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Genotoxicity biomarkers for monitoring occupational exposure to antineoplastic drugs

[Biomarcadores de genotoxicidad para el monitoreo de la exposición ocupacional a fármacos antineoplásicos]

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Abstract

Context: The Institute of Oncology and Radiobiology (INOR) is the leading institution for the diagnosis, treatment and follow-up of cancer in Cuba. The main methods used in cancer treatment are surgery, radiotherapy and chemotherapy. The last one involves the handling of hazardous substances, such as cytostatics, which implies a health risk to persons occupationally exposed to it. There are two sites where a considerable among of cytostatic is handled (Ambulatory Chemotherapy Room (ACR) and the Central Unit of Cytostatic Mixture Preparation (CUCM)). Genotoxicity biomarkers of exposure and effects have been widely used to detect occupational environment hazards.

Aims: To evaluate genotoxicity biomarkers indicative of exposure and effects to cytostatics.

Methods: In this study were tested samples taken from the surfaces of biological safety cabinets located in the Central Unit of Cytostatic Mixture using SOS – Chromotest. We also evaluated samples of oral mucosa exfoliated cells from exposed and control subjects, by micronucleus test.

Results: All subjects were exposed and subjects who administered the mixes in the institution had an increased of DNA damage in comparison with the pharmaceutical staff that prepared it and wear the primary protection barriers properly.

Conclusions: These results underline the efficiency of genotoxicological biomarkers in detecting the exposure levels and the deleterious effect of cytostatics on occupationally exposed personal.

Keywords: Biosafety; cytostatic handling; micronucleus test; occupational health; SOS Chromotest.

Resumen

Contexto: El Instituto de Oncología y Radiobiología (INOR) es la institución líder en el diagnóstico, tratamiento y seguimiento del cáncer en Cuba. Los principales métodos usados en el tratamiento son: la cirugía, la radio y quimioterapias. Esta última involucra el manejo de sustancias peligrosas, como los son los citostáticos que implican un riesgo a la salud de las personas que lo manipulan. Existen dos sitios donde se manipulan grandes volúmenes de estas sustancias la Sala de Quimioterapia Ambulatoria (ACR) y la Unidad Central de Mezclas Citostáticas (CUMC). Los marcadores de genotoxicidad han sido ampliamente empleados para detectar peligros en el ambiente laboral.

Objetivos: Evaluar biomarcadores de genotoxicidad indicativos de exposición y efecto a citostáticos.

Métodos: Las investigaciones farmacognósticas y fitoquímicas se llevaron a cabo en relación con los parámetros macroscópicos, microscópicos y fitoquímicos preliminares.

Resultados: Se evaluaron muestras tomadas de las superficies de las cabinas de seguridad biológica de la CUCM usando el SOS – Chromotest. También se evaluaron muestras de células exfoliadas de la mucosa bucal de sujetos expuestos y controles mediante el ensayo de micronúcleos.

Conclusiones: Estos resultados ponen de manifiesto la eficacia de los biomarcadores genotoxicológicos en la detección de los niveles de exposición y el efecto nocivo de los citostáticos en el personal expuesto ocupacionalmente.

Palabras Clave: Bioseguridad; ensayo SOS; manejo de citