Mutagenesis, 2016, 31, 669–675
doi:10.1093/mutage/gew038
Original Manuscript
Advance Access publication 16 August 2016

OXFORD

Original Manuscript

Assessment of DNA damage in underground coal miners using the cytokinesis-block micronucleus assay in peripheral blood lymphocytes

Maxim Yu. Sinitsky[1,2,*], Varvara I. Minina[1,3], Nikolay I. Gafarov[4], Maxim A. Asanov[3], Aleksey V. Larionov[1], Anastasia V. Ponasenko[2], Valentin P. Volobaev[1] and Vladimir G. Druzhinin[1,3]

[1]Department of Genetics, Kemerovo State University, Krasnaya St 6, 650043 Kemerovo, Russian Federation, [2]Laboratory of Genome Medicine, Research Institute for Complex Issues of Cardiovascular Diseases, Sosnovy Boulevard 6, 650002 Kemerovo, Russian Federation, [3]Laboratory of Cytogenetics, The Federal Research Center of Coal and Coal Chemistry of Siberian Branch of the Russian Academy of Sciences, Leningradsky Ave 10, 650065 Kemerovo, Russian Federation and [4]Laboratory of Population Genetics, Research Institute for Complex Problems of Hygiene and Occupational Diseases, Kutuzova St 23, 654041 Novokuznetsk, Russian Federation

*To whom correspondence should be addressed: Tel: +7 3842 580166; Fax: +7 3842 583885; Email: sinitsky.maxim@gmail.com

Received 4 June 2016; Revised 5 July 2016; Accepted 18 July 2016.

Abstract

Coal miners are exposed to coal dust, containing mineral particles, inorganic compounds and polycyclic aromatic hydrocarbons, and to ionizing radiation. These factors can induce oxidative stress and promote inflammation that leads to DNA damage. The aim of this investigation is to analyse the degree of DNA damage in miners working in underground coal mines in Kemerovo Region (Russian Federation) using the cytokinesis-block micronucleus assay (CBMN) in peripheral blood lymphocytes. The exposed group included 143 coal miners (mean age = 50.11 ± 7.36 years; mean length of service in coal mining conditions = 23.26 ± 9.66 years). As a control group, we have used venous blood extracted from 127 healthy non-exposed men. The mean age in this group was 47.67 ± 8.45 years. We have discovered that coal miners are characterized by a significant increase in the frequency of binucleated lymphocytes with micronuclei (MN), nucleoplasmic bridges (NPBs) and protrusions (NBUDs) compared to non-exposed donors. In addition, we report, for the first time, a reduction of cell proliferation in a cohort of coal miners. These data are evidence of the genotoxic and cytostatic effects of occupational harmful factors of the coal mining industry. No correlation between the level of chromosome damage and age, smoking status or length of service in coal mining conditions were discovered. We suggest that the CBMN assay would be useful in biomonitoring studies to monitor hygiene and prevention strategies in occupational settings in coal mining countries.

Introduction

Coal is one of the most abundant minerals in nature and constitutes the largest fossil fuel source used for the generation of energy (1). Coal production is prominent in various countries, such as the Russian Federation, Brazil, South Africa, Australia, China and others. However, its extraction and use is associated with pollution of the environment, which represents a major risk for human health (2,3). During coal mining, a large quantity of coal dust particles is released into the atmosphere of mines. In addition, due to the high caloric power of this mineral, when it is exposed to ambient oxygen and sunlight, a spontaneous combustion process may be initiated,

generating the release of a high concentration of polycyclic aromatic hydrocarbons (PAHs) into the environment (4). Coal wastes consist of a mixture of substances, containing carbon, hydrogen, nitrogen, oxygen and sulphur (2), mineral particles of smaller size and inorganic compounds in the ashes (4).

In addition to various organic and inorganic chemical compounds and mineral particles, an increased level of ionising radiation may be detected in underground coal mines. Radon (^{222}Rn), generated from uranium and seeped into the atmosphere from rocks and soil, and its decay products are the main sources of radiation in coal mines (5–7). Coal miners are exposed to external β- and γ-radiation as well. The synergistic influence of coal dust and ionising radiation leads to an increase in exposure. Radioactive isotopes adsorbing on dust particles can be inhaled into the lung, where they may penetrate the epithelial cells that cover the bronchi and alveoli. These radioactive sources emit alpha particles that can interact with biological tissues in the lungs and induce DNA damage (8,9). It is also known that radon is soluble in water and fat and thus can circulate in the human body and interact not only with lungs and nearby tissues but also with other tissues (e.g. bone marrow).

Complexes of these harmful agents are considered hazardous due to synergistic, additive and enhancing effects (10). The main route of coal mining wastes exposure is through inhalation. Today it is known that chronic inhalation of complex harmful mixtures containing substances such as heavy metals, ash, iron, PAHs and sulphur can result in pulmonary diseases like coal workers' pneumoconiosis, progressive massive fibrosis, bronchitis, loss of lung function, emphysema and lung cancer (11,12). Some researchers have proposed that some of these diseases may be a result of activation of macrophages, interaction with epithelial and other cells, finally leading to generation of oxidative stress (13). Biomonitoring studies in peripheral blood lymphocytes of coal employees demonstrated increased formation of adducts and an increase in non-cellular and cellular sources of reactive oxygen species (ROS) that can induce oxidative DNA damage (14,15).

