Biomarkers, 2011; 16(8): 642–648

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2011.620744

informa
healthcare

RESEARCH ARTICLE

Assessment of oxidative status and genotoxicity in photocopier operators: a pilot study

Elisa C. Kleinsorge[1], Melina Erben[1], María Gimena Galan[1], Caterina Barison[1], María Eugenia Gonsebatt[3], and María Fernanda Simoniello[1,2]

[1]Cátedra de Toxicología, Farmacología y Bioquímica Legal, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina, [2]Citogenética Humana y Genética Toxicológica (CIGETOX), Departamento Bioquímica Clínica (INFIBIOC), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina and [3]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México

Abstract

Occupational exposure to photocopiers has been indicated as being responsible for a number of health complaints, particularly effects on the respiratory, immunological, and nervous systems. In this study, we investigated oxidative and genotoxic damage in photocopier operators by assessing catalase activity (CAT), reduced vs. oxidized glutathione ratio (GSH/GSSG), level of lipid peroxidation (TBARS), damage index by Comet assay (DICA), and buccal cells with micronuclei (BCMN). Our results reveal that the TBARS levels in operators were increased (27%; $p<0.05$) but that no significant alterations to GSH/GSSG or CAT activity were observed. The DICA and the number of BCMN were significantly increased (134% and 100%, respectively; $p<0.05$) in the exposed group. There was a significant association between the time in months spent at work and DNA damage in lymphocytes ($r_s = 0.720$; $p<0.001$) and buccal cell with MN ($r_s = 0.538$; $p<0.001$). Because laser printers and photocopiers have become increasingly used, it is important to control human exposure using reliable biomarkers.

Keywords: Photocopier operators, oxidative stress, Comet assay, buccal micronucleus

Introduction

Black toner contains black carbon or iron oxide as pigments. In addition to these main constituents, toner contains not only various additives, such as wax and silica, but also small amounts of specific metal salts to control the electromagnetic properties. Typically, about 75% of the toner is transferred to the photoconductive drum. However, toner particles that do not adhere to the drum become available for emission in the indoor air. This may lead to users being exposed to different concentrations of emitted particles (Bai et al. 2010). Particulate matter, volatile organic compounds (VOC) and toxic gases such as ozone and nitrogen dioxide are typical components of indoor air pollution (Perez-Padilla et al. 2010). There is ample evidence associating their presence with adverse health effects, including increased cardiovascular episodes (disease), oxidative stress and genotoxicity (Limón-Pacheco & Gonsebatt 2009).

The emission of fine and ultrafine particles as well as VOCs by photocopy machines and laser printers has been clearly documented in several reports (Uhde et al. 2006, Wensing et al. 2006, Kagi et al. 2007, Lee & Hsu 2007). Photocopier operators are exposed to toner particles (when machines are reloaded), toxic gases (e.g. ozone and nitrogen dioxide), VOCs, semi-volatile organic compounds (SVOCs), particles (emitted by the paper processed during printing and copying) and extremely low-frequency electromagnetic fields (ELF-EMFs) during the operation of these machines (Selway et al. 1980, Stefaniak et al. 2000, Tuomi et al. 2000). The emission rates reported from photocopy machines are much higher than for printers and multi-functional devices; however, the variability between studies is

Address for Correspondence: Maria Fernanda Simoniello, Cátedra de Toxicología, Farmacología y Bioquímica Legal, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Santa Fe, Argentina. E-mail: fersimoniello@yahoo.com.ar

(Received 26 April 2011; revised 15 August 2011; accepted 01 September 2011)

high, ranging over three orders of magnitude for some chemicals (e.g. toluene and styrene; Destaillats et al. 2008). Recent studies have shown that exposure to toner particles and volatile compounds that are emitted in indoor environments during laser printing and photocopier operation or maintenance has been indicated as being responsible for a number of health complaints, particularly those related to effects on the respiratory, immunological, and nervous systems (Lee & Hsu 2007).

Ozone and particulate matter have been associated with occupational symptoms such as eye, nose or throat irritation; headache and fatigue in photocopier operators (Mendell et al. 2002, Wolkoff et al. 2006, Yang & Haung 2008); and oxidative damage to cultured cells (Halliwell & Whiteman 2004). Furthermore, multiple investigations have demonstrated the genotoxic potential of photocopying components (Löfroth et al. 1980, IARC: International Agency for Research on Cancer 1996, Goud et al. 2001, 2004, Gadhia et al. 2005).

