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DNA damage assessment with buccal micronucleus cytome assay in Turkish coal miners

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The aim of this study was to assess DNA damage in Turkish coal miners with the buccal micronucleus cytome (BMCyt assay as the least invasive and therefore most practical method that may find wider application in coal miner biomonitoring. Buccal epithelial cell samples were taken from 54 coal miners and 42 controls from Zonguldak, Turkey to establish their micronucleus (MN), binucleus (BN), condensed chromatin (CC), karyorrhectic (KHC), karyolytic (KYL), nuclear bud (NBUD), and pyknotic (PYC) frequencies. We also analysed the effects of confounding factors such as age, years of work at the mine, smoking, alcohol drinking, and use of protective equipment on differences in MN frequencies. Two miners had confirmed and three suspect pneumoconiosis, whereas 49 displayed normal chest radiographs. MN, BN, KHC, and NBUD frequencies were significantly higher in coal miners than controls. Years of work at the mine also showed a significant effect on buccal MN frequencies in coal miners, but we found no correlation between MN frequencies and age, smoking, and alcohol consumption. In conclusion, BMCyt assay proved itself an accurate and practical screening method, as it can detect DNA damage much earlier than pneumoconiosis develops.

KEY WORDS: binucleus frequency; coal dust; condensed chromatin frequency; epithelial cells; genotoxicity; industrial health; karryorrhexis; karyolysis; micronucleus frequency; nuclear bud frequency; pyknosis; occupational toxicology

Open-cast coal miners are exposed to coal dust which contains quartz, trace metals, inorganic minerals, and polycyclic aromatic hydrocarbons (PAHs). This exposure can lead to a number of diseases such as pneumoconiosis (black lung disease), progressive massive fibrosis, bronchitis, lung dysfunction, emphysema, and stomach, liver, or lung cancer (1–5).

Several studies of genotoxic and cytogenetic effects of coal dust have shown significantly higher DNA and chromosomal damage in coal miners than in general population (6–9). Coal contains many organic and inorganic compounds. Quartz, one of its major compounds, has been classified as "carcinogen" (Group 1) by the International Agency for Research on Cancer (IARC) due to ample evidence in animals and humans, but coal dust is still "not classifiable as to its carcinogenicity to humans" (Group 3) due to contradictory findings of *in vivo* and *in vitro* studies (10). This calls for further research to characterise the risk of coal dust exposure.

A wide range of methods is currently available for the detection of DNA damage due to occupational exposure to chemicals, but most of them are expensive and impractical. One of the recent methods that has seen a growing use in assessing the risk of DNA damage and mutation that can evolve into cancer is the buccal micronucleus cytome (BMCyt) assay (11). It is a relatively non-invasive method assessing changes in buccal epithelial cells, which are easy to collect. Buccal epithelial cells are in constant contact with environmental/occupational chemicals such as those in coal dust and epithelial cells in general are the point of origin of most cancers. The BMCyt assay can be used to investigate a variety of toxicological endpoints (12).

Our primary aim was therefore to use it to assess the genotoxic effects of coal dust exposure in Turkish coal miners and see how age, years of work at the mine, smoking, and alcohol consumption might affect micronucleus (MN) frequencies. Our secondary aim was to determine exposure by measuring respirable coal dust levels and to determine the lung function of the exposed miners with chest X-ray imaging and spirometry tests.

PARTICIPANTS AND METHODS

This study included 54 Turkish male coal miners from the Kozlu Coal Mine, 8 km to the south-west from the city of Zonguldak. This is the biggest coal mine in Turkey that employs over 6800 miners (13). Our participants were involved in different operations such as surface mining,

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coal beneficiation, dragline operations, and transporter of extracted coal. All of them were exposed to coal dust.

The study also included a control group of 42 men from Zonguldak who were not exposed to any coal dust or chemicals. The two groups were matched in age and lifestyle habits. Possible confounding factors such as smoking, alcohol consumption, years of work at the mine, medical background, and nutritional habits were established in face-to-face interviews with every participant.

The study was approved by the Zonguldak Bulent Ecevit University Clinical Research Ethics Committee (approval no. 2018-111-11-04 of 25 April 2018). All participants were volunteers who signed a written informed consent before they entered the study.

Biological sampling

All participants rinsed their mouth with water before sampling. Exfoliated buccal epithelial cells were obtained by gently scraping the inside of both cheeks with a wet tongue depressor. The collected cells were smeared directly onto wet slides and then left to dry at room temperature.

Chemicals and equipment

The chemicals used in this study were purchased from the following suppliers: activated coal, ethanol, fast green, HCl, methanol, pararosaniline, sodium bisulfite, and xylene from Merck Chemicals (Darmstadt, Germany), water bath from Isolab (Eschau, Germany), and light microscope from Olympus (Tokyo, Japan).

Buccal micronucleus cytome (BMCyt) assay

Slides with collected buccal samples were first fixed in 80 % methanol for 10 min and dried at room temperature. They were then immersed in 1 N HCl at room temperature for 2 min, at 60 °C for 10 min, and again at room temperature 2 min, followed by washing in distilled water and drying. They were then stained with Feulgen stain (1 g pararosaniline in 100 mL of distilled water) at room temperature in the dark for 90 min and washed in distilled water for 5 min. Followed staining in fast green solution (0.5 g of fast green in 95 % ethanol) for 10 s, fixed in xylene for 10 min, and finally air-dried in a fume hood. For each participant, 2000 buccal cells (1000 from each of the duplicate slides) were scored by light microscopy as recommended by Sarto et al. (14) and Thomas et al. (15). Cell frequencies were expressed as per thousand (‰).

Radiological examination and spirometry test

Posteroanterior chest (PA) radiographs of the exposed miners were taken in the radiology clinic of the Turkish Hard Coal Enterprises using short exposure time with high voltage (Dongmun, South Korea). The radiographs were read by a specialist according to the International Labour Office (ILO) classifications (16). The miners were also tested for standard spirometry parameters with a dry-seal spirometer (MIR SpiroLab III, Rome, Italy). Pulmonary function tests were interpreted in accordance with the American Thoracic Society standards (17). They involved forced expression volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and forced mid-expiratory flow rate ($FEF_{25–75\ \%}$). Pulmonary restriction is indicated when FVC and FEV_1 values are lower but FEV_1/FVC ratio is higher than the expected value, while obstruction is indicated when the FEV_1/FVC ratio is lower than the expected value. Obstructive pattern is also defined as an FEV_1/FVC ratio below 75 % (18).

Exposure measurements

Coal dust samples were collected at the respiration level during the daily 8-hour shift with a badge dosimeter (SKC AirCheck 3000 Deluxe, Dorset, United Kingdom). Respirable total dust concentrations were measured in mg/m^3 by gravimetry according to the Health and Safety Executive method MDHS 14/4 (19).

Statistical analysis

Statistical analysis was done using the computer program SPSS 20.0 for Windows (IBM, Armonk, New York, USA). The normality of data distribution and homogeneity of the variance were tested with the Kolmogorov Smirnov and Levene tests, respectively. Pearson's chi-square test was used to analyse demographical data. Student's *t*-test and Mann Whitney U test were used to determine differences between the groups with and without normal distribution, respectively. Multiple linear regression analysis was used to determine the effects of parameters on the results. Data are expressed as a mean ± standard deviation for continuous variables and the percentage (%) for categorical variables. Power of $p<0.05$ was considered statistically significant.

RESULTS

Study group demographics

Table 1 shows the demographic and lifestyle information about the coal miners and control group. The two did not significantly differ in age, smoking, and alcohol habits. The miner group most often complained of ear disorders (such as otitis media and hearing loss) and respiratory disorders (such as asthma, bronchitis, and shortness of breath), but they also complained of waist and neck hernia, callus, and stomach diseases.

BMCyt assay findings

Micronucleus (MN) frequencies in the epithelial cells of the coal miners were significantly higher than in the control group ($p<0.05$). This is also true for the frequencies

Table 1 General characteristics of coal miners and controls

Factors	Controls (n=42)	Workers (n=54)	p values
Age (years)[a]	39.21±6.23	39.24±5.34	0.98
Min-max	19–48	30–56	
19–30	4	2	
≥31	38	52	
Smokers	18	37	**0.01**
Non-smokers	24	17	
Number of cigarettes smoked[a]	16.0±6.0	17.02±9.21	
Alcohol consumption			0.07
No	40	45	
Yes	2	9	
Years of work at the mine (mean± SD)		11.93±5.14	
Min-max		2–27	
1–15		41	
≥16		13	
Using Protective Equipment			
Gloves			
No		1	
Yes		53	
Mask			
No		3	
Yes		51	
Safety goggles			
No		14	
Yes		40	
Special clothing			
No		1	
Yes		53	
ILO category			
Silicosis		2	
Suspicion of silicosis		3	
Normal		49	
Spirometric evaluation			
Obstructive pattern		4	
Restrictive pattern		1	
Normal		49	
Medicine use			**0.01**
No	42	46	
Yes	0	8	
Vitamin-mineral supplementation use			**0.03**
No	42	51	
Yes	0	3	

[a]mean ± standard deviation

of binucleated (BN) cells, karyorrhectic (KHC) cells, and nuclear buds (NBUD) (Table 2).

Older coal miners had significantly higher buccal MN frequencies than younger coal miners ($p<0.05$), but smoking, alcohol consumption, and use of protective equipment did not correlate with their MN frequencies (Table 3).

Multiple regression analysis showed significant effects of years of work at the mine on buccal MN frequencies (Table 4).

Chest X-ray and spirometry findings

Two workers had confirmed (diagnosed) and three suspect pneumoconiosis, while the remaining 49 workers had normal chest X-rays. According to the ILO classification, chest X-rays of the two miners with pneumoconiosis were category 1 and 2.

Table 2 Abnormal buccal epithelial cell frequencies (mean±SD per 1000 cells)

	Controls (n=42)	Coal miners (n=54)	p values
MN	3.07±2.50	**8.46±6.81***	**0.00***
BN	4.21±3.08	**10.44±6.99**	**0.00***
CC	2.38±2.68	5.14±7.16	0.58
KHC	2.42±3.68	**5.46±8.19**	**0.00***
KYL	7.88±10.52	8.00±7.46	0.06
PYC	4.45±3.42	7.50±9.18	0.26
NBUD	0.35±0.61	**0.81±1.02**	**0.01***

*p<0.05 compared to controls; BN cells – binucleated; CC – condensed chromatin; KHC – karyorrhectic; KYL – karyolytic; NBUD – nuclear bud; PYC – pyknotic; SD – standard deviation

Spirometric measurements showed FEV_1/FVC ratios below 75 % in two coal miners. Four showed signs of mild obstruction and one of mild restriction. Mean FEV_1/FVC and $FEF_{25-75\ \%}$ were 96.48±9.98 and 83.44±20.66, respectively.

Exposure measurements

Respirable total dust levels varied with mining operations involved (sections of the mine) and ranged from 0.57 to 2.81 mg/m^3. The mean concentration of total dust was 1.52±0.37 mg/m^3, which is lower than the 5 mg/m^3 respirable dust limit for coal mines in Turkey (20).

DISCUSSION AND CONCLUSION

Miners in our study had significantly higher MN, BN, KHC, and NBUD frequencies in buccal epithelial cells than controls, and MN frequencies significantly correlated with years of work at the mine. However, we found no significant effect of smoking and alcohol drinking on buccal MN frequencies. These findings are in line with earlier reports of increased DNA damage in coal workers from different countries (7, 8, 21–27) and contribute to the limited knowledge about the genotoxic effects of coal dust exposure in Turkish coal miners. One Turkish study (7) with 39 coal workers from the Armutcuk coal mine in the Zonguldak region found significantly higher sister chromatid exchange (SCE), chromosome aberration (CA), and MN frequencies in miners than controls. They also found significant effects of smoking on SCE and MN frequencies and of years of exposure on SCE and CA frequencies. In contrast, another Turkish study (28), which compared lymphocyte SCE and MN frequencies between coal workers (n=29), coal workers with diagnosed pneumoconiosis (CWP, n=23), and controls reported no significant differences between coal workers and controls but did report significantly higher SCE and MN frequencies in CWPs than in miners and controls. It also reported no effect of smoking, duration of exposure, and age.

The formation of MN in both lymphocytes and buccal epithelial cells has been used as a useful biomarker to evaluate the genotoxic damage in epidemiological studies (29–31), and our results support that MN formation in buccal epithelial cells is consistent with the model in lymphocytes (32). Most importantly, our findings have pointed out that the BMCyt assay can be very practical and clinically valuable as a method of early detection of DNA damage, long before coal miners develop pneumoconiosis. The majority of our workers had normal chest radiographs and spirometry, even though their buccal MN frequencies were significantly higher than in controls. While it has its limitations, its relatively non-invasive nature allows for more extensive screening of exposed workers to coal dust, even when respirable mean dust levels in the mine are below the Turkish occupational limit of 5 mg/m^3, which does not define the content of chemical ingredients in respirable dust. Of course, buccal findings could then be verified by other, more specific and more expensive genotoxicity assays such as comet assay, SCE, and CA tests to better predict DNA damage.

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Conflict of interest statement

None to declare.

Table 3 Buccal MN frequencies in coal miners and controls (mean±SD per 1000 cells)

Factors	Controls (n=42)	Workers (n=54)
Age (years)		
19–30	4.40±7.70	1.50±0.70
≥31	2.89±2.66	**8.73±6.80**[*,**]
Smokers	3.05±2.33	**8.51±6.61**[*]
Non-smokers	3.08±4.22	**8.35±7.45**[*]
Alcohol consumption		
No	3.10±3.54	**8.44±6.95**[*]
Yes	2.50±3.53	8.44±6.59
Duration of exposure (years)		
1–15		9.51±2.61
≥16		9.41±5.75
Using Protective Equipment		
Gloves		
No		9.00
Yes		8.43±6.90
Mask		
No		6.00±2.64
Yes		8.58 6.99
Safety goggles		
No		7.00±5.33
Yes		8.95±7.28
Special clothing		
No		9.00
Yes		8.43±6.90
ILO category		
Silicosis		10±1.41
Suspicion of silicosis		10.33±5.03
Normal		8±7.08
Spirometric evaluation		
Obstructive pattern		6.75±4.99
Restrictive pattern		4
Normal		9±7.02

[*]p<0.05 compared to controls; [**]p<0.05 compared between miners. ILO – International Labour Organization; SD – standard deviation

Table 4 Multiple linear regression analysis of studied parameters in miners and controls

	Buccal MN frequencies (‰) B (95 % CI)
Age	-0.1 (-0.3 to 0.1)
Alcohol consumption	0.0 (-4.9 to 2.9)
Smoking	0.0 (-2.8 to 2.1)
Years of work at the mine	**0.4 (0.2 to 0.5)***

*p<0.05; B – regression coefficient (slope); CI – confidence interval

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Procjena oštećenja DNA bukalnih epitelnih stanica primjenom *Cytome* inačice mikronukleus-testa

Cilj je ovoga istraživanja bio procijeniti oštećenje DNA u turskih rudara primjenom tzv. *Cytome* inačice mikronukleus-testa na bukalnim epitelnim stanicama (engl. *buccal micronucleus cytome assay*, krat. BMCyt) kao najmanje invazivne i stoga najpraktičnije metode koja bi mogla naći svoju primjenu u biomonitoringu rudara u ugljenokopima. Uzorci bukalnih epitelnih stanica prikupljeni su od 54 rudara i 42 kontrolna ispitanika iz Zonguldaka u Turskoj radi utvrđivanja učestalosti mikronukleusa (MN), binuklearnih stanica (BN), zgusnutoga kromatina (CC), kariooreksije (KHC), kariolize (KYL), jezgrinih pupova (NB) i piknoze (PYC). Osim toga, analizirali smo koliko čimbenici poput dobi, godina staža u rudniku, pušenja, pijenja alkohola i uporabe zaštitne opreme utječu na razlike u nalazima učestalosti MN-a. Dva su rudara imala potvrđenu dijagnozu pneumokonioze, u tri je rudara postojala sumnja na nju, a ostalih 49 rudara imalo je uredne rendgenske nalaze. Učestalosti MN-a, BN-a, KHC-a i NB-a bile su značajno više u rudara nego u kontrolnih ispitanika. Godine staža u rudniku također su značajno pridonijele povišenoj učestalosti MN-a u rudara, ali takva korelacija nije utvrđena za dob, pušenje i pijenje alkohola. BMCyt test pokazao se preciznim i praktičnim probirnim testom jer otkriva oštećenje DNA mnogo ranije nego što se pojavi pneumokonioza.

KLJUČNE RIJEČI: binuklearne stanice; epitelne stanice; genotoksičnost; karioliza; karioreksija; mikronukleus; piknoza; jezgrini pupovi; toksikologija radne sredine; ugljena prašina; zdravlje u industriji; zgusnuti kromatin