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Cytogenetic biomonitoring of road tunnel construction workers: buccal micronucleus cytome assay

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Key words: Road tunnel construction workers, Occupational exposure, Genotoxic hazard, Biomonitoring, Buccal micronucleus cytome (BMCyt) assay, Micronuclei, Nuclear buds

Parole chiave: Escavazione di tunnel stradali, Esposizione occupazionale, Rischio genotossico, Biomonitoraggio, Test del micronucleo (citoma) su cellule buccali (BMCyt), Micronuclei, Anomalie nucleari

Abstract

Introduction. *Tunnel construction workers are exposed to complex mixtures of toxic agents, some of which are known to be genotoxic. The aim of this study was to evaluate the genotoxic risk in this occupational setting by comparing tunnel workers with a control group for frequencies of nuclear aberrations in oral exfoliated cells.*

Methods. *To evaluate the genotoxic effects of tunnel air pollutants, we conducted a cross-sectional, molecular epidemiological study (35 tunnel workers and 35 healthy controls) using the buccal micronucleus cytome assay. A questionnaire was administered to obtain information about demographic variables, lifestyle, dietary habits, anthropometric data, and occupational history. Buccal mucosa cells were collected by scraping the buccal mucosa with a small-headed toothbrush. Coded slides were examined blind by trained scorers for micronuclei (MN), nuclear buds (NBUD), and other nuclear abnormalities.*

Results. *Road tunnel construction workers revealed higher frequencies of cells with genotoxic damage (i.e., MN and NBUD). MN and NBUD resulted to be Poisson distributed and counts of these genotoxicity biomarkers were then analysed by Poisson regression. The frequency ratio (FR) for MN was 1.31 (95% CI: 0.84-2.04), with an increase in the exposed subjects; this finding, though indicating a higher genotoxic risk in the exposed subjects, did not reach statistical significance. On the other hand, the FR for NBUD was 3.49 (95% CI: 1.86-6.56), with a statistically significant increased risk of chromosomal damage. Even the frequencies of binucleated cells (a marker of cell proliferation) and pyknotic cells (a cell death biomarker) were significantly higher in tunnel workers.*

Conclusions. *Our observations provide further knowledge and understanding of the occupational hazards of tunnel workers and confirm the complexity of effects (cytotoxic and genotoxic) probably induced by fumes and dust produced in underground operations.*

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Introduction

Underground tunnelling activities consist in excavating a tunnel by means of machines and/or explosives, and in supporting and finishing the tunnel using concrete and steel. Even though the presence of a ventilation system, providing localised removal of dust, heat or fumes during excavation, post-blasting, or other activities, tunnelling activity represents a particular indoor confined environment where the workers are exposed to a variety of continuously renewed sources of air contaminants, including silica dust, emissions from diesel engines, oil mist and gases (*i.e.*, nitrogen oxides, carbon monoxide and ammonia) (1-3). Particulate and gaseous air contaminants are related to the geology of the work site, and are generated by explosives, diesel-powered machines, concrete and products used for the lubrication of the machinery (4, 5). Studies on the health impact of dust, gas, and particulate matter (PM) exposure in tunnel construction workers have shown an increased risk of long- and short-term lung function decline and chronic obstructive pulmonary diseases (6-11), as well as an increase in cardiovascular morbidity and mortality (12).

Moreover, most of the indoor pollutants, typically present in this occupational setting, are genotoxic/carcinogenic (13, 14). Crystalline silica, in the form of quartz or cristobalite dust, has been classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1) (15). Based on sufficient evidence that such exposure is associated with an increased risk for lung cancer, the IARC has classified diesel engine exhaust in Group 1 (carcinogenic to humans) (16). Diesel exhaust is a complex mixture, consisting of thousands of compounds present as gases and PM, such as carbon oxide (CO), carbon dioxide (CO_2), nitrogen oxide (NO), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), volatile organic compounds (VOCs; including aldehydes, benzene, and toluene), and polycyclic aromatic hydrocarbons (PAH) (5). The IARC has reviewed experimental data for more than 60 individual PAHs and several of them have been classified as "probably" (Group 2A) or "possibly" (Group 2B) carcinogenic to humans (*e.g.*, dibenz[a,h]anthracene, and dibenzo[a,l]pyrene, classified in Group 2A) (17). Benzo[a]pyrene has been classified as carcinogenic to humans (Group 1) (18). PM, almost the most studied outdoor/indoor air pollutant, consists of particles commonly gathered into three size groups according to its nominal median aerodynamic diameter (AD): coarse particles with AD 2.5-10 μm (PM_{10}), fine particles with AD less than 2.5 μm ($PM_{2.5}$), and ultrafine particles with AD less than 0.1 μm ($PM_{0.1}$). PM (in outdoor air pollution) has been classified by the IARC as carcinogenic to humans (Group 1) (19); strong mechanistic evidence indicated that PM is mutagenic and carcinogenic to humans via genotoxicity.

Although tunnel construction workers are exposed to genotoxic compounds, few molecular epidemiology studies have been conducted to evaluate their genotoxic hazard. To the best of our knowledge, only three studies have been undertaken to evaluate the genotoxic risk associated with underground work activities (20-22), and none of these studies has focused on buccal epithelial cells. Human biomonitoring provides valuable information on environmental exposure from multiple sources and routes and helps to identify potential health risks. Cytogenetic methods play a crucial role in human biomonitoring studies and the buccal micronucleus cytome (BMCyt) assay is one of these most frequently used approaches (23-26).

The BMCyt assay is a minimally invasive method to determine the impact of exposure to toxic and genotoxic agents. This method relies on exfoliated cells rubbed

off from the mucosa of the oral cavity and allows to determine the frequency of cells with micronuclei (MN) and other nuclear abnormalities such as: nuclear buds (NBUD), binucleated cells (BN), abnormally condensed chromatin (CC), karyorrhexis (KR), karyolysis (KL), and pyknotic nuclei (PN). MN and NBUD are the main biomarkers for chromosomal damage and instability. MN originate during mitosis and can be induced by clastogens or aneugens. NBUD are indicative of gene amplification and originate from the process of elimination of amplified DNA, DNA repair complexes and possibly excess chromosomes from aneuploid cells; NBUD are characterised by having the same morphology as MN, with the exception that they are connected to the nucleus by a narrow or wide stalk of nucleoplasmic material, depending on the stage of the budding process. BN cells occur as a consequence of a failure in cytokinesis during cell division, and together with basal cells (BC) are considered as biomarkers for proliferative activity. PN may represent a mechanism of nuclear disintegration, whereas CC, KR, KL cells are manifestations of cytotoxic effects and appear as a consequence of processes leading to cell death (apoptosis and/or necrosis) (27). The rationale for the use of oral cavity epithelial cells in human biomonitoring studies is based on the evidence that: (i) about 90% of human cancers are of epithelial origin (28); (ii) buccal epithelial cells are the first target site for numerous potential hazardous substances ingested and inhaled (29); (iii) buccal epithelial cells reflect genotoxic events occurring in the dividing basal cell layer of the target organ 1–3 weeks earlier (30).

In our previous study we have evaluated primary and oxidative DNA damage (comet assay), sister-chromatid exchanges (SCE), and micronuclei (MN) in peripheral blood lymphocytes of 39 workers from three different tunnel construction sites in Central Italy (20). The results showed a significant increase of MN frequency in exposed subjects compared with controls, and data obtained suggested that a genotoxic risk in tunnel construction workersw could not be excluded.

The aim of the present study was to further evaluate the genotoxic risk in this occupational setting by comparing tunnel workers with a control group for frequencies of MN, NBUD and other nuclear abnormalities in buccal exfoliated cells, by applying the BMCyt assay.

Materials and Methods

1. Study design, workplace, and population characteristics

The present approach represents a cross-sectional, molecular epidemiologic study. The study was conducted between June 2013 and April 2014. Biological samples were collected from workers operating inside a road tunnel (final length 2,391 m) under construction near the village of Pale – Municipality of Foligno, Umbrian Apennines – along the State Highway 77 Var "Val di Chienti" which was conceived to connect the cities of Foligno (Umbria Region) and Civitanova Marche (Marche Region). Layout of the State Highway 77 Var "Val di Chienti" and geographic coordinates of the Pale tunnel are shown in Figure 1.

The Pale tunnel was excavated by the common technique of drilling and blasting. Sampling sessions were performed in the West yard (Foligno side) during dislodging of rocks which were not completely released during the blasting procedure (after the loose rocks have been dislodged from the working face by an excavator, blasted materials and rubbles are loaded onto dump trucks and carried out from the tunnel) and in the East yard (Civitanova Marche side) (i) during drilling and (ii) during scaling (removing of loose rocks from the walls) and grouting with

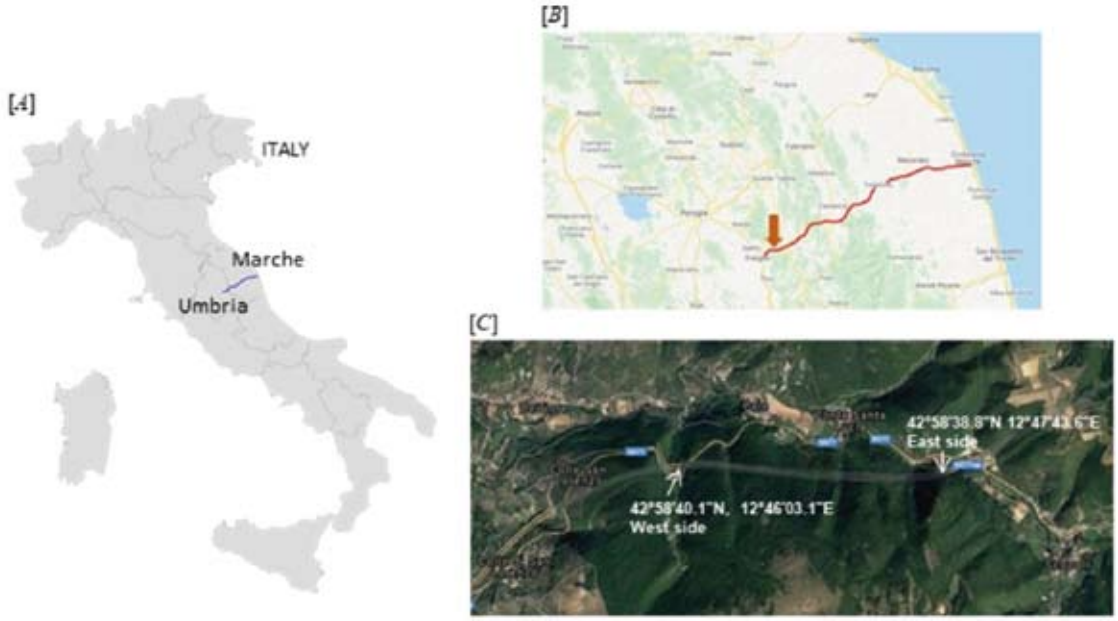

Figure 1 - Layout of the State Highway 77 Var "Val di Chienti" [A, B] and geographic coordinates of the Pale tunnel [C].

quick-drying shotcrete. A forced ventilation system was installed inside the tunnel.

All the workers (n = 40) involved in the construction of the Pale road-tunnel were invited to participate in the study. Workers who have been exposed to radiations six months before biological sampling (n = 5) were excluded and the final analytical sample included 35 male tunnel construction workers. The control group comprised 35 non-exposed male subjects recruited from the staff of the University of Perugia (academic and administrative personnel); the control subjects had no history of exposure to potential genotoxic substances. Exposed subjects were matched to controls for exposure to the principal confounders (*i.e.*, age and smoking) on an individual basis by pairing each road tunnel worker with a control of the same age (± 1 year) and smoking habits. Complete matching for other important confounding factors for genoma damage (*e.g.*, alcohol intake, dietary habits, BMI) was not possible because of the limited pools of potential controls. For these other confounding factors we researched the overall similarity of exposed and control groups and no particular importance was placed on the matched pairs themselves.

The Ethics Committee (*i.e.*, Comitato Etico Aziende Sanitarie della Regione Umbria, CEAS) approved the study (Cod. BioNP2012). All participants were informed about the objectives of the study and provided their written informed consent.

2. Questionnaire

Trained personnel administered a questionnaire designed to obtain information about demographic variables (age, ethnicity and educational attainment), lifestyle (smoking/e-smoking and alcohol use), dietary habits, anthropometric data, and occupational history (including tunnel jobs title, tasks, duration of work).

Body mass index (BMI) was calculated (weight /height^2) and nutritional status was classified according to the following cut-points: BMI 18.5-24.9 = normal weight; BMI 25-29.9 = overweight; BMI 30 or

greater = obesity.

About smoking, we have considered: (i) smokers, persons who reported smoking every day or subjects who had quit smoking less than 6 months before; (ii) non-smokers, subjects that have never smoked or subjects who had quit smoking more than 6 months before and did not smoke since then.

Alcohol consumption was assessed asking type of beverages (beer, wine, and liquor) and quantity consumed during the previous week. The amount of ethanol in each drink was calculated by using an ethanol conversion factor as described in the NESARC study (31) and the total ethanol consumption was estimated by summing ethanol contents in individual beverage types. The resulting volume of ethanol intake was divided by 7 to yield average daily ethanol intake (ADEI).

Because numerous potential carcinogens are formed in meat or fish cooked at high temperatures (*i.e.*, heterocyclic amines and polycyclic aromatic hydrocarbons), subjects were asked about the weekly frequency of consumption of meat and fish grilled or fried. Data about consumption of processed meat were also recorded. As a plant-based diet protects against oxidative stress, recruited subjects were asked to provide information on number of servings of fruits and vegetables consumed weekly.

3. Chemicals and media

All reagents used were of analytical grade. Ethanol was obtained from J.T. Baker Chemicals (Deventer, The Netherlands); Dulbecco's phosphate-buffered saline pH 7.3 (PBS), polyethylene glycol and DePex mounting medium were from VWR International PBI Srl (Milan, Italy); acetic acid, methanol, potassium phosphate monobasic (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4) and Giemsa stain solution were purchased from Carlo Erba Reagents Srl (Milan, Italy). Nylon filters (100 μm) were from Merck Spa (Milan, Italy); 18G needles were obtained from Becton Dickinson Italia SpA (Milan, Italy). Conventional microscope slides and cover-slips were supplied by LLG Labware (Meckenheim, Germany). Distilled water was used throughout the experiments.

4. Sample collection and buccal micronucleus cytome (BMCyt) assay

Tunnel construction workers were sampled after at least a 4-weeks working period. The enrolled subjects (exposed subjects and controls) were asked to rinse their mouth twice with mineral water to remove excess of debris and unwanted particles. Exfoliated cells were collected by scraping gently the buccal mucosa from both cheeks with a small-headed toothbrush. As the frequency of BC may be influenced by sampling intensity, to avoid this bias sampling was always performed using the same protocol by the same operator. After scraping, the head of the toothbrush was then placed into Saccomanno's fixative (50% ethanol and 2% polyethylene glycol, vol/vol, diluted in water) and rotated repeatedly such that the cells were released into the buffer. The collected buccal cells were centrifuged for 10 minutes at 1,800 rpm, the supernatant was discarded, and the cells were washed twice with 5 mL phosphate-buffered saline (PBS; 0.01 M, pH 7.3). The pellet was resuspended in 5 mL of PBS, with cells drawn by a syringe fitted with an 18-gauge needle. The suspension was then passed through a 100 μm nylon filter in order to increase the number of single cells in suspension. The cells were further spun at 1,800 rpm for 10 minutes and finally resuspended in a mixture of cold methanol and acetic acid (3:1) to obtain 2×10^6 cells/mL. For each individual, two slides were prepared by smearing 100 μL of cell suspension onto pre-cleaned slide.

The slides were stained with 4% Giemsa in Sörensen phosphate buffer (0.06 M, pH 6.8), air-dried, and then mounted in DePex

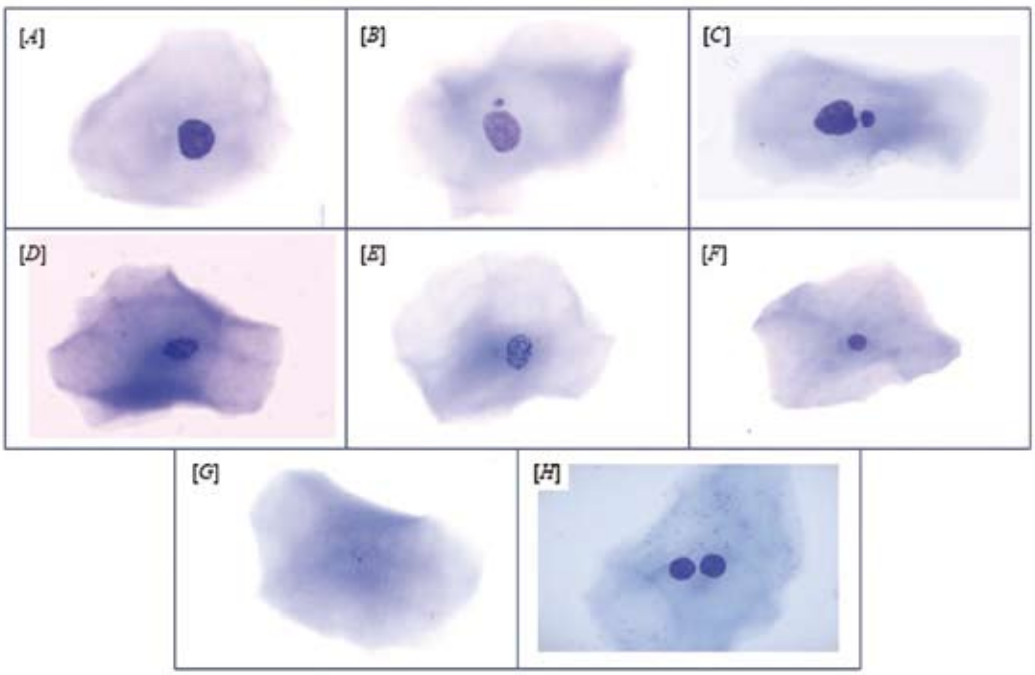

Figure 2 - Representative images (!1,000 magnification) of cells scored using the buccal micronucleus cytome assay: [A] differentiated cell, [B] differentiated cell with micronucleus, [C] differentiated cell with nuclear bud, [D] cell with condensed chromatin, [E] karyorrhectic cell, [F] pyknotic cell, [G] karyolytic cell; [H] binucleated cell.

(32, 33). For cytome analysis, microscope observation was performed by following the criteria proposed by Bolognesi et al. (34). Coded slides were examined blind at ×1,000 magnification by trained scorers supervised by an experienced scorer. The frequency of all the various cell types was determined in a minimum of 1,000 cells, then the frequency of DNA damage biomarkers (MN and NBUD) was scored in a minimum of 2,000 differentiated cells with well-preserved cytoplasm (35). Photomicrographs showing typical examples of cells scored in the BMCyt assay are shown in Figure 2.

5. Statistical analysis

Data from tunnel construction workers were compared with those from the reference group matched for age and smoking habits. Before conducting statistical analysis, the distribution of variables was tested for normality by the Kolmogorov-Smirnov test with Lilliefors correction. Quantitative variables were summarised using mean and standard deviation (SD) for normally distributed data, median and interquartile range (IQR) otherwise. Differences for nuclear anomalies between tunnel workers and control subjects were analysed by applying the Student's *t*-test or the ANOVA with Bonferroni adjustments when subgroups were considered. Variables not normally distributed were analysed by the non-parametric Mann-Whitney *U*-test. Categorical variables were expressed as percentage and compared by Pearson χ^2 test.

The risks of chromosomal damage (MN and NBUD) associated with occupational exposure were estimated by computing frequency ratios (*i.e.*, FR = e^{β}, with e = 2.71828, and β = regression coefficient) and 95% confidence intervals (CI) from univariate and multivariate Poisson regression models. For categorical variables, the FR indicates the proportional increase/decrease of nuclear anomalies frequency in a comparison group relative to the referent; whereas, for continuous variables the FR represents the proportional increase of nuclear anomalies frequency due to the increase of one unit of the variable evaluated (36). Potential confounding variables included in the final models were age (continuous variable) and smoking and drinking status (dichotomous variables).

IBM SPSS Statistics 20.0 for Windows (IBM CO., Armonk, NY, USA) was used for data analysis; $p < 0.05$ was considered as statistically significant.

Results

Demographic and other general characteristics of the studied groups are shown in Table 1. There were no statistically significant differences in age, ethnicity, BMI, smoking and drinking between tunnel workers and controls. As regards dietary habits, tunnel construction workers have shown to consume greater amounts of fried foods, with a statistically significant difference. The education level in tunnel workers was significantly lower than that in the control group ($p < 0.05$).

Table 2 gives an overview of occupational characteristics of tunnel workers included in this study, work history included present occupation and length of service. Each underground worker was assigned to one of the following five sets of exposure: 1) drilling and blasting workers; 2) excavator, wheel loader and truck operators; 3) shotcrete workers; 4) carpenters; 5) support workers. The majority of participants were drilling and blasting workers (42.8%) and their work consisted in drilling boreholes in the rock to place charges or explosives for blasting. After blasting, loose rocks are dislodged from the working face by excavators, and blasted materials and rubbles are loaded onto dump trucks with wheel loaders and carried out from the tunnel. Unsafe rock is then fastened with steel bolts and sealed by spraying wet concrete (shotcrete workers and carpenters). Support workers are employed for mounting ventilation systems, maintenance and repair of machines, and installation of electrical power supply. All the workers reported proper use of personal protective equipment, such as filtering facepiece respirators, shoes with protective toecaps, helmets, gloves, and earmuffs.

The results of BMCyt assay in tunnel workers and controls are summarized in Table 3. Tunnel construction workers revealed a higher frequency of cells with nuclear damage (MN and NBUD) as compared with control subjects. Similarly, the frequencies of BC (a marker of cell-proliferation) and of PN (with about a two-fold increase) were significantly higher in tunnel workers than in controls. No statistically significant differences were observed for frequencies of biomarkers of cytokinetic-defects (BN cells) and other cytotoxicity parameters (KR, KL and CC cells). Stratification by age, ethnicity, level of education, smoking habits, alcohol intake, dietary patterns, and BMI did not show differences for cytome abnormality frequencies between exposed and controls, as well as among the groups (data not shown).

MN and NBUD resulted to be Poisson distributed and counts of these genotoxicity biomarkers were then analysed also by Poisson regression. Occupational exposure was used in the primary analysis as an independent variable (Table 4). The FR for MN was 1.31 (95% CI: 0.84-2.04), with an increase in the exposed subjects; this finding,

Table 1 - General characteristics of the study population and statistical analysis of the differences.

Variables	Tunnel workers	Controls	*p*-value[4]
Age (years)[1]	40.31 ± 10.45	39.11 ± 10.60	n.s. [a]
< 40 years[2]	18 (51.4%)	20 (57.1%)	n.s.[b]
≥ 40 years[2]	17 (48.6%)	15 (42.9%)	
Ethnic groups[2]			
Italian	31 (88.6%)	33 (94.3%)	n.s.[b]
European	4 (11.4%)	1 (2.9%)	
Extra-European	0 (0.0%)	1 (2.9%)	
Level of education[2]			
Primary	25 (71.4%)	4 (11.4%)	<0.001[b]
Secondary or higher	10 (28.6%)	31 (88.6%)	
Smoking habits[2]			
Non-smokers	19 (54.3%)	19 (54.3%)	n.s.[b]
Smokers	16 (45.7%)	16 (45.7%)	
Alcohol intake[2]			
Abstemious	20 (57.1%)	16 (45.7%)	n.s.[b]
Drinkers	15 (42.9%)	19 (54.3%)	
ADEI (g/day)*	7.94 ± 7.69	8.09 ± 5.79	n.s.[a]
Dietary habits			
Fried foods[2]	20 (57.1%)	16 (45.7%)	n.s.[b]
- servings/week[1]	2.50 ± 1.10	1.38 ± 0.98	0.020[a]
Grilled foods[2]	18 (51.4%)	19 (54.3%)	n.s.[b]
- servings/week[1]	2.07 ± 1.33	1.40 ± 0.70	n.s.[a]
Processed meat[2]	29 (82.9%)	32 (91.4%)	n.s.[b]
- servings/week[1]	2.66 ± 1.59	2.75 ± 1.78	n.s.[a]
Vegetables[2]	34 (97.1%)	34 (97.1%)	n.s.[b]
- servings/week[1]	13.00 ± 5.79	13.06 ± 7.89	n.s.[a]
BMI[2,3]	26.88 ± 3.16	25.58 ± 3.55	n.s.[a]
Normal weight	9 (25.7%)	17 (48.6%)	n.s.[b]
Overweight	21 (60.0%)	13 (37.1%)	
Obese	5 (14.3%)	5 (14.3%)	

[1] Group mean ± standard deviation.

[2] Number of subjects with characteristics and % (between brackets), respectively.

[3] Body mass index (BMI) was calculated as weight/height^2 (kg/m^2) and nutritional status was classified according to the following cut-points: normal weight, BMI between 18.5-24.9; overweight, BMI between 25-29.9; obesity, BMI of 30 or greater.

[4] Differences between road tunnel workers and controls were investigated using the Student's *t*-test, comparisons among proportions were performed using the Pearson's χ^2 test.

even indicating a higher genotoxic risk in the exposed subjects did not reach statistical significance. On the other hand, the FR for NBUD was 3.49 (95% CI: 1.86–6.56), with a statistically significant increased risk of chromosomal damage. Multivariate Poisson regression analyses were performed with age, smoking habits, and alcohol consumption included as potential confounders. MN and NBUD frequencies were found not to be associated with age, smoking, or alcohol use (data not shown).

Table 2 - Main characteristics of exposed subjects (road tunnel workers).

Work characteristic	
Years working in underground (tunnels)[1]	12.46 ± 10.24
≤ 20 years[2]	29 (82.9%)
> 20 years[2]	6 (17.1%)
Job tasks[2]	
Drilling and blasting workers	15 (42.9%)
Excavator, wheel loader and truck operators	7 (20.0%)
Shotcrete workers	2 (5.7%)
Carpenters	7 (20.0%)
Support workers	4 (11.4%)

[1] Group mean ± standard deviation.
[2] Number of subjects with characteristics and % (between brackets), respectively.

Table 3 - Rates of nuclear abnormalities in buccal epithelial cells in road tunnel construction workers and controls. Group values are summarized as the mean ± SD or the median and [IQR], for data distributed normally or not-normally, respectively.

Nuclear abnormalities (‰)[a]	Tunnel workers	Controls	*p*-value[b]
Genotoxicity biomarkers			
MN	2.0 [1.0]	1.0 [1.0]	0.029
NBUD	2.0 [2.0]	0.5 [1.5]	<0.001
Cell proliferation biomarkers			
BC	15.60 ± 11.59	9.17 ± 5.28	0.004
BN	6.09 ± 3.19	5.54 ± 3.17	n.s.
Cell death biomarkers			
KR	43.43 ± 27.15	33.69 ± 19.93	n.s.
KL	77.77 ± 42.74	83.06 ± 39.90	n.s.
PN	5.26 ± 3.88	2.60 ± 1.65	<0.001
CC	65.23 ± 21.54	70.86 ± 24.92	n.s.

[a] MN, micronuclei; NBUD, nuclear buds; BC, basal cells; BN, binucleated cells; KR, karyorrhectic cells; KL, karyolitic cells; PN, pyknotic nuclei; CC, condensed chromatin cells; nuclear anomalies per 1,000 buccal cells.
[b] Differences between road tunnel construction workers and controls were investigated using the Student's *t*-test or the Mann-Whitney *U*-test (two-tailed).

Table 4 - Influence of occupational exposure (*i.e.*, working in underground yards, road tunnel construction) on the frequency of genotoxicity biomarkers (*i.e.*, MN and NBUD): Poisson regression analysis.

	MN (‰)[a]		NBUD (‰)[a]	
	FR (95% CI)	*p*-value	FR (95% CI)	*p*-value
Controls	1 (reference)		1 (reference)	
Tunnel workers	1.31 (0.84–2.04)	n.s.	3.49 (1.86–6.56)	<0.001

[a] MN, micronuclei; NBUD, nuclear buds; nuclear anomalies per 1,000 buccal cells.

Table 5 - Influence of job tasks of road tunnel construction workers on the frequency of genotoxicity biomarkers (*i.e.*, MN and NBUD): Poisson regression analysis.

	MN (‰)[a]		NBUD (‰)[a]	
	FR (95% CI)	*p*-value	FR (95% CI)	*p*-value
Excavator, wheel loader and truck operators Shotcrete workers Carpenters	1 (reference)		1 (reference)	
Drilling and blasting workers Support workers	1.50 (0.90–2.50)	n.s.	0.82 (0.53–1,28)	n.s.

[a] MN, micronuclei; NBUD, nuclear buds; nuclear anomalies per 1,000 buccal cells.

Furthermore, we have evaluated the influence of job tasks on all nuclear anomalies. Length of service did not significantly affect the frequency of DNA damage biomarkers, even though the frequencies of MN, NBUD, and some parameters of cell proliferation and cell death (BN, KL, PN, CC) were higher in subjects with a length of service longer than 20 years, with respect to those having less than 20 years of employment (data not shown). Influence of job tasks on the frequency of genotoxicity biomarkers (*i.e.*, MN and NBUD) was evaluated also by Poisson regression analysis (Table 5). Because of the relatively small sample size, exposed workers were gathered in only two sub-groups by considering working activities located at the digging face or along the excavated tunnel. Even though the results did not reach statistical significance, the trends indicate different kinds of genotoxic effects associated with different job tasks/activities. At the digging face, drilling and blasting workers showed a higher frequency of MN; whereas, excavator, wheel loader and truck operators, shotcrete workers, and carpenters, mainly working along the tunnel barrel, showed a higher frequency of NBUD.

Discussion and Conclusions

In our study, we investigated the frequencies of nuclear abnormalities in exfoliated buccal cells of tunnel workers and control subjects. As above mentioned, tunnel workers might be exposed to complex mixtures of toxic materials that have been identified as potential health hazards. Tunnel workers enrolled in the present study were involved mainly in drilling and blasting operations. Other important tasks were operating excavators, wheel loaders and trucks, and carpentry activities. Hence, contaminants mainly present in the work environment were diesel exhaust, particulate matter and blasting fumes (as NO, ammonia, and VOCs) (4).

The aim of this study was to evaluate the genotoxic risk in this occupational setting by comparing tunnel workers with a control group for frequencies of nuclear aberrations in oral exfoliated cells. We have applied the BMCyt assay that in recent decades was proposed to determine nuclear abnormalities due to toxic or genotoxic substances (6, 35) on the basis of that more than 90% of cancers have an epithelial origin (37) and that buccal mucosa is the main targets of the genotoxic agents (38).

Studies evaluating one of the potential genotoxic effects of exposure to aerosols and gases among tunnel construction workers are still scarce; most of studies characterized and assessed exposure to contaminants during tunnel activities (39, 40), and analysed relationship between exposure to air contaminants and respiratory or cardiovascular diseases (8, 41).

The present study is an explorative

approach for the biomonitoring of occupational exposure to air contaminants during tunnel operations. Both exposed and control groups showed a high inter-individual variability for all the evaluated endpoints, probably due to the small number of studied subjects and/or to confounding factors not identified in our work. Anyway, the main result of our study was a significant higher frequency of NBUD, BC and PN cells in tunnel workers compared with controls.

The NBUD frequency is a sensitive biomarker of DNA damage and probably indicates chromosome breakage/translocation. In a case-control study, the Authors reported an increase of NBUD frequencies in peripheral lymphocytes associated with an increase of lung cancer risk (42). In our study, the significant increase of NBUD might be indicative of occupational exposure to mixtures of potentially carcinogenic substances. This finding is in agreement with the results reported by Duan et al. (43) and Zhang et al. (44), who observed an increased NBUD frequency associated to exposure to polycyclic aromatic hydrocarbons. In our work, NBUD frequency reaches the highest values in subjects with 20 years or more of job seniority, thus suggesting that exposure duration might be relevant for the development of health effects.

We also observed a higher, even if non statistically significant, MN frequency in epithelial buccal cells of tunnel workers compared with controls; the difference did not reach the level of statistical significance probably because of the high inter-individual variation. In any case, the observed trend corroborates our previous findings showing a significant higher MN frequency in peripheral blood lymphocytes of tunnel construction workers compared with controls (20). In a large prospective study, the Authors have provided evidence that MN frequency (the only biomarker allowing the simultaneous evaluation of both clastogenic and aneugenic effects) in lymphocytes is a predictive biomarker of cancer risk within a population of healthy subjects (45). The BMCyt assay is a well-validated method and a recent review showed a significant correlation between the increase in the frequency of MN in peripheral lymphocytes and buccal cells in genotoxic-exposed or diseased groups (46). This observation supports the hypothesis that similar genotoxic events might cause MN formation in both tissues.

BMCyt assay also provides endpoints for cell death events and cellular proliferation. In our study, we observed significant higher PN (indicative of cell death) and BC (indicative of proliferation) frequencies in tunnel workers compared with controls. Therefore, it could be supposed that fumes and dusts in tunnel construction environment might be cytotoxicants thus inducing cellular death in buccal mucosa. As consequence, we observed an increase in the regenerative capacity of this tissue, an activity that could be linked to an enhanced carcinogenesis risk.

When the exposed subjects were grouped by job categories, we observed that excavator, wheel loader and truck operators had the greatest NBUD frequency. Within each of the underground operations, generally the highest exposure levels were found for workers driving diesel-powered equipment, and for workers located at the digging face (47). Thus, it could be supposed that the increase in NBUD frequency observed in tunnel workers operating excavators, wheel loaders and trucks might be due to a potential exposure to greater concentrations of a number of contaminants. In a recent, *in vitro* approach the same cytogenetic effects (NBUD) were mainly caused by fine PM fractions (AD < 0.25 μm) sampled 25 m far from the digging face during drilling (13).

In this study, we have considered individual factors that might have had confounding effects on frequencies of all nuclear abnormalities, such as age, alcohol use, and smoking habits, and have not found significant effects of either

of those variables.

The present study is characterised by both positive (strengths) and negative (weaknesses) aspects.

A weakness of the study could be the limited number of subjects enrolled. However, this limit was the consequence of the size of the workforce in this occupational setting. Moreover, Giemsa staining has been recently proposed as being suboptimal for exfoliated buccal mucosa cells (48), because of the risk of an overestimation of MN formation with non-specific stain. We consider the adopted scoring procedure, supervised by an experienced scorer (*e.g.*, in case of major differences, the coded slides were scored again), was adequate for controlling this bias.

As regards the strengths, to the best of our knowledge, this is the first study applying the BMCyt assay to assess MN and other nuclear abnormalities associated with exposure to cyto- or genotoxic contaminants during tunnelling activities.

In conclusion, underground tunnelling activities provide significant challenges in terms of exposure to dusts and fumes. Over the years, management of occupational risks has led to a reduction of exposure to contaminants during tunnel construction work: ventilation was improved, new types of explosive were employed, and diesel-powered machines were equipped with particle filters.

In our study, even though the totality of the workers used protective measures which can reduce the exposure, we observed a significant increase in toxic and genotoxic damage. Our findings provide further knowledge and understanding to the occupational hygiene and confirm the complexity of effects (toxic and genotoxic) induced by fumes and dust produced in underground operations.

On the basis of our results, we recommend the implementation of measures to reduce exposure to as low as is reasonably practicable and to ensure a safe working environment. Appropriate interventions might concern the increase of use of battery powered vehicles and equipment, and the implementation of health surveillance activities.

We highlight that our results, even obtained on a limited number of subjects, showed that the exfoliated buccal cells (first contact site between airborne contaminants and body), and the BMCyt assay could be useful in the surveillance of populations potentially exposed to cyto-genotoxic contaminants.

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Authors' contributions: MM and MV designed the study, applied for Research Ethics Board approval, and analyzed the data. EG identified and recruited suitable participants, collected biological samples and questionnaire data. CF, LD, and SV performed the micronucleus cytome assay. MM, MV, MA and VG reviewed literature. MV wrote the first draft of the manuscript. All authors contributed to the interpretation of the results, performed a critical revision of the manuscript, and approved the final version before submission. The corresponding author had full access to the totality of data in the study and had the responsibility for guaranteeing the integrity of the data and the decision to submit the manuscript for publication.

Riassunto

Valutazione degli effetti citogenetici in lavoratori impegnati nella escavazione di un tunnel stradale: test del micronucleo (citoma) su cellule esfoliate buccali

Introduzione. I lavoratori impiegati nella escavazione di tunnel stradali sono esposti a miscele complesse di agenti tossici, alcuni dei quali con spiccate proprietà genotossiche. La presente ricerca è stata finalizzata a valutare il rischio genotossico in questo particolare comparto occupazionale analizzando la frequenza di alterazioni nucleari nelle cellule esfoliate della mucosa buccale in un gruppo di lavoratori in attività in un cantiere nel sottosuolo in confronto ad un gruppo di controllo.

Metodi. L'approccio di epidemiologia molecolare utilizzato per la valutazione degli effetti genotossici conseguenti alla esposizione ad inquinanti aerodispersi è stato condotto applicando un disegno *cross-sectional*: in 35 lavoratori esposti e 35 soggetti di controllo la frequenza di alterazioni nucleari è stata valutata utilizzando il test del micronucleo (citoma) su cellule buccali esfoliate. A tutti i soggetti partecipanti allo studio è stato somministrato un questionario per raccogliere informazioni riguardo le principali caratteristiche demografiche, gli stili di vita, le abitudini alimentari, dati antropometrici e storia occupazionale. Per la determinazione della frequenza di micronuclei (MN), gemmazioni nucleari (NBUD), e altre anomalie nucleari, le cellule esfoliate della mucosa buccale sono state raccolte spazzolando delicatamente la superficie interna delle guance; l'analisi al microscopio è stata condotta in cieco su vetrini codificati.

Risultati. I lavoratori impegnati nel cantiere nel sottosuolo per la escavazione di un tunnel stradale hanno mostrato una frequenza maggiore di cellule con anomalie nucleari (MN e NBUD), in confronto al gruppo di controllo. MN e NBUD mostrano tipicamente una distribuzione poissoniana ed i dati relativi a questi biomarcatori di genotossicità sono stati quindi analizzati mediante la regressione di Poisson. Il rapporto di frequenza (FR) per i MN è risultato pari a 1.31 (95% CI: 0.84–2.04), con un aumento nei soggetti esposti; il risultato, pur indicando un più alto rischio genotossico nei lavoratori esposti, non raggiunge la significatività statistica. D'altro canto, il FR per le gemmazioni nucleari (NBUD) è risultato pari a 3.49 (95% CI: 1.86–6.56), con un rischio genotossico significativamente più elevato nei soggetti esposti, rispetto al gruppo di controllo. Anche le frequenze di cellule binucleate (biomarcatore di proliferazione cellulare) e picnotiche (biomarcatore di citotossicità) sono risultate significativamente più elevate nei lavoratori impegnati nella escavazione del tunnel stradale.

Conclusioni. I risultati del nostro studio forniscono ulteriori evidenze riguardo i rischi espositivi occupazionali connessi con la escavazione di tunnel stradali, e confermano la complessità degli effetti (sia citotossici che genotossici) probabilmente indotti dai fumi e dalle polveri prodotti durante le attività del cantiere nel sottosuolo.

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