DNA damage in coal miners resulting from exposure to genotoxic agents can be registered at the chromosomal level using various cytogenetic methods (16–18). Due to the fact that genotoxic effects in coal miners are the result of a complex of harmful factors having both the chemical and the physical nature, the cytokinesis-block micronucleus assay (CBMN) is the most useful method for the assessment of such exposure (19). The cytogenetic markers measured using CBMN are easy to recognise and score, and the results can be obtained in a shorter period of time. In addition, information regarding other cellular events such as mitotic rate and cell death by apoptosis and necrosis can be simultaneously obtained from the same slides (20). Despite the urgency of this problem, there are a limited number of studies describing the occupational hazard effects in coal miners. However, some studies have been conducted in animals of mining regions. For example, cytotoxic and genotoxic effects of coal dust have been shown *in vivo* in wild rodents in coal mining areas in Brazil (21,22) and in Colombia (23) as well as in bats (24) and in land snails (25).

The aim of the present study was to evaluate the potential genotoxic effects in peripheral blood lymphocytes in coal miners working in underground coal mines in Kemerovo Region (Russian Federation) using CBMN. The results described in this study present the opportunity to use CBMN for evaluating potential occupational health risks in coal miners.

Materials and Methods

Group characteristics

Blood samples were obtained from 143 underground coal miners (only men) working in coal mines of Kemerovo Region (Russian Federation) and undergoing medical examination at the Research Institute for Complex Problems of Hygiene and Occupational Diseases (Novokuznetsk, Kemerovo Region, Russian Federation). The mean time of service in coal mining conditions in the exposed group is 23.26 ± 9.66 years; mean age is 50.11 ± 7.36 years. In the exposed group, 56 donors were smokers, 23 were ex-smokers and 64 individuals have not smoked (Table 1).

The exposed group was divided into five subgroups according to the type of activities performed in the mine: (i) drift miners (n = 42) providing access to coal seams by adits driven into the surface outcrop of the coal bed; (ii) stope miners (n = 39) extracting the desired ore or other mineral from an underground mine; (iii) drivers of extracted coal transport (n = 28) that destroy rock and load it into special machines for transporting; (iv) electricians (n = 19) serving underground mine equipment; and (v) steigers (n = 15) who are mine managers responsible for part of the mine and the people subordinated to them.

Blood samples obtained from 127 healthy unexposed men (donors of the Kemerovo Centre for Blood Transfusion, Kemerovo, Russian Federation), 49 smokers, 50 non-smokers and 28 ex-smokers, were included in the non-exposed control group. The mean age in this group was 47.67 ± 8.45 years (Table 1).

Exposed workers were matched to non-exposed controls by age, similar social–economic status and dietary habits. All donors completed a detailed questionnaire that included data about health and smoking status, cancer history, medicine intake, allergies, occupation and time of service and previous exposure to medical X-rays or treatment with known mutagenic effects. Exclusion criteria for exposed and non-exposed groups were age over 60 years, intake of medicine with known mutagenic effects or receiving an X-ray examination up to 3 months prior to collection of the material and infectious diseases or cancer. All data were recorded in databases. All participants were informed about the aim, benefits, risks and methodology details of the study; informed consent was obtained for each donor. The research was performed in accordance with the requirements of the Ethics Committee of Kemerovo State University.

Table 1. Age, length of service and smoking status in studied groups

Group	Number	Age, years		Time of service in coal mining conditions, years		Smoking status		
		μ ± SD	Min-max	μ ± SD	Min-max	Smokers	Non-smokers	Ex-smokers
Exposed	143	50.11 ± 7.36	24–60	23.26 ± 9.66	4–39	56	64	23
Non-exposed control	127	47.67 ± 8.45	25–60	0	0	49	50	28

Cytogenetic investigation

Cytogenetic investigation was performed using the routine protocol of CBMN (20,26) with slight modifications (27). The whole blood obtained from the ulnar vein was sampled using vacutainers with heparin before culturing and was stored at 4°C for no more than 24 h. Volumes of 0.2 ml blood, 3 ml RPMI-1640 (PanEco, Moscow, Russian Federation), 0.8 ml embryonic veal serum (PanEco, Moscow, Russian Federation) and 30 µl phytohaemagglutinin (PanEco, Moscow, Russian Federation) were added to culture flasks and incubated for 44 h at 37°C. Then, cytochalasin B (PanEco, Moscow, Russian Federation) was poured into each culture at a final concentration of 6 µg/ml and incubated another 24 h at 37°C. At the end of the cultivation cycle, the cultures were centrifuged for 10 min at 1000 rpm, the supernatant was removed, and the pellet was resuspended. Then, 6 ml of a cold, freshly prepared hypotonic solution of 0.125 M KCl was poured into the tubes. After this, 1 ml of cold, freshly prepared Carnoy's fixer (a compound of methanol and acetic acid in a ratio of 3:1) was poured into the tubes and pellet was stirred. The suspension was centrifuged for 10 min at 1000 rpm. The supernatant was removed, the pellet was resuspended and another 10 ml of cold Carnoy's fixer was added. The samples were stored at –20°C for 1 h until the next centrifugation step. This procedure was repeated several times until the pellet appeared clean and the cell suspension was clear. After the last centrifugation step, the majority of the supernatant was removed, leaving a volume not exceeding 200 µl. Next, the suspension was pipetted onto dry, cold glass slides. The slides were encoded and stained with 2% Giemsa solution for 15 min. The slides were analysed using a Nikon Eclipse 80i microscope with transmitted light and a full filter at ×1000 magnification.

On each slide, 1000 binucleated (BN) lymphocytes were analysed, and abnormalities such as micronuclei (MNs), nucleoplasmic bridges (NPBs) and protrusions (NBUDs) were scored. For the identification of these abnormalities, we used the criteria described by Fenech (20,28).

In addition, 500 cells were scored to determine the frequency of apoptotic cells (28) as well as cells with 1, 2, 3 and 4 nuclei. The nuclear division index (NDI) was calculated using the following formula:

$$NDI=[M1+2(M2)+(M3)+4(M4)/N,$$

where M1–M4 represent the number of cells with one to four nuclei and *N* is the total number of viable cells scored (29).

Statistical analysis

Statistical analysis was performed using the software StatSoft STATISTICA 7.0. We used the Kolmogorov–Smirnov test to verify the consistency of the data with the normal distribution. The data analysis was performed using the non-parametric statistics block. Group comparisons were performed using the *U*-rank Mann–Whitney test. Spearman's correlation coefficient was used to calculate correlation.

Results

The results of cytogenetic investigation of the exposed (coal miners) and non-exposed groups are presented in Table 2. We discovered some significant differences in the level of cytogenetic markers between coal miners and the non-exposed control group. The coal miners are characterised by the increased frequency of BN lymphocytes with 1 MN (9.92 ± 3.37‰ vs. 6.83 ± 1.71‰, $P < 0.0001$) and the total frequency of cells with MN (11.15 ± 3.81‰ vs. 7.51 ± 1.83‰, $P < 0.0001$) in comparison with non-exposed controls. Other cytogenetic indictors also showed increases in the group of coal miners. For example, the frequency of BN lymphocytes with NPBs is 4.04 ± 2.82‰ and the frequency of those with NBUDs is 7.23 ± 2.54‰, whereas the frequency of these indicators in the control group are 2.36 ± 1.27‰ and 5.83 ± 2.55‰, respectively. These differences were all statistically significant, evaluated using a Mann–Whitney *U* test at $P < 0.0001$ (for the frequency of cells with NPBs) and $P < 0.01$ (for the frequency of cells with NBUDs).

The results of counting 500 lymphocytes and scoring cells with 1, 2, 3 and 4 nuclei as well as apoptotic cells are presented in Table 3. We discovered that only the frequency of mononucleated lymphocytes were increased in coal miners (34.72 ± 9.60%) compared with non-exposed controls (21.86 ± 6.44%, $P < 0.0001$). Other indicators, such as the frequency of bi-, three- and four-nucleated cells and NDI, were increased in the control group compared with the exposed group. The frequency of BN lymphocytes was the most varied parameter—50.29 ± 3.97% in the control group in comparison with coal miners (42.80 ± 7.45%, $P < 0.0001$). The frequencies of cells

Table 2. Results of cytogenetic investigation

Group	BN lymphocytes with MN, ‰ (µ ± SD)					BN lymphocytes with NPBs, ‰ (µ ± SD)	BN lymphocytes with NBUDs, ‰ (µ ± SD)
	1 MN	2 MN	3 MN	> 3 MN	Total MN		
Exposed	9.92 ± 3.37*	0.93 ± 1.00	0.05 ± 0.38	0.07 ± 0.28	11.15 ± 3.81*	4.04 ± 2.82*	7.23 ± 2.54**
Non-exposed control	6.83 ± 1.71	0.62 ± 0.77	0.02 ± 0.14	0.04 ± 0.20	7.51 ± 1.83	2.36 ± 1.27	5.83 ± 2.55

*$P < 0.0001$: significant differences in comparison with non-exposed control.
**$P < 0.01$: significant differences in comparison with non-exposed control.

Table 3. Results of investigation of proliferation activity and frequency of apoptotic cells

Group	NDI	Cells with different number of nucleus (%), µ ± SD				Apoptotic cells (%), µ ± SD
		1	2	3	4	
Exposed	1.91 ± 0.19*	34.72 ± 9.60*	42.80 ± 7.45*	4.99 ± 1.94**	10.45 ± 4.12*	1.71 ± 1.04
Non-exposed control	2.16 ± 0.15	21.86 ± 6.44	50.29 ± 3.97	5.81 ± 1.63	15.32 ± 3.94	1.34 ± 0.55

*$P < 0.0001$: significant differences in comparison with non-exposed control.
**$P < 0.01$: significant differences in comparison with non-exposed control.

with three and four nuclei in the control group were 5.81 ± 1.63% and 15.32 ± 3.94%, while in the exposed group the frequencies were 4.99 ± 1.94% ($P < 0.01$) and 10.45 ± 4.12% ($P < 0.0001$), respectively. A similar relationship was discovered in NDI—in the exposed group this indicator was 1.91 ± 0.19, in comparison with 2.16 ± 0.15 in non-exposed controls ($P < 0.0001$). We found no significant differences in the frequency of apoptotic cells ($P > 0.05$).

To determine the level of genetic damage in relation to the type of activities performed in coal mines, we assessed the frequency of cytogenetic markers in subgroups divided by the type of work that employees perform in mines. The full results are shown in Table 4. We found only two significant differences: steigers were characterized by the increased frequency of BN lymphocytes with MN (12.70 ± 3.13‰ vs. 10.39 ± 4.54‰ in drift miners) and with NPBs (6.10 ± 1.91‰ vs. 3.04 ± 2.27‰ in drivers of extracted coal transport). The rest of the results were not statistically different ($P > 0.05$).

The Spearman correlation coefficient for the cytogenetic marker frequencies and the indexes of proliferation, such as NDI and cells with different number of nucleus with age (for the exposed and the control group), and length of service in coal mining conditions (for the exposed group), were not significant ($P > 0.05$). We also found no significant distinctions in the studied markers in donors with different smoking status or in the correlation with length of smoking and number cigarettes/day.

Discussion

Kemerovo Region is the largest coal mining area in the Russian Federation, and the coal mining industry is the leading industrial sphere in this region. According to a report by the Federal Department of National Statistics, 88 765 people (11.1% of the total working men) worked in coal mines in Kemerovo Region in February 2016. It is known that coal miners have a high relative risk of initiation of pulmonary diseases—3.2 cases per 1000 workers. The greatest contribution to occupational diseases in coal miners is from diseases such as chronic dust-induced bronchitis and coal workers' pneumoconiosis (CWP) (30,31).

It has been suggested that ROS are involved in the pathogenesis of lung diseases and cancer risk (11,14,32) in workers exposed to some harmful agents. The primary target cells of inhaled coal dust particles are macrophages and epithelial cells. Activated macrophages produce excessive amounts of ROS. ROS also may be generated by cell-independent mechanisms due to intrinsic chemical properties of the coal dust (e.g. surface radicals and iron). Epithelial cells and fibroblasts, which are the main producers of components of the extracellular matrix, can also produce ROS upon stimulation. Additional phagocytic cells (neutrophils and monocytes) may be recruited by chemokines produced by the alveolar macrophages as well as epithelial cells and may amplify local production of ROS. ROS may cause damage or proliferation of local epithelial and mesenchymal tissue and may as such have consequences to lung tissue morphology, cell turnover and deposition of extracellular matrix components (4,12).

When there is excessive production of ROS, or when there are insufficient *in vivo* defence mechanisms, oxidative stress may occur. This stress may initiate DNA damage, lipid peroxidation, protein modification, membrane disruption and mitochondrial damage (33). It is also known that ROS potentially promote inflammatory processes (34,35). A recent pilot investigation performed by Volobaev *et al.* (36) in a group of coal miners working in underground coal mines of Kemerovo Region and suffering from occupational pulmonary diseases showed that inflammation can modify the overall level of cytogenetic damage in miners.

In addition to oxidative stress and inflammation, large amounts of mutagenic compounds such as PAHs (chrysene, benzo(k)fluoranthene, pyrene and fluoranthene) are produced during spontaneous coal combustions (37). PAHs can induce DNA damage such as single-strand breaks (38–40) and can become electrophilic metabolites that covalently interact with DNA (41), forming adducts with purines, especially guanine, after metabolic activation by enzymatic complex P450.(42)

Ionizing radiation from natural sources is another factor that can induce DNA damage in underground coal miners. This type of exposure leads to the occurrence of double-strand DNA breaks (DSBs) (43–46).

Thus, in addition to the increased risk of initiation of pulmonary occupational diseases, coal miners are exposed to genotoxic risks resulting from the influence of chemical and physical agents on the organism. Cytogenetic tests permitting the detection of various types of DNA damage at the cellular and chromosomal level can be used for the assessment of such genotoxic risk. Currently, many cytogenetic tests are used for the investigation of coal miners. A number of articles describe the increased level of MN and other cytogenetic abnormalities discovered using CBMN on peripheral blood lymphocytes (4,47–49) and the MN assay on exfoliated cells (48,50,51), as well as DNA damage, discovered using the DNA-comet assay(1,4) and the sister chromatid exchange (SCE) test (47,49) in coal miners in comparison with people who do not work in coal mines.

At the first stage of our research, we scored the frequency of cytogenetic damage, such as MN, NPBs and NBUDs in BN lymphocytes. We discovered a significant increase in the frequency of BN lymphocytes with one MN and in the total frequency of cells with MN (Table 2). It is known that MN can originate from chromosome fragments as a result of clastogenic influence to the organism. Studies

Table 4. The level of cytogenetic markers in coal miners with different types of activity in mines

Group (*N*)	BN lymphocytes with MN, ‰ (μ ± SD)					BN lymphocytes with NPBs, ‰ (μ ± SD)	BN lymphocytes with NBUDs, ‰ (μ ± SD)
	1 MN	2 MN	3 MN	> 3 MN	Total MN		
Drift miners (*N* = 42)	9.39 ± 4.02	0.71 ± 0.82	0.15 ± 0.38	0.16 ± 0.37	10.39 ± 4.54	4.26 ± 3.21	6.83 ± 2.03
Stope miners (*N* = 39)	10.64 ± 3.56	1.14 ± 1.10	0.17 ± 0.45	0.03 ± 0.17	11.97 ± 4.07	4.53 ± 3.26	5.92 ± 2.31
Drivers of extracted coal transport (*N* = 28)	9.53 ± 2.91	0.71 ± 1.15	0.25 ± 0.44	0.04 ± 0.19	10.54 ± 3.05	3.04 ± 2.27	6.21 ± 2.27
Electricians (*N* = 19)	10.00 ± 2.76	0.82 ± 0.66	0.14 ± 0.35	0.05 ± 0.21	11.00 ± 2.79	3.64 ± 2.30	5.82 ± 2.40
Steigers (*N* = 15)	11.50 ± 2.92	1.20 ± 1.03	0	0	12.70 ± 3.13*	6.10 ± 1.91**	5.83 ± 2.55

*$P < 0.01$: significant differences in comparison with drift miners.

**$P < 0.01$: significant differences in comparison with drivers of extracted coal transport.

over several decades have shown that misrepair of DNA DSBs can lead to symmetrical and asymmetrical chromatid and chromosome exchanges as well as chromatid and chromosome fragments. Furthermore, DNA breaks, which lead to MN formation, may be left unrepaired if repair enzymes in the non-homologous end joining pathway are defective. Other mechanisms that could lead to MN formation from acentric fragments include simultaneous excision repair of damaged (e.g. 8-oxo-deoxyguanosine) or inappropriate bases (e.g. uracil) incorporated in DNA that are in proximity and on opposite complementary DNA strands. Such simultaneous excision repair events, particularly if the gap-filling step is not completed, leads to DNA DSBs and MN formation. In fact, this process can be exploited to greatly enhance the lymphocyte MN assay response to genotoxic agents that mainly induce DNA adducts (19). Another way for MN to originate is from whole chromosome loss events. There are a range of possible molecular mechanisms that could cause chromosome malsegregation at anaphase, resulting in MN formation. One of the mechanisms that may lead to MN from chromosome loss events is hypomethylation of cytosine in centromeric and pericentromeric repeat sequences such as classical satellite repeats at pericentromeric regions and higher order repeats of satellite DNA in centromeric DNA (52,53).

Results of an *in vitro* investigation, performed in lymphocyte cultures treated by various clastogenic and aneugenic agents, showed an increasing number of cells with more than one (i.e. two or three) MN in cultures treated with aneugens and berenil. In contrast, the vast majority of cells in clastogen-treated cultures were associated with only one MN (54). Therefore, we suggest that clastogenic effects contribute substantially to the observed increase in cytogenetic damage in coal miners because we have discovered no significant increase in cells with multiple MN and have detected differences in the level of lymphocytes with one MN.

At the same time, Speit *et al.* (55) reported that the process of cultivation may influence the level of MN. In our research, we cultivated lymphocytes with the same protocol in the both studied groups, therefore we minimized the influence of this factor to the observed results.

Our results conform to those obtained by other researchers. For example, León-Mejía *et al.* (4) and Rohr *et al.* (1) studied open-cast coal miners and discovered a significant increase in the frequency of BN lymphocytes with MN in the exposed groups. The overall level of MN described in these studies was slightly lower than similar markers in our research. This discordance can be explained by the fact that we have studied underground coal miners (not open-cast workers) who work in conditions characterized by a higher concentration of harmful genotoxic agents, which can accumulate in the enclosed space of underground coal mines. However, the frequency of BN lymphocytes with NPBs was greatly increased in miners studied by researchers from Brazil (1) in comparison with our results (12.33 ± 7.48 vs. 7.23 ± 2.54). Taking into account that the NPBs (biomarkers of DNA misrepair and/or telomere end-fusions) are formed from dicentric chromosomes, which are a marker of radiation exposure, we can assume that the differences in these results are due to disparities in the exposure to radiation, probably related to the different radioactivity of coal extracted from different coal mining areas (56). A study by Turkish researchers demonstrated a similar trend of increased levels of MN in workers of underground bituminous coal mines compared with non-exposed people from managerial jobs (47). It should be noted that the frequency of cells with MN in the work of Donbak *et al.* was considerably higher in exposed as well as non-exposed groups in comparison with results discovered in our research.

In an article by Ulker *et al.* (49) contrasting results were reported. They studied three groups: CWP patients, healthy coal miners and non-exposed people. They found no significant differences in the frequency of BN lymphocytes with MN or the percentage of SCE in coal miners and non-exposed controls, whereas the CWP patients were characterized by the significant increase of studied cytogenetic markers. These results suggest that inflammatory processes accompanying CWP have an important influence on the level of cytogenetic indicators in coal miners.

We have discovered no significant differences in the frequency of MN, NPBs and NBUDs between smokers, ex-smokers and non-smokers. No correlation was found between the length of smoking, number of cigarettes/day, age or length of service and the different parameters of CBMN. This tendency is typical for both studied groups (coal miners and non-exposed donors) and conforms to literary data (1,4,47,49).

At the second stage of our research, we scored the percentage of cells with 1, 2, 3 and 4 nuclei and apoptotic cells. We discovered a significant decrease in the percentage of lymphocytes with two, three and four nuclei and NDI with a synchronous increase of mononucleated cells in coal miners compared with non-exposed controls. These data suggest that coal miners are characterised by inhibited proliferation, probably due to cytostatic effects of harmful components of mixtures presenting in underground coal mines. Some researchers have reported the reduction of NDI in cultures of lymphocytes treated *in vitro* by various cytostatic and mutagenic substances like diethylstilbestrol, doxorubicin and mitomycin C (54,55). At the same time, eukaryotic organisms have several checkpoints where the validity of the cell cycle is controlled and where the arrest of proliferation in the case of DNA damage or aberrant DNA structures occurs. Checkpoints activated when DNA damage occurs in the G1 and G2 phases lead to cell cycle arrest in the same phases (57). DNA damage occurring in the S phase leads to the delay of DNA replication and its stopping at G2 phase (58).

Analysis of the level of cytogenetic damages in coal miners differentiated by type of activities performed in mines showed an increase of the BN lymphocytes with MN and NPBs in steigers. These workers are responsible for planning, organising, directing and controlling working processes to accomplish goals, and they spend more time in mines than other workers. This work therefore corresponds to increased time of exposure to genotoxic agents and, consequently, the degree of DNA damage. On the other hand, León-Mejía *et al.* (4) studied activities such as extracted coal transport, equipment field maintenance, coal stripping and coal embarking and reported no significant differences between these activities.

Conclusions

As a result of the study performed, we discovered genotoxic and cytostatic effects in coal miners working in underground coal mines of Kemerovo Region (Russian Federation) compared with healthy non-exposed donors. Because it is impossible to single out one leading genotoxic factor, we suggest that DNA damage in the exposed employees may be a consequence of oxidative damage resulting from exposure to coal residue mixtures containing traces of iron, sulphur, coal ash, heavy metals and PAHs, as well as ionizing radiation (radon and external β- and γ-radiation). Our results present novel data for the genotoxic damage induced by coal mining exposure in the Russian Federation and contribute to the assessment of the usefulness of the CBMN assay in biomonitoring studies to monitor hygiene and prevention strategies in occupational settings in coal

mining countries. We also report the first data concerning cytostatic effects in coal miners.

Funding

This work was supported by RFBR grants (no. 16-34-00441 mol_a, no. 16-44-420926 r_a) and an RSF grant (no. 16-15-00034).

Acknowledgements

We are particularly indebted to the staff of Kemerovo Center for Blood Transfusion (Kemerovo, Russian Federation) for their help in forming a group of non-exposed donors.
Conflict of interest statement: None declared.

References

1. Rohr, P., Kvitko, K., da Silva, F. R., *et al*. (2013) Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal. *Mutat. Res*., 758, 23–28.
2. Chen, Y., Shah, N., Huggins, F. E. and Huffman, G. P. (2005) Transmission electron microscopy investigation of ultrafine coal fly ash particles. *Environ. Sci. Technol*., 39, 1144–1151.
3. Zakrzewski, S. (1991) *Principles of Environmental Toxicology*. ACS, Washington, DC.
4. León-Mejía, G., Espitia-Pérez, L., Hoyos-Giraldo, L. S., Da Silva, J., Hartmann, A., Henriques, J. A. and Quintana, M. (2011) Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. *Sci. Total Environ*., 409, 686–691.
5. Dantas, A. L., Dantas, B. M., Lipsztein, J. L. and Spitz, H. B. (2007) In vivo measurements of 210Pb in skull and knee geometries as an indicator of cumulative 222Rn exposure in a underground coal mine in Brazil. *Radiat. Prot. Dosimetry*, 125, 568–571.
6. Dixon, D. W., Page, D. and Bottom, D. A. (1991) Estimates of doses from radon daughters in UK mines. *Radiat. Prot. Dosimetry*, 36, 137–141.
7. Skowronek, J. (1999) Radiation exposures to miners in Polish coal mines. *Radiat. Prot. Dosimetry*, 82, 293–300.
8. Bilban, M. and Jakopin, C. B. (2005) Incidence of cytogenetic damage in lead-zinc mine workers exposed to radon. *Mutagenesis*, 20, 187–191.
9. Rafique, M., Rahman, S.U., Rahman, S., *et al.* (2010) Assessment of indoor radon doses received by the students in the Azad Kashmir schools, Pakistan. *Radiat. Prot. Dosimetry*, 142, 339–346.
10. White, P. (2002) The genotoxicity of priority polycyclic aromatic hydrocarbons in complex mixtures. *Mutat. Res. Genet. Toxicol. Environ. Mutagen*, 515, 85–98.
11. Beckman, K. B. and Ames, B. N. (1997) Oxidative decay of DNA. *J. Biol. Chem*., 272, 19633–19636.
12. Schins, R. P. and Borm, P. J. (1999) Mechanisms and mediators in coal dust induced toxicity: a review. *Ann. Occup. Hyg*., 43, 7–33.
13. Kamp, D. W., Graceffa, P., Pryor, W. A. and Weitzman, S. A. (1992) The role of free radicals in asbestos-induced diseases. *Free Radic. Biol. Med*., 12, 293–315.
14. Schins, R. P., Schilderman, P. A. and Borm, P. J. (1995) Oxidative DNA damage in peripheral blood lymphocytes of coal workers. *Int. Arch. Occup. Environ. Health*, 67, 153–157.
15. Schoket, B., Poirier, M., Mayer, G., Török, G., Kolozsi-Ringelhann, Á., Bognár, G., Bigbee, W.L. and Vincze, I. (1999) Biomonitoring of human genotoxicity induced by complex occupational exposures. *Mutat. Res. Genet. Toxicol. Environ. Mutagen*, 445, 193–203.
16. Hsu, T. C. (1983) Genetic instability in the human population: a working hypothesis. *Hereditas*, 98, 1–9.
17. Hsu, T. C., Johnston, D. A., Cherry, L. M., Ramkissoon, D., Schantz, S. P., Jessup, J. M., Winn, R. J., Shirley, L. and Furlong, C. (1989) Sensitivity to genotoxic effects of bleomycin in humans: possible relationship to environmental carcinogenesis. *Int. J. Cancer*, 43, 403–409.
18. Snigiryova, G. P. (2011) Biological dosimetry on the strength of cytogenetic analysis. *Bull. Russian Scientific Center Russian Ministry Health Radiol*, 11, 18–24.
19. Fenech, M., Kirsch-Volders, M., Natarajan, A. T., Surralles, J., Crott, J. W., Parry, J., Norppa, H., Eastmond, D. A., Tucker, J. D. and Thomas, P. (2011) Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*, 26, 125–132.
20. Fenech, M. (2007) Cytokinesis-block micronucleus cytome assay. *Nat Protoc*., 5, 1084–1104.
21. Da Silva, J., de Freitas, T., Heuser, V., Marinho, J., Bittencourt, F., Cerski, C., Kliemann, L.M. and Erdtmann, B. (2000) Effects of chronic exposure to coal in wild rodents (*Ctenomys torquatus*) evaluated by multiple methods and tissues. *Mutat. Res. Genet. Toxicol. Environ. Mutagen*, 470, 39–51.
22. da Silva, J., de Freitas, T. R., Heuser, V., Marinho, J. R. and Erdtmann, B. (2000) Genotoxicity biomonitoring in coal regions using wild rodent Ctenomys torquatus by Comet assay and micronucleus test. *Environ. Mol. Mutagen*., 35, 270–278.
23. León, G., Pérez, L. E., Linares, J. C., Hartmann, A. and Quintana, M. (2007) Genotoxic effects in wild rodents (*Rattus rattus* and *Mus musculus*) in an open coal mining area. *Mutat. Res*., 630, 42–49.
24. José Zocche, J., Dimer Leffa, D., Paganini Damiani, A., Carvalho, F., Avila Mendonça, R., Dos Santos, C. E., Appel Boufleur, L., Ferraz Dias, J. and de Andrade, V. M. (2010) Heavy metals and DNA damage in blood cells of insectivore bats in coal mining areas of Catarinense coal basin, Brazil. *Environ. Res*., 110, 684–691.
25. Leffa, D. D., Damiani, A. P., da Silva, J., Zocche, J. J., dos Santos, C. E., Boufleur, L. A., Dias, J. F. and de Andrade, V. M. (2010) Evaluation of the genotoxic potential of the mineral coal tailings through the *Helix aspersa* (Müller, 1774). *Arch. Environ. Contam. Toxicol*., 59, 614–621.
26. Fenech, M. (1993) The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. *Mutat. Res*., 285, 35–44.
27. Ingel, F. I. (2006) Perspectives of micronuclear test in human lymphocytes cultivated in cytogenetic block conditions. Part 1: Cell proliferation. *Ecol. Genet*., 4, 7–19.
28. Fenech, M. (2000) The in vitro micronucleus technique. *Mutat. Res*., 455, 81–95.
29. Eastmond, D. A. and Tucker, J. D. (1989) Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. *Environ. Mol. Mutagen*., 13, 34–43.
30. Laney, A. S., Petsonk, E. L. and Attfield, M. D. (2010) Pneumoconiosis among underground bituminous coal miners in the United States: is silicosis becoming more frequent? *Occup. Environ. Med*., 67, 652–656.
31. Ross, M. H. and Murray, J. (2004) Occupational respiratory disease in mining. *Occup. Med*., 54, 304–310.
32. Cooke, M. S., Evans, M. D., Dizdaroglu, M. and Lunec, J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J*., 17, 1195–1214.
33. Zhai, R., Liu, G., Ge, X., Yang, C., Huang, C., Wu, C. and Christiani, D. C. (2002) Genetic polymorphisms of MnSOD, GSTM1, GSTT1, and OGG1 in coal workers' pneumoconiosis. *J. Occup. Environ. Med*., 44, 372–377.
34. Duval, C., Cantero, A. V., Auge, N., Mabile, L., Thiers, J. C., Negre-Salvayre, A. and Salvayre, R. (2003) Proliferation and wound healing of vascular cells trigger the generation of extracellular reactive oxygen species and LDL oxidation. *Free Radic. Biol. Med*., 35, 1589–1598.
35. Kopprasch, S., Pietzsch, J. and Graessler, J. (2003) Validation of different chemilumigenic substrates for detecting extracellular generation of reactive oxygen species by phagocytes and endothelial cells. *Luminescence*, 18, 268–273.
36. Volobaev, V. P., Sinitsky, M. Y., Larionov, A. V., Druzhinin, V. G., Gafarov, N. I., Minina, V. I. and Kulemin, J. E. (2016) Modifying influence of occupational inflammatory diseases on the level of chromosome aberrations in coal miners. *Mutagenesis*, 31, 225–229.
37. Sai, X. C. (1995) The pollution of PAH. *Environ. Protect*., 10, 31–33.
38. Brescia, G., Celotti, L., Clonfero, E., Neumann, G. H., Forni, A., Foà, V., Pisoni, M., Ferri, G. M. and Assennato, G. (1999) The influence of

cytochrome P450 1A1 and glutathione S-transferase M1 genotypes on biomarker levels in coke-oven workers. *Arch. Toxicol*., 73, 431–439.
39. Pavanello, S., Pulliero, A., Siwinska, E., Mielzynska, D. and Clonfero, E. (2005) Reduced nucleotide excision repair and GSTM1-null genotypes influence anti-B[a]PDE-DNA adduct levels in mononuclear white blood cells of highly PAH-exposed coke oven workers. *Carcinogenesis*, 26, 169–175.
40. Rojas, M., Cascorbi, I., Alexandrov, K., Kriek, E., Auburtin, G., Mayer, L., Kopp-Schneider, A., Roots, I. and Bartsch, H. (2000) Modulation of benzo[a]pyrene diolepoxide-DNA adduct levels in human white blood cells by CYP1A1, GSTM1 and GSTT1 polymorphism. *Carcinogenesis*, 21, 35–41.
41. Singh, R., Sram, R. J., Binkova, B., Kalina, I., Popov, T. A., Georgieva, T., Garte, S., Taioli, E. and Farmer, P. B. (2007) The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. *Mutat. Res. Fundam. Mol. Mech. Mutagen*, 620, 83–92.
42. Baird, W. M., Hooven, L. A. and Mahadevan, B. (2005) Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *Environ. Mol. Mutagen*., 45, 106–114.
43. Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A. and Ellenberger, T. (2006) *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.
44. Jain, V., Kumar, P. R., Koya, P. K., Jaikrishan, G. and Das, B. (2016) Lack of increased DNA double-strand breaks in peripheral blood mononuclear cells of individuals from high level natural radiation areas of Kerala coast in India. *Mutat. Res*. 2016 Mar 25 [Epub ahead of print], doi:10.1016/j.mrfmmm.2016.03.002
45. Lieber, M. R. (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu. Rev. Biochem*., 79, 181–211.
46. Robertson, A., Allen, J., Laney, R. and Curnow, A. (2013) The cellular and molecular carcinogenic effects of radon exposure: a review. *Int. J. Mol. Sci*., 14, 14024–14063.
47. Donbak, L., Rencuzogullari, E., Yavuz, A. and Topaktas, M. (2005) The genotoxic risk of underground coal miners from Turkey. *Mutat. Res*., 588, 82–87.
48. Rohr, P., da Silva, J., da Silva, F. R., Sarmento, M., Porto, C., Debastiani, R., Dos Santos, C. E., Dias, J. F. and Kvitko, K. (2013) Evaluation of genetic damage in open-cast coal mine workers using the buccal micronucleus cytome assay. *Environ. Mol. Mutagen*., 54, 65–71.
49. Ulker, O. C., Ustundag, A., Duydu, Y., Yucesoy, B. and Karakaya, A. (2008) Cytogenetic monitoring of coal workers and patients with coal workers' pneumoconiosis in Turkey. *Environ. Mol. Mutagen*., 49, 232–237.
50. Kvitko, K., Bandinelli, E., Henriques, J. A. P., *et al.* (2012) Susceptibility to DNA damage in workers occupationally exposed to pesticides, to tannery chemicals and to coal dust during mining. *Genet. Mol. Biol*., 35, 1060–1068.
51. León-Mejía, G., Quintana, M., Debastiani, R., Dias, J., Espitia-Pérez, L., Hartmann, A., Henriques, J. A. and Da Silva, J. (2014) Genetic damage in coal miners evaluated by buccal micronucleus cytome assay. *Ecotoxicol. Environ. Saf*., 107, 133–139.
52. Pironon, N., Puechberty, J. and Roize`s, G. (2010) Molecular and evolutionary characteristics of the fraction of human alpha satellite DNA associated with CENP-A at the centromeres of chromosomes 1, 5, 19, and 21. *BMC Genomics*, 11, 195–203.
53. Schueler, M. G. and Sullivan, B. A. (2006) Structural and functional dynamics of human centromeric chromatin. *Annu. Rev. Genomics Hum. Genet*., 7, 301–313.
54. Rosefort, C., Fauth, E. and Zankl, H. (2004) Micronuclei induced by aneugens and clastogens in mononucleate and binucleate cells using the cytokinesis block assay. *Mutagenesis*, 19, 277–284.
55. Speit, G., Linsenmeyer, R., Schütz, P. and Kuehner, S. (2012) Insensitivity of the in vitro cytokinesis-block micronucleus assay with human lymphocytes for the detection of DNA damage present at the start of the cell culture. *Mutagenesis*, 27, 743–747.
56. Lauer, N. E., Hower, J. C., Hsu-Kim, H., Taggart, R. K. and Vengosh, A. (2015) Naturally occurring radioactive materials in coals and coal combustion residuals in the United States. *Environ. Sci. Technol*., 49, 11227–11233.
57. Michelson, R. J. and Weinert, T. (2000) Closing the gaps among a web of DNA repair disorders. *Bioessays*, 22, 966–969.
58. Tercero, J. A. and Diffley, J. F. (2001) Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. *Nature*, 412, 553–557.