Proximity can be particularly important in the case of SVOCs, ultrafine particles, and reactive species that can interact with different surfaces where rapid transformations can occur. Furthermore, the accumulation of SVOCs on inside epithelial surfaces may involve other exposure routes (e.g. dermal). The mode of operation can influence temperature and air circulation under ambient conditions, which in turn can impact the emission rates of pollutants.

One of the assays that is used to assess DNA damage in humans resulting from occupational exposure is the Single Cell Gel Electrophoresis (SCGE) assay, also known as the Comet assay, that enables the assessment of DNA fragmentation resulting from a variety of types of DNA damage (e.g. single- and double-strand breaks and alkali labile sites including abasic sites and oxidized bases; Valverde & Rojas 2009). In addition, the micronucleus assay in exfoliated buccal cells (BCMN) is a useful and minimally invasive method that can be used to monitor exposure to genotoxic agents in humans (Kirsch-Volders et al. 2003, Holland et al. 2008). The collection of buccal cells is arguably the least invasive method available for measuring DNA damage in humans, especially in comparison to the collection of blood samples or tissue biopsies (Majer et al. 2001).

To monitor early biomarkers of effects in a group of individual photocopy machine operators, we analyzed catalase activity (CAT), the reduced vs. oxidized glutathione ratio (GSH/GSSG), and levels of lipid peroxidation (TBARS) to monitor oxidative stress in erythrocytes and the measured damage index by Comet assay (DICA) in peripheral blood leukocytes, while the frequency of cells with micronuclei was analyzed in buccal cells (BCMN). Data obtained from exposed individuals were compared with age- and sex-matched controls, and significantly higher levels of TBARS, DICA and BCMN were observed in the exposed group. The levels of these biomarkers were also associated with exposure time.

Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO, USA).

Photocopy sites

The study exposed group (photocopier operators) consisted of men and women who worked at five different small-dimensioned photocopy centers that were approximately 30 ± 10 m^3 in size (on average) with poor ventilation and without extraction systems. Each center had four photocopy machines supplied with powder toner that processed approximately 32,000 sheets of paper each 8-h working day.

Subjects

The purpose of the study was explained to each of the participants, and all volunteers accepted an informed consent protocol previously that had been previously reviewed by the Institutional Ethics Committee (Universidad Nacional del Litoral).

The study group comprised 26 photocopy machine operators and 52 control subjects (2 unexposed subjects were sought for each exposed one) who were matched according to age, sex, socioeconomic status, and other personal habits from the Littoral National University campus, Santa Fe, Argentina. The photocopy machine operator group included 14 men and 12 women with an average age of 23.5 ± 2.52 years, and the average duration of their employment was 25.69 ± 21.6 months (with a range of 4–78 months). Each of the subjects worked 6 days a week for either 4 or 8 working hours per day. The control group (52 subjects) included 24 men and 28 women with an average age of 23.9 ± 3.33 years, who were selected from the same University campus with no history of occupational exposure to photocopy machines or chemical laboratories.

Volunteers were asked to complete a questionnaire regarding their age, area of residence, general health conditions, medications used, smoking habits, and exposure to X-rays and other genotoxic agents in the preceding 3 months. Criteria taken for the assessment of smoking and alcohol consumption habits were as follows for both the study and control groups: subjects who smoked more than one cigarette or consumed at least one glass of any alcoholic beverage a day for 1 year were considered to be smokers and alcohol drinkers, respectively. Subjects were classified as participating in regular physical training if they exercised at least 2 days per week. Table 1 lists the main characteristics of both groups.

Sample collection

Sampling was performed from October to November 2009. To avoid possible bias, the samples were collected and coded for blind analysis.

A blood sample from each subject was obtained from the brachial vein using a heparinized syringe and

Table 1. Demographic variables and ambient PM of the studied groups.

