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Evaluation of genotoxic and cytotoxic effects in buccal mucosa cells of welders in the cities of Cubatão and Santos, state of São Paulo, Brazil

Avaliação de danos genotóxicos e citotóxicos em células da mucosa bucal de soldadores nas cidades de Cubatão e Santos, São Paulo

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ABSTRACT | Background: Approximately 5 million workers are estimated to be occupationally exposed to welding fumes worldwide. Nickel and chromium are genotoxic metals found in welding fumes, therefore welders are exposed to these metals at the workplace. **Objective:** The objective of the present study was to investigate the frequency of cytotoxic and genetic damage in cells harvested from the oral mucosa of welders and also from a group of workers not exposed to metallic fumes. **Methods:** A total of 44 individuals, divided into 2 groups — welders and non-welders — were compared using the micronucleus assay technique and cell death (pyknosis, karyorrhexis and karyolysis) on buccal mucosa cells of welding workers. The examined cells were stained with Feulgen/Fast Green. **Results:** Welders exhibited higher frequency ($p<0.05$) of cytotoxicity than the group of volunteers not exposed to metallic fumes. **Conclusion:** The results of this preliminary study suggest that the frequency of cytotoxic damage in buccal mucosa cells might be higher among welders compared to non-welders.

Keywords | chromosome-defective micronuclei; DNA damage; oxidative stress; genotoxicity; cell death.

RESUMO | Contexto: Estima-se que no mundo existam, aproximadamente, 5 milhões de trabalhadores expostos ocupacionalmente à fumaça de soldagem. Níquel e Cromo são metais que podem ocasionar danos ao material genético, e soldadores, por força do ofício, são rotineiramente expostos a eles. **Objetivo:** O objetivo do presente estudo foi investigar a frequência de danos citotóxicos e genotóxicos em células da mucosa bucal de um grupo de soldadores. M**étodos:** Um total de 44 indivíduos, distribuídos em 2 grupos — soldadores e não soldadores —, foi comparado utilizando a técnica do ensaio do micronúcleo e morte celular (picnose, cariorrexe e cariólise) em células da mucosa oral de trabalhadores de soldagem. As células examinadas foram coradas com Feulgen/Fast-green. **Resultado:** Os soldadores apresentaram maior frequência ($p<0,05$) de alterações indicativas de citotoxicidade quando comparados ao grupo de indivíduos não expostos a fumos metálicos. **Conclusão:** Os resultados deste estudo preliminar sugerem que soldadores apresentam maior frequência de danos citotóxicos e morte celular em células da mucosa bucal que trabalhadores não expostos.

Palavras-chave | micronúcleos com defeito cromossômico; dano no DNA; estresse oxidativo; genotoxicidade; morte celular.

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INTRODUCTION

According to estimates, about 5 million workers are exposed to welding fumes[1] at the workplace.

Such workers might be exposed to chromium (Cr) and nickel (Ni) contained in welding fumes[2]. Although the type and concentration of metals vary as a function of the type of welds and materials to be welded, Cr and Ni are fundamental components, because they afford metals high resistance to corrosion[3].

Environmental — physical, biological or chemical — contaminants are able to modify and/or damage the human genetic material, thus causing mutations[4]. There are several routes for exposure to occupational contaminants, as e.g., through the skin or ingestion, but inhalation is the main one for welders[5]. The effects of metals such as Ni and Cr (VI) can be direct or indirect. The former involve enzyme inhibition, chelation of vital elements and binding to protein sulfhydryl groups. Indirect effects commonly involve activation of the NF-κB, AP-1 and P53 signaling pathways and oxidative biomolecule damage[6]. Cr (VI) is found in welding procedures[7]; it gives rise to intermediate forms in cells involving Fenton's reaction, with consequent generation of free radicals through the interaction of water metabolites with metals, which might favor the occurrence of structural DNA damage, such as strand breaks, chromosomal aberrations and crosslinking, as well as signaling cells to death[8-10].

As a result, one of the prophylactic measures to reduce the occurrence of occupational intoxication is biological monitoring[11]. Methods for detection, and consequently measurement of the risk for human populations exposed to various, even suspected, carcinogens are increasingly being looked for. Such assays were successfully applied to the identification of dietary, occupational, environmental and genetic factors that have significant impact on the genome stability[12]. Micronucleus assay of the oral mucosa is a biomarker used in tests assessing mutagenic events, also because the oral epithelium is one of the first areas of contact with substances arriving to the body through the oral and inhalation routes[13,14].

The aim of the present study was to investigate the frequency of mutagenic abnormalities and cytotoxicity on buccal mucosa cells of welders as a function of the latter's exposure to metal particles generated during welding.

METHODS

STUDY PARTICIPANTS

The sample comprised 44 male welders working at various metallurgical companies in the cities of Cubatão and Santos, São Paulo, Brazil from July through September 2016. The sample was divided in two groups — exposed and not exposed to metals — with 22 participants each. The group of participants not exposed to metals was selected as a function of their occupational status, the priority being given to individuals working in education, commerce, health care and research, to ensure a population without contact with known genotoxic agents to reduce possible confounding factors. Data on the personal characteristics of the participants were collected and are described in Table 1. We included individuals in apparent good state of health, not exposed to x-rays 15 days before data collection, did not use medications for therapeutic purposes, did not exhibit infectious or neoplastic lesions on the oral mucosa and without history of neoplasms. The participants were matched per sex, age and smoking habit always in compliance with the inclusion criteria to ensure the reproducibility of the study. The study

Table 1. Demographic characteristics of individuals occupationally exposed or not to metals, Cubatão (SP, Brazil), 2016 (N=44)

Characteristics	Welders (N=22)	Non-welders (N=22)
Average age (years)	41.34	34.05
Working hours/day	8	7
Length of work in the profession (years)	14.10	-
Use of protective equipment	20 (90.90%)	

was approved by the research ethics committee of Paulista University (UNIP), ruling no. 1.555.96, CAAE (Certificate of Presentation for Ethical Appraisal) no. 55441816.2.0000.5512.

SAMPLE COLLECTION, SLIDE PREPARATION AND ANALYSIS

The micronucleus assay was conducted with buccal mucosa cells according to the method described by Meireles et al.[15]. The participants were requested to rinse their mouths with tap water. Next samples were collected with a wooden spatula, smoothly rubbing the cheek mucosa, placed in Falcon tubes containing cooled normal saline and fixated with 3:1 acetic acid/methanol solution. For staining, slides were immersed in 5N HCl solution for 15 minutes, rinsed and placed in distilled water for 15 minutes. Next they were stained with Schiff's reagent (Merck, Darmstadt, Germany) for 90 minutes and counterstained with 1% Fast Green (Merck, Darmstadt, Germany) for about 30 seconds. After staining, the slides were dehydrated through 3 absolute alcohol baths, clarified with 2 xylene baths and mounted with coverslips using Entellan mounting medium (Sigma). The slides were examined under microscope at 400x magnification; 1,000 cells were assessed per individual.

The parameters adopted for cell counting were:

- normal cells with oval or round nucleus, largest size and smallest nucleus/cytoplasm ratio — such cells contain no other DNA source but the nucleus;
- micronuclei, characterized by presence of a main and one or more smaller nuclei, usually round, with diameter 1/3 to 1/6 of the one of the main nucleus;
- pyknosis, characterized by a smaller, but strongly stained nucleus, with diameter varying 1/3 to 1/6 of the one of normal cells;
- karyorrhexis, which presents more extensive nuclear chromatin aggregation, causing fragmentation and disintegration of the nucleus;
- karyolysis, defined as complete loss and absence of genetic material; Feulgen staining, indicative of delayed cell death, is not visible[16] (Figure 1).

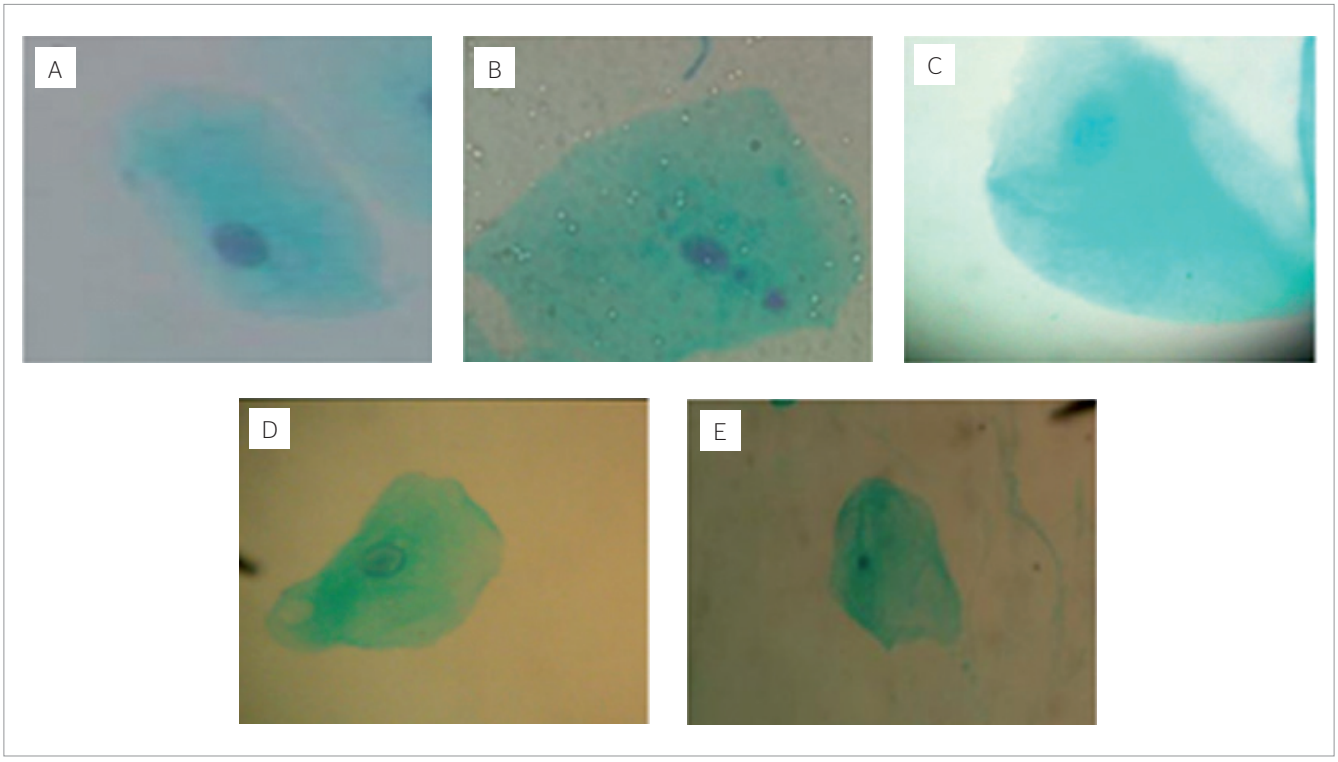

Figure 1. Nuclear abnormalities of exfoliative buccal mucosa cells: (A) differentiated normal cell, (B) cell with micronucleus, (C) cell with karyorrhexis, (D) cell with karyolysis, (E) cell with pyknosis.

STATISTICAL ANALYSIS

The data are described as mean and standard deviation of the mean (SD). Comparison between groups was performed by means of the Mann-Whitney U test. Differences were considered to be significant when $p<0.05$. Data analysis was performed with software GraphPad Prism 7.0.

RESULTS

We analyzed the participants as to average age, length of work in the profession and daily working hours. All the participants were male. The average age of welders was slightly higher compared to the non-exposed group. All welders worked the same number of hours per day (Table 1).

The values of all the cytotoxicity parameters were significantly higher among the participants in the group exposed to welding. The average number of normal cells was smaller among welders (963.40) compared to the non-exposed group (984.31). Cell abnormalities (pyknosis, karyorrhexis and karyolysis) exhibited significantly different patterns between the groups (Table 2).

In regard to genotoxic damage (frequency of cells with micronuclei) there was not significant difference between the groups (Table 3).

Table 2. Frequency of metanuclear abnormalities (pyknosis, karyorrhexis and karyolysis) in the participants' buccal mucosa cells, Cubatão (SP, Brazil), 2016 (N=44)

Group	Pyknosis	Karyorrhexis	Karyolysis
Welders (n=22)	26.0±9.0**	4.4±5.6*	7.2±6.0*
Non-welders (n=22)	13.9±7.0	1.5±3.5	2.4±2.5

Mean±SD: *p<0.05; **p<0.01.

Table 3. Frequency of micronuclei in the participants buccal mucosa cells, Cubatão (SP, Brazil), 2006 (N=44)

Group	Number of participants	Micronuclei
Welders	22	0.07±0.30
Non-welders	22	0.12±0.30

Mean±SD (for 1,000 cells): p<0.05.

DISCUSSION

Continuous contact of the oral epithelium with environmental agents via inhalation and oral exposure makes application of the investigated method advantageous for occupational biomonitoring tests[16]. Low-cost, easy obtainment of samples through a minimally invasive procedure and investigation of DNA damage and cell death in the epithelium make it interesting, as 90% of neoplasms are originated in the epithelium[17]. Similar occupational studies were performed globally: in Poland with workers exposed to copper and arsenic[18]; in India with battery manufacturing workers exposed to lead[19]; in Colombia with miners[20]. We should mention our difficulty to select a non-exposed group fully free from this type of exposure, since the non-exposed volunteers were constantly exposed to several substances, even if in low concentration, a phenomenon typical of the modern urban life, in which exposure to contaminants is ubiquitous even if in minimal levels.

In the present study, comparison between welders and non-welders evidenced higher frequency of cytotoxicity among the former. The results suggest that the cytotoxic effects found among welders were very probably due to exposure to metals such as Cr and Ni in their work activities. However, this hypothesis could not be confirmed based on the methods used, since the present was an exploratory study. The data represent preliminary results with no thorough investigation. We did not find data suggesting occurrence of mutagenesis.

In a study performed in Austria with 22 welders, Wultsch et al.[21] found higher cytotoxic, but no mutagenic effect, which agrees with the results of the present study. Differently, a study conducted with welders in Mexico did not find correlation between exposure and mutagenesis or cytotoxicity parameters[22]. Two studies performed in India[23,24], also with welders, found increased frequency of mutagenesis markers compared to non-exposed individuals. However these findings might be put into question, because the staining technique used, i.e., the Giemsa method, might increase the occurrence of false-positive results[21,22]. We found increased frequency of nuclear abnormalities — such

as pyknosis, karyorrhexis and karyolysis, characterizing cell death — which occur in both necrosis and apoptosis[25]. These abnormalities denote repair mechanisms for genetic damage and might indicate genome instability, as the latter leads to apoptosis[26]. This phenomenon might be further explained by the efficiency of the cell cycle checkpoints[27], which might also result in the occurrence of micronuclei, since cells with micronuclei might be induced to death and consequently are not visualized[28].

Not all the mechanisms related with heavy metals have been fully elucidated; however, their ability to generate reactive oxygen species is well known[19,29]. OH^- radicals might interfere with repair mechanisms for DNA, RNA, proteins, lipids and the nuclear and mitochondrial membrane[30]. These events might be involved in carcinogenesis, teratogenesis and premature aging[18,28,29]. Relative to carcinogenesis, for instance, cytotoxicity might induce chronic injury followed by compensatory increase of cell multiplication, i.e., hyperplasia, resulting in tumor development[27]. Therefore, there is correlation between cytotoxicity and genetic damage, and accumulation of damage in the genome might contribute to the malignant transformation of normal cells.

Studies on genotoxic and cytotoxic damage among welders are rare and controversial. Protective measures, exposure levels and metal concentrations vary among different work environments, but are factors deserving consideration, because they might account for the findings in definite environments[23].

In Brazil, scientific investigation of health hazards derived from occupational exposure gained momentum together with the passing of laws on work safety at the end of the past century[31]. The Brazilian legal frame for work safety is based on Regulatory Norms[32,33], which establish a rather high level of demands and require use of personal and collective protective equipment against hazards found at the workplace. However, informal workers do not usually comply with such measures, as a function of cultural and educational reasons. In the present study, although 90% of the welders reported to use mandatory protective equipment, we could empirically establish, based on observation, that this was not completely true. During visits to some facilities, we found that employees, especially informal workers, did not use safety equipment, or used it irregularly or incompletely. Facing this scenario, companies perform more rigorous control of use of safety equipment.

The biomarkers analyzed in the present study were also used in the investigation of the risk of occurrence of upper respiratory and digestive tract cancer. However, also other diseases, such as diabetes and breast cancer, had been associated with higher frequency of abnormalities relative to these markers[34-36]. More thorough understanding of these issues, as well as automated methods of analysis — the method used in the present study analysis is subjective and depends on the examiner's expertise — will contribute to the comprehension of the direction of studies and to more rapid and precise investigation.

As to our findings, additional studies with more advanced techniques for assessment of genome damage or repair, and longitudinal studies might elucidate better the potential for mutagenesis and cytotoxicity of the welders' work environment.

CONCLUSION

In the present study we did not find increased frequency of genotoxic damage among the workers exposed to welding fumes compared to group of non-welders. However, the results of this preliminary study showed a trend for exposed welders to exhibit more cytotoxic damage in buccal mucosa cells compared to non-exposed workers.

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