due to small number of subjects in analysed subgroups and lack of knowledge on real personal exposure to NP (personal NP samplers were not available at this stage of study). Therefore, they were not included into the manuscript and will be subjected to further detailed investigation.

The results of bivariate (A) and multivariate (B) regression of the studied parameters and their impact on the level of DNA damage in relation to long-term and acute exposure to NP are summarised in Table 9. The data on the total DNA damage and DNA-SB obtained in 2016 did not allow this analysis as even their lowest values found in the exposed subjects still exceeded the highest values in the control group (i.e. perfect separation). Therefore, only the results for 2017 are presented. This analysis revealed the long-term (chronic) and the acute exposure to NP as the only factor affecting the levels of total DNA damage or DNA-SB alone. Regarding the oxidative damage induction, the multivariate regression analysis confirmed that the gender could play an important role during both long-term and acute exposure to NP. In the case of long-term exposure, also BMI seemed to influence the levels of FPG- and ENDO III-sensitive DNA sites. Although in 2016 no significant differences between the average BMI values in the exposed and control group were noted, the exposed group involved relatively high number of overweight males compared to the controls. Accordingly, only the exposed males examined in 2016 showed the positive correlation between the BMI and the levels of oxidatively damaged DNA (Figure 2). No similar relationship was detected as regards the data from 2017, when the majority of obese males did not participate in the study.

Discussion

The workplace selected for this study has long been focussed on the experimental development, production and processing of nanocomposite materials. This provides a unique opportunity to analyse the biological and health effects of chronic exposure to NP in the working environment. In 2016, the workers involved into the study were exposed on average for almost 18 years and the levels of both DNA-SB and oxidised bases in their leukocytes from pre-shift samples considerably exceeded those detected in the controls. The results of comet assay thus clearly demonstrated strong genotoxic potential of NP present in the workplace aerosol. This corresponded with an elevation of markers of nucleic acid and protein oxidation found in the pre-shift samples of exhaled breath condensates (21). In contrast, the total number of micronuclei did not differ between the exposed and control group. However, the sorting of micronuclei into those with and without centromere revealed in pre-shift samples a considerable increase of chromosomal breaks at the expense of centromeric micronuclei (23). Acute exposure to NP during the shift led in workers to an additional increase of DNA breaks (but

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Table 5. Exposure characteristics of the study subjects: comparison of workshops 1 and 2 in the year 2016

Exposure time	Workshop 1		Workshop 2		P^a
	N	Mean ± SD (range)	*N*	Mean ± SD (range)	
Total (years)		16.2 ± 7.7 (5–30)		19.8 ± 12.5 (8–40)	0.757
Common daily (min)	11	84.5 ± 42.0 (30–150)	9	121.7 ± 73.7 (30–240)	0.290
On the monitoring day (min)		148.2 ± 51.7 (60–240)		166.1 ± 74.6 (75–330)	0.757

N, number of subjects; SD, standard deviation; workshop 1, welding and smelting; workshop 2, machining of surfaces including milling and grinding.
[a]Workshop 1 vs. workshop 2.

Table 6. DNA damage (expressed as a percentage of Tail DNA) in peripheral leukocytes of controls and workers occupationally exposed to nanoparticles

Year	Group	*N*/(*N*)	Total DNA damage (mean ± SD)	P^a	P^b	P^c	DNA-SB (mean ± SD)	P^a	P^b	P^c	Oxidized bases (mean ± SD)	P^a	P^b	P^c
2016	Exposed pre-shift	20	6.46 ± 0.99	***	***	***	5.05 ± 0.92	***	***	***	1.42 ± 0.72	0.519	**	**
	Exposed post-shift	20	7.46 ± 0.90		***	***	5.96 ± 0.82		***	***	1.50 ± 0.90		**	*
	Controls	21	2.78 ± 0.72			**	2.07 ± 0.54			*	0.71 ± 0.42			0.180
	Internal standard	(5)					1.19 ± 0.22			0.145				
2017	Exposed pre-shift	20	3.95 ± 1.23	***	***		3.08 ± 1.04	***	***		0.88 ± 0.41	0.675	*	
	Exposed post-shift	20	4.47 ± 1.43		***		3.56 ± 1.13		***		0.92 ± 0.61		0.082	
	Controls	20	2.19 ± 0.57				1.63 ± 0.50				0.57 ± 0.25			
	Internal standard	(5)					0.97 ± 0.15							