Parameter	Controls ($n=52$)	Workers ($n=26$)
Age (X±SD)	23.9 ± 3.33	23.5 ± 2.52
Sex (n (%))		
Female	28 (53.8)	12 (46.2)
Male	24 (46.2)	14 (53.8)
Smoking (n (%))		
Yes	5 (9.6)	3 (11.5)
No	47 (90.4)	23 (88.5)
Alcohol (n (%))		
Yes	9 (17.3)	4 (15)
No	43 (82.7)	22 (85)
Training (n (%))		
Yes	33 (63.5)	4 (15.4)
No	19 (36.5)	22 (84.6)
$PM_{0.45}$ (X±SD; μg/m³)	25.55 ± 15.68	88.94 ± 40.40*

Average ± SD and the number of individuals (%) per age, sex and smoking, alcohol and physical exercise (training) habits, and PM. *$p<0.01$, Student's t-test.

was divided into two vials. All of the samples were collected in the morning, before the photocopier operators began their work, in the same laboratory where they were later processed. The contents of one vial taken from each subject were centrifuged at 1000 x g for 10 min to separate erythrocytes. The buffy coat was then removed, and the remaining erythrocytes were drawn from the bottom and washed three times in cold saline solution (9.0 g/l NaCl). Aliquots of erythrocytes were stored at 70°C. The second vial of whole blood was used for the study of DNA damage by means of the Comet assay. In addition, the subjects were asked to rinse their mouth with water, and buccal cell samples were obtained by rubbing the inside of both cheeks with a toothbrush; after collection, these samples were then shaken in a conical tubes containing 10 ml of buffer solution A (0.1 M EDTA, 0.01 Tris–HCl, and 0.02 M NaCl; pH 7).

GSH and GSSG in erythrocytes

Erythrocytes were hemolyzed through the addition of ice-cold demineralized ultrapure water (Milli Q plus reagent grade) at a 1:3 dilution prior to mixing the solutions with TCA 10% (1:1). Cell homogenates were centrifuged at 10,000 x g for 10 min at 4°C. Reduced GSH was measured in the supernatants according to the method described by Ellman et al. (1961) with some modifications: 0.1 ml of supernatant was dissolved in 1 ml of 1.5 mM dithiobis-nitrobenzoate (DTNB) in 0.25 M phosphate buffer pH 8.0. The mixture was incubated at room temperature for 5 min, and the absorbance at 412 nm was measured. The GSH concentration in samples was estimated using a standard curve of absorbance and was expressed as μmol Glutathione mg^{-1} Hb.

Aliquots from the initial supernatant were used to determine GSSG and GSH: Total GSH (TGSH; Venturino et al. 2001). Briefly, in each sample (at pH 8.0), GSSG was reduced to GSH with 0.6 U glutathione reductase (EC 1.6.4.2.) and 0.21 mM NADPH in 143 mM potassium phosphate buffer plus 6.3 mM EDTA (pH 7.5). The reaction was incubated for 30 min at room temperature and was stopped with an equal volume of 10% TCA. TGSH was measured in the supernatant as indicated above. The concentration of GSSG was estimated by subtracting TGSH-GSH and was expressed as μmol of GSSG mg^{-1} Hb.

Hemoglobin levels were determined using Drabkin reagent (Drabkin 1971).

Catalase activity of erythrocytes

Erythrocytes were hemolyzed by adding ice-cold demineralized ultrapure water (Milli Q plus reagent grade) at a 1:100 dilution. The CAT activities of the erythrocyte hemolysates were measured spectrophotometrically by monitoring the decrease in H_2O_2 concentration over time (Aebi 1984). Sample aliquots (10 μl) were added to 3 ml 50 mM phosphate buffer, pH 7.0, in a quartz cuvette. Subsequently, H_2O_2 was added to a final concentration of 54 mM, and absorbance at 240 nm was measured in a spectrophotometer at 25°C for 60 s. One unit of enzyme activity was defined as the activity required to degrade 1 mole of hydrogen peroxide during a period of 60 s per gram of Hb.

Lipid peroxidation in erythrocytes

Lipid peroxidation in erythrocytes was determined by measuring the production of color generated during the reaction of TBA with malondialdehyde (TBARS assay) according to the method of Beuge and Aust (1978) with some modifications. Erythrocytes were lysed (dilution 1:4) in ice-cold demineralized ultrapure water (Milli Q plus reagent grade) and mixed thoroughly with four volumes of reaction solution (15% w/v TCA, 0.375% w/v TBA, 0.25 mol/l HCl acid) and 4% BHT to inhibit peroxidation stimulated by Fe^{3+} without affecting the formation of the MDA-TBA chromogen. The mixture was then heated in a glycerine bath at 95°C for 45 min. After cooling, the flocculent precipitate was removed by centrifugation at 12,000 g for 10 min at 4°C. The sample absorbance at 535 nm was determined, and the TBARS concentration was calculated using the extinction coefficient 1.56×10^5/M/cm. To avoid interference, iron-free tubes and deionized water were used for these assays. The MDA concentration in erythrocytes was expressed as nmol/g Hb. To obtain precision parameters, 1,1,3,3′-tetraethoxy propane was used.

Micronucleus test in buccal epithelial cells (BCMN)

Buccal cell suspensions were washed three times in buffer solution A followed by centrifugation at 1500 rpm for 10 min. The washing procedure helped to remove bacteria and cell debris, which confound the scoring. The cells were carefully dropped onto preheated (50°C) slides with a pipette and dried. The slides were then fixed in 80% cold methanol for 30 min, air-dried overnight (Titenko-Holland et al. 1998) and stored at –20°C until analysis. They were then stained with acridine orange (DNA

specific stain) and analyzed using an epifluorescence microscope at 400× magnification. A total of 2000 cells/subject were scored. The total number of cells with MN per 1000 buccal cells was determined for each volunteer.

Alkaline Comet assay

Cell viability was determined using acridine orange/ethidium bromide (Simoniello et al. 2010); the Alkaline Comet assay (pH>13) was then performed according to the method described by Singh et al. (1988), with the following modifications: 50 µl of fresh blood was added to 950 µl of RPMI 1640 at 37°C and, subsequently, centrifuged at 1000 g for 4 min. Cell pellets (containing roughly 60,000 cells) were mixed with 200 µl of low melting point agarose 1% and two slides were prepared. To lyse the cellular and nuclear membranes of the embedded cells, the key-coded slides were immediately immersed in freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM trizma base, 1% Triton X-100, and DMSO 10%; pH 10) and left at 4°C overnight. The slides were then immersed in freshly prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA; pH>13), first for unwinding (20 min) and then for electrophoresis (0.7–1 V/cm, 300 mAmp, 20 min at 4°C). All of the steps were carried out under conditions of minimal illumination and low temperature (on ice). Once electrophoresis was completed, the slides were neutralized. The dried microscope slides were then stained with ethidium bromide. One hundred randomly selected Comet assays from each of two duplicate gels were visually analyzed on a scale of 0–4 (zero category is considered without damage and not included in the final score. One to four categories, which depend on DNA damage level, are included). The overall score, between 100 and 400 arbitrary units, is related to DNA break frequency, a comet-like image indicates the presence of DNA breaks, and the Damage Index Comet assay (DICA) was calculated (Simoniello et al. 2008).

DICA = $n_1+2n_2+3n_3+4n_4$. Where, n_1 = number of cells with level 1 of damage, n_2 = number of cells with level 2 of damage, n_3 = number of cells with level 3 of damage, and n_4 = number of cells with level 4 of damage (Rodriguez-Ferreiro et al. 2002).

Environmental monitoring

Concentrations of particulate matter were measured using 7 m^3/h air samplers with 0.45 µm filters and gravimetric analysis. The sampling was performed during the 8-h working period for 5 days, inside the photocopier centers and outside them or in the different locations of the University campus building where the non-exposed participants worked. The results were expressed as the 8-h working-day average concentrations.

Statistical analysis

Analysis was performed using the SPSS 14.0 software package for Windows. All experiments were carried out in duplicate. Median values and interquartile ranks were calculated for each biomarker. Contingency tables were used to test differences in questionnaire information such as smoking, alcohol consumption, physical exercise, sex, and age between controls and exposed. To compare oxidative stress and DNA damage indicators between controls and photocopier operators, the Mann–Whitney *U*-test was performed. A multivariate regression analysis was performed to examine the relationships between variables and to examine the contribution of confounding factors such as age, sex, alcohol consumption, and smoking. Spearman rank order correlations were performed to investigate the association between exposure (duration of work) and the biomarkers tested. *p* Values of less than 0.05 were considered to represent significance.

Results

The demographic features of both control and photocopier operator groups are summarized in Table 1, which shows that the groups were similar in terms of age and sex. In addition, according to the information from the questionnaires, they were also similar in the habits of smoking and alcohol consumption. Both group smoked in average less than five cigarettes and consumed less than two glasses of any alcoholic beverage per day in the last year. More than 60% of the individuals in the control group and 15% in the photocopier operator group engaged in regular physical activity (training).

We did not observe significant differences in relation to confounding factors such as age: $F(1, 51) = 0.20$, Prob>F = 0.65; smoking: $F(2, 43) = 2.97$, Prob>F = 0.06; or drinking alcohol: $F(2, 41) = 2.44$, Prob>F = 0.09 using a multivariate regression analysis in either the oxidative or genotoxicity assays.

The results show that photocopier operators exhibit a 25% decrease in the levels of GSH/GSSG, a difference that was not statistically significant; a similar observation was obtained with respect to CAT activity (Table 2;

Table 2. Oxidative stress and genotoxicity biomarkers in controls and photocopy operators.

	Controls (n = 52)		Photocopiers (n = 26)		
Biomarker	Median	IQR[a]	Median	IQR[a]	p value[b]
CAT	252.26	202.07–287.59	246.99	205.13–305.23	0.656
TBARS	279.19	222.52–369.03	355.87	253.74–422.50	0.041
GSH/GSSG	21.52	12.24–33.45	16.04	9.78–27.57	0.255
DICA	135.00	129.25–145.50	181.00	170.00–190.00	<0.001
BCMN	0.00	0.00–1.00	1.00	1.00–2.00	0.004

[a]IQR: interquartile range; [b]Mann–Whitney *U*-test.

Table 3. Spearman's Rank Correlation analysis of the different biomarkers in the studied groups ($n = 78$).

Variables	TBARS	GSH/GSSG	Duration of exposure (months)	DICA	BCMN	
CAT	0.310	−0.161	0.0834	0.116	0.262	r_s
	0.02	0.24	0.55	0.41	**0.05**	p value
TBARS		−0.141	0.187	0.241	0.183	r_s
		0.31	0.18	0.08	0.19	p value
GSH/GSSG			−0.0811	−0.0783	0.214	r_s
			0.56	0.57	0.12	p value
Duration of exposure (months)				0.720	0.538	r_s
				<0.001	**<0.001**	p value
DICA					0.369	r_s
					0.006	p value
BCMN						

r_s: Spearman rank order correlation coefficient; $n = 78$.

$p>0.05$). Nevertheless, the levels of TBARS were significantly higher (27%) in the photocopier operator group ($p = 0.041$).

In blood samples, cell viability was determined as recommended by Singh (2000) and was greater than 95 %. Levels (median and interquartile rank) of the Comet assay in lymphocytes are presented in Table 2. Photocopier operators exhibited a 134% increase in DICA ($p<0.001$). In addition, the number of cells with MN was increased by 100% in the exposed group (Table 2; $p = 0.004$). To investigate the association between exposure and these indicators, we used the Spearman correlation test. We identified a significant association between the time in months spent at work and DNA damage in lymphocytes ($r_s = 0.720$; $p<0.001$) and the occurrence of buccal cell MN ($r_s = 0.538$; $p<0.001$; Table 3). Furthermore, those individuals with higher DICA values exhibited the higher number of cells with MN ($r_s = 0.369$; $p = 0.006$; Table 3), and the activity of CAT was significantly associated with TBARS ($r_s = 0.310$; $p = 0.02$) and BCMN ($r_s = 0.262$; $p = 0.05$; Table 3).

Discussion

Photocopiers have been found to generate ozone in varying amounts (Kagi et al. 2007, Lee & Hsu 2007); furthermore, toner and paper dust from printing devices may become airborne, generating respirable particles that include ultrafine aerosols. It has also been reported that photocopiers emit nitrogen dioxide and volatile organic compounds such as 1,1,biphenyl, p-dichlorobenzene, styrene, propylbenzene, tetrachloroethylene aldehydes, and nitrated PAHs (Destaillats et al. 2008). Thus, during the operation and reloading of the photocopy machines, individuals are exposed to mutagenic substances as well as ELF-EMFs.

In this study, workers were exposed to photocopy equipment emissions for 4–8 h during a 6-day working week in closed spaces with poor ventilation. The levels of PM inside the photocopy centers were significantly higher compared with control ambient (Table 1).

The detection of lipid peroxidation has relied largely on indirect methods; TBARS assay has been employed to a uniquely great degree over the last decades to detect and quantify lipid peroxidation in a variety of chemical as well as biological matrices (Janero 1990). We observed a significant increase in TBARS in erythrocytes, a result similar to that reported in photocopy operators by Zhou et al. (2003a,b), who demonstrated an increase in lipoperoxidation levels in both plasma and erythrocytes. In the process of photocopier operation, oxidative stress increases via ozone emission and UV irradiation, leading to increases in apoptosis and cellular damage and the stimulation of autoimmune processes (Bar-Sela & Shoenfeld 2008). Also, Bai et al. (2010) demonstrated inflammatory responses in the lung caused by toner particles when the toner suspension was instilled into the lungs of the male mice through intratracheal instillation. UV and ozone in indoor environments can generate reactive oxygen species in exposed tissues, increasing production of highly reactive hydroxyl radicals (·OH) and, therefore, chronic oxidative stress in exposed subjects (Bar-Sela & Shoenfeld 2008).

Diet certainly plays an important role in preventing cancer, but the mechanisms involved in this protection are still unclear. Convincing epidemiological evidence links consumption of fruits and/or vegetables with decreased risk of cancer of the lung, mouth and pharynx, oesophagus, stomach, colon, and rectum (WCRF/AICR 1997). In a previous study of the same group (controls and photocopy operators), a nutritional evaluation was performed. Results showed that consumption of food sources of antioxidant micronutrients was inadequate, and a large percentage of subjects did not meet the required daily amount (RDA) in both photocopy operators and controls (Erben et al. 2010). We also observed increased DNA damage levels in peripheral leucocytes using the Comet assay and in buccal cells using the MN test. Similar studies have demonstrated that occupational exposure to compounds used and/or emitted by photocopy machines induces genotoxic effects (Goud et al. 2001, 2004).

A Spearman's correlation analysis revealed that the damage observed in leucocytes by the Comet assay was positively associated with the presence of MN in buccal

cells, suggesting that both tissues are affected by the working conditions. In addition, the levels of damage assessed by the Comet assay and the number of observed cells with MN were both significantly associated with exposure time (Table 3), suggesting a cumulative genotoxic effects.

In occupational biomonitoring studies, genotoxic alterations in epithelial cells and peripheral blood lymphocytes are of particular interest because these cells are frequently the targets of toxic agents. Most studies use human blood cells because they have been considered adequate surrogate tissues to assess environmental exposure (Collins & Dusinska 2009). The cellular, nuclear, and metabolic state of blood cells can reflect the overall extent of body exposure. Although the Comet assay is capable of evaluating levels of DNA damage, additional studies are necessary to identify the molecular mechanisms underlying damage (Valverde & Rojas 2009). Some of this damage could originate from increased oxidation of DNA due to the increased levels of TBARS observed in the exposed group.

Epithelial cells of the respiratory tract represent a direct uptake route for airborne pollutants in situations of occupational exposure. Photocopier operators may frequently inhale different substances nasally or orally during the processing and reloading of machines. In addition, it remains unknown whether an elevated MN frequency in buccal cells is predictive of cancer risk, and whether the relationship is primarily site-specific (i.e. the oral cavity) or extends to overall cancer risk (Holland et al. 2008).

Previous studies revealed the relationship between oxidative stress and potential free radical damage associated with photocopying (Zhou et al. 2003a,b). An increase in chronic oxidative stress, which may affect autoimmunity by leading to the oxidation and nitration of proteins and rendering them to the immune system as neo-antigens, has been demonstrated in photocopy machine operators exposed to these emissions (Bar-Sela & Shoenfeld 2008).

The increases in BCMN and DICA indicate the presence of genotoxic components. Some of these components, either directly or through their metabolites, may form DNA adducts. These adducts could likely open up and form single strand breaks during faulty repair processes, which, after replication, could result in double strand breaks. Their interaction with DNA can result in various types of genotoxic damage. An increase in DNA damage and a decrease in nucleic acid repair have also been described in photocopy machine operators using Comet assay (Goud et al. 2001). A significant increase in the frequency of MN in buccal epithelial cells and peripheral blood lymphocytes, as well as chromosomal aberrations have been also reported in photocopiers (Goud et al. 2004).

Because laser printers and photocopiers are increasingly used not only in offices but also in houses, and considering that much remains to be understood regarding the sources and nature of emitted pollutants, it is critical for human exposure studies to be conducted using reliable biological markers and epidemiological surveys for human risk assessment.

Acknowledgements

The authors are especially grateful to Lic. Gisela L. Poletta and Mg. Jorge Scagnetti, who provided expert help during the research.

Declaration of interest

This work was supported by Elisa C. Kleinsorge grant CAI+D UNL 12B/420. The authors declare no competing interests.

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