N, number of subjects; (*N*), number of electrophoresis runs; SD, standard deviation.
[a]Pre-shift vs. post-shift values of DNA damage in the exposed subjects.
[b]DNA damage in the exposed vs. control group.
[c]2016 vs. 2017.
*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

not oxidised bases) above the pre-shift values. This was associated with post-shift increase of lipid oxidation markers in EBC (21). Experiments on mice have indicated that oxidised DNA bases in peripheral lymphocytes exposed to compounds inducing oxidative stress may be masked by rapid removal of altered bases from DNA (38,39). Additional increase of DNA-SB detected by comet assay in post-shift vs. pre-shift samples of workers handling nanomaterials could thus reflect an increased number of transient repair sites in the DNA as well as single- and double-SB resulting from unrepaired or misrepaired DNA lesions. This seemed to be mainly associated with welding and smelting of nanocomposites because the comparison of both workshops revealed the significant effect of acute exposure on the levels of DNA-SB only in subjects from workshop 1. Similar results were obtained with micronucleus test where again only the workers from workshop 1, but not those from workshop 2, exhibited a significant increase of chromosomal breaks in post-shift samples compared to controls (23).

Analysis of aerosol in the working environment revealed great differences between the workshops, both in quantity and chemical composition of the nanosized fractions. In 2016, welding and smelting (workshop 1) generated about 100× and 10× higher mass concentration of NP in working environment than machining (workshop 2). An iron dominated in aerosol NP regardless of the type of working process. In workshop 1, however, this metal accounted for about 80% from the total amount of nanosized fraction, while in the

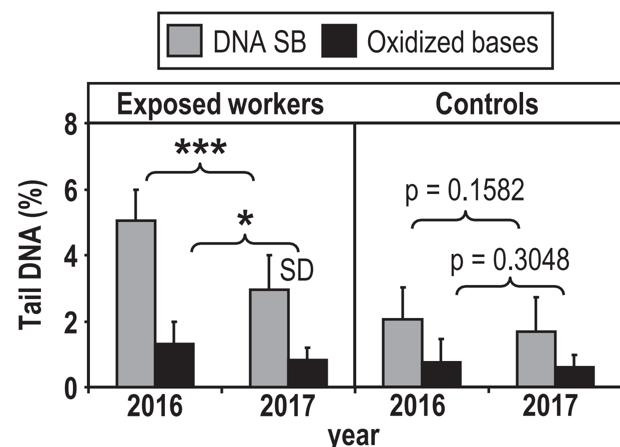

Figure 1. DNA damage in the leukocytes of controls and workers long-term exposed to nanoparticles: comparison of pre-shift values obtained from subjects examined repeatedly in the years 2016 and 2017. Number of exposed workers—14; number of controls—11; *$P \leq 0.05$; ***$P \leq 0.001$.

workshop 2, this metal accounted for <50%. Photocopier operators chronically exposed to printer emitted NP also exhibited significant DNA damage in lymphocytes and increased number of buccal cells with micronuclei (40) as well as chronic upper airway inflammation

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Table 7. DNA damage (expressed as a percentage of Tail DNA) in peripheral leukocytes of workers occupationally exposed to nanoparticles: comparison of workshops 1 and 2 in the year 2016

Group	Total damage (mean ± SD)	P^a	P^b	P^c	DNA-SB (mean ± SD)	P^a	P^b	P^c	Oxidized bases (mean ± SD)	P^a	P^b	P^c
Exposed pre-shift												
Workshop 1	6.26 ± 1.04	0.406	**	***	5.03 ± 0.92	0.986	***	***	1.22 ± 0.74	0.209	0.966	*
Workshop 2	6.72 ± 0.93		*	***	5.07 ± 0.98		0.099	***	1.65 ± 0.67		0.261	***
Exposed post-shift												
Workshop 1	7.35 ± 0.84	0.448		***	6.17 ± 0.93	0.259		***	1.18 ± 0.77	0.066		0.056
Workshop 2	7.60 ± 1.01			***	5.71 ± 0.63			***	1.89 ± 0.95			***
Controls	2.78 ± 0.72				2.07 ± 0.54				0.71 ± 0.42			

N, number of subjects; SD, standard deviation; workshop 1, welding and smelting; workshop 2, machining of surfaces including milling and grinding.
[a]Workshop 1 vs. workshop 2.
[b]Pre-shift vs. post-shift values of DNA damage.
[c]DNA damage in the exposed vs. control groups.
*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

and systemic oxidative stress (15). A recent study analysed relative contribution of various fractions in toner-based laser printer and photocopier emissions (LPE) in relation to the amount of generated ROS. The results showed that the transition metals/metal oxides, albeit a minor constituent of the LPE $PM_{0.1}$ emissions, are the species responsible for the majority of induced short-lived ROS and H_2O_2 (41). Experiments on cell cultures have also demonstrated strong genotoxic potential of manufactured iron oxide NP as well as their capability to increase lipid peroxidation and decrease activity of enzymes functioning as ROS scavengers (34,42). Rats intravenously exposed to iron oxide NP exhibited 2 weeks post-treatment signs of inflammation in the lungs, liver and kidney (6). These results suggest that the high mass concentration of iron NP in aerosol in workshop 1 is responsible for greater genotoxic effects among the workers from this workshop. The absence of iron in the nanosized fraction collected in 2017 associated with the decrease of DNA damage in the exposed subjects seem to support this assumption. However, whether this is a role of iron NP alone and/or other components of aerosol remains to be identified.

Also, silicon was relatively abundantly generated, especially during grinding and milling of nanocomposites. In 2017, silicon even represented the predominant component in the nanosized fraction of aerosol. At present, silica NP are widely used in biomedical applications and consumer products, but the data on their possible genotoxic risks remain controversial (43). A comprehensive testing in both *in vitro* and *in vivo* systems under OECD guidelines detected no genotoxicity of silica NP (44). On the other hand, *in vivo* comet/micronucleus combination assay revealed a small but reproducible increase of DNA damage and micronucleated reticulocytes when silica NP were tested at their maximum tolerated dose (45). Concurrently, an increased neutrophilic infiltration, necrosis and apoptotic cells in the liver as well as an induction of inflammatory markers (tumour necrosis factor-α, interleukin-6) in plasma were observed. The authors suggested that 'silica NP initiate secondary genotoxic effects through release of inflammatory cell-derived oxidants' (45). Hence, we cannot exclude that the presence of silicon in nanosized fraction of aerosol could also contribute to DNA damage in workers long-term handling nanocomposites.

Multivariate regression analysis suggested that, in addition to NP exposure alone, gender and BMI could influence genotoxic effects in nanocomposite workers. The response to the chronic exposure manifested as an increase of DNA damage over the control values was basically the same in both sexes. However, only the male workers exhibited an additional rise of DNA-SB after the acute exposure to NP, although their time spent in the workplace on the monitoring day was comparable to females (152 and 168 min, respectively). On the other hand, in 2016, only 2 from 5 females, but 9 from 15 males in the exposed group worked in the workshop 1 which seemed to be associated with a higher genotoxic risk.

Numerous papers have reported gender-related differences in the basal DNA damage assessed by comet assay in leukocytes of healthy population (e.g. 46–47), but according to recent comprehensive review of literature, the influence of sex on DNA damage in the comet assay may be mediated by lifestyle factors or external exposures rather than direct effects of sex (48). Nevertheless, Fortoul *et al.* (49) used comet assay to analyse the genotoxic effects in subjects residing in a highly polluted area and sharing comparable activity pattern and they found a higher DNA damage in nasal cells and leukocytes of males compared to females and control individuals. A question remains whether the higher 'sensitivity' of males vs. females to acute exposure observed in our study reflects the differences in working activities (and, therefore, the different exposures), real gender differences mediated by sexual hormone intervention or simply an error of small numbers.

Concerning BMI, only the exposed males showed on average the values at the upper border of overweight (BMI = 29 kg/m^2), while the control males had this value only slightly elevated above the norm (BMI = 26 kg/m^2) and the females from both exposed and control groups were within the norm (BMI = 24 and 23 kg/m^2, respectively). A great number of studies have already documented that obesity is characterised, among other, by lowered antioxidant defence, permanently increased oxidative stress and chronic low-grade inflammation (50–53), and these changes seem to be more pronounced in males (54). In our study, we demonstrated a close association between the BMI and oxidative damage to DNA in a group of exposed males with a high proportion of overweight and obese individuals. This supports an assumption that the differences in the lifestyle and/or dietary habits could be at least partially responsible for the observed gender differences in the response to NP. In any case, high value of BMI appeared to be an important factor contributing to DNA oxidation in workers handling nanocomposites.

In 2017, some workers examined in the previous year were replaced by new ones with shorter working history in the field, so the average total exposure time was only 12 years. On the other hand, the common daily exposure and the current exposure on the monitoring day were even slightly longer compared to 2016. The comet

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Table 8. DNA damage (expressed as a percentage of Tail DNA) in peripheral leukocytes of controls and workers occupationally exposed to nanoparticles: comparison of male and female subjects

Year/ gender	Group	*N*	Total DNA damage (mean ± SD)	*P*[a]	*P*[b]	DNA-SB (mean ± SD)	*P*[a]	*P*[b]	Oxidized bases, (mean ± SD)	*P*[a]	*P*[b]
2016/ males	Exposed pre-shift	15	6.39 ± 1.10	**	***	5.11 ± 0.98	**	***	1.28 ± 0.70	0.720	*
	Exposed post-shift	15	7.25 ± 0.82		***	5.95 ± 0.82		***	1.30 ± 0.72		*
	Controls	15	2.73 ± 0.60			2.04 ± 0.48			0.69 ± 0.42		
2017/ males	Exposed pre-shift	13	3.99 ± 1.25	***	***	3.04 ± 1.04	**	***	0.95 ± 0.43	0.649	*
	Exposed post-shift	13	4.51 ± 1.30		***	3.49 ± 0.98		***	1.02 ± 0.62		*
	Controls	13	2.29 ± 0.63			1.74 ± 0.57			0.55 ± 0.27		
2016/ females	Exposed pre-shift	5	6.69 ± 0.57	0.063	**	4.87 ± 0.78	0.124	**	1.82 ± 0.70	0.625	*
	Exposed post-shift	5	8.10 ± 0.91		**	6.00 ± 0.92		**	2.10 ± 1.21		*
	Controls	6	2.92 ± 1.03			2.14 ± 0.72			0.78 ± 0.46		
2017/ females	Exposed pre-shift	7	3.89 ± 1.29	0.296	***	3.14 ± 1.13	0.075	**	0.74 ± 0.34	0.265	0.621
	Exposed post-shift	7	4.41 ± 1.76		**	3.69 ± 1.45		***	0.72 ± 0.57		0.933
	Controls	7	2.01 ± 0.45			1.42 ± 0.27			0.59 ± 0.22		

N, number of subjects; SD, standard deviation.
[a]Pre-shift vs. post-shift values of DNA damage in the exposed subjects.
[b]DNA damage in the exposed vs. control groups.
*$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$.

Table 9. Bivariate (A) and multivariate regression (B) of the studied parameters and their impact on the level of DNA damage related to long-term and acute exposure to nanoparticles in 2017

	Total DNA damage	*P*	DNA-SB	*P*	Oxidized bases	*P*
	OR (95% CI)		OR (95% CI)		OR (95% CI)	
Long-term exposure						
(A) Bivariate regression						
Age (values)	0.98 (0.92–1.05)	0.616	0.97 (0.91–1.04)	0.438	1.02 (0.95–1.09)	0.545
Gender (males/females)	1.00 (0.27–3.67)	1.000	1.56 (0.42–5.76)	0.508	2.45 (0.64–9.39)	0.190
BMI (values)	0.89 (0.77–1.04)	0.136	0.95 (0.83–1.1)	0.496	0.93 (0.81–1.07)	0.317
Exposure: pre-shift/controls	16.00 (3.40–75.34)	***	9.00 (2.15–37.66)	**	3.45 (0.94–12.65)	0.062
(B) Multivariate regression						
Age (values)	1.01 (0.92–1.12)	0.787	0.98 (0.9–1.07)	0.641	1.09 (0.98–1.22)	0.101
Gender (males/females)	1.77 (0.27–11.83)	0.555	2.04 (0.37–11.4)	0.415	8.22 (1.19–56.78)	*
BMI (values)	0.87 (0.69–1.09)	0.224	0.97 (0.79–1.19)	0.781	0.79 (0.62–1)	*
Exposure: pre-shift/controls	15.4 (3.13–75.81)	**	9.03 (2.05–39.65)	**	3.7 (0.85–16.14)	0.082
Acute exposure						
(A) Bivariate regression						
Age (values)	0.99 (0.92–1.06)	0.716	1.02 (0.95–1.1)	0.523	1.03 (0.96–1.1)	0.438
Gender (males/females)	1.56 (0.42–5.76)	0.508	2.45 (0.64–9.39)	0.190	4.00 (0.98–16.27)	0.053
BMI (values)	0.95 (0.83–1.1)	0.505	1.03 (0.9–1.19)	0.657	1.00 (0.87–1.14)	0.969
Exposure: pre-shift/controls	32.11 (5.66–182.18)	***	16.00 (3.4–75.34)	***	3.45 (0.94–12.65)	0.062
(B) Multivariate regression						
Age (values)	1.00 (0.9–1.12)	0.953	1.05 (0.94–1.18)	0.384	1.08 (0.97–1.20)	0.148
Gender (males/females)	2.82 (0.33–24.45)	0.346	4.25 (0.51–35.8)	0.183	9.49 (1.38–65.27)	*
BMI (values)	0.96 (0.75–1.24)	0.768	1.02 (0.81–1.3)	0.848	0.87 (0.70–1.07)	0.189
Exposure: pre-shift/controls	34.95 (5.53–220.76)	***	27.76 (4.08–189.11)	***	4.22 (0.94–19.04)	0.061

OR, odds ratio; 95% CI, 95% confidence interval.
*$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$.

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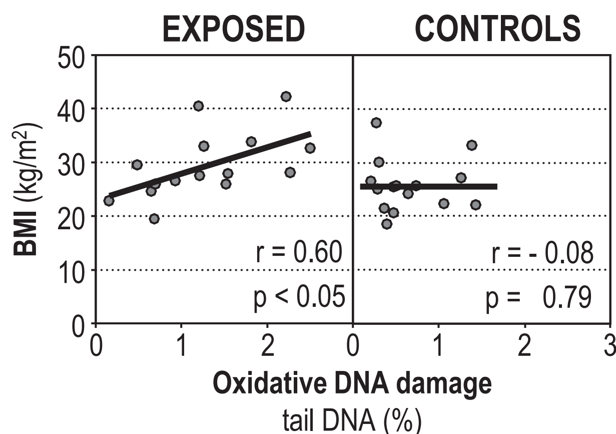

Figure 2. Relationship between BMI and DNA oxidation in the males from exposed and control groups examined in the year 2016. Number of exposed males—15, number of control males—15, *r*—Spearman correlation coefficient.

assay again confirmed in workers strong genotoxic effect of long-term exposure to NP compared to control subjects although the detected levels of the DNA damage were considerably lower than those in 2016. This could be related to shortening the overall exposure time because some of the most exposed workers from 2016 did not participate in the repeated study. In addition, no welding and melting were carried out in 2017 and the remaining operations were concentrated in one workshop, which was associated with a reduction in the total amount of nanosized fraction generated. However, also the controls analysed in 2017 showed lower basal levels of DNA damage compared to previous year. The genotype, i.e. inherited properties responsible for the metabolism of xenobiotics and DNA damage repair, may significantly influence the susceptibility or resistance of a subject to adverse health impacts (55,56). Indeed, when only the subjects examined in both years were compared (in this case, neither the length of exposure time nor genotype played a role), the controls did not differ between 2016 and 2017, while the workers in 2017 still exhibited lower DNA damage than in the previous year. Thus, the lower exposure to NP along with the change in elemental composition of nanosized aerosol fraction are probably the main causes of differences in DNA damage level between workers examined in 2016 and 2017.

In addition, the results from 2017 seemed to confirm a greater 'susceptibility' of males to the action of NP in working environment as the males, unlike females, exhibited an increase of DNA oxidation in response to chronic exposure. However, the current data did not allow to analyse the genotoxic effects in relation to the true personal exposures during the different working activities. The use of personal monitors could help to solve some issues such as an identification of aerosol component mainly responsible for genotoxic effects in workers handling nanocomposites, as well as the clarification of the observed gender differences. Therefore, this approach will be applied in the next step of our research.

Conclusions

We demonstrated adverse effects of long-term inhalation exposure to NP on genetic material. The workers exposed during processing and manufacturing nanocomposite materials exhibited high levels of DNA-SB and oxidised bases compared to controls. High values of BMI appeared to be a factor contributing to an increased risk of oxidative damage to DNA. The results underline an urgent need for further studies aimed at a detailed analysis of the mechanisms responsible for harmful biological effects of NP and their health consequences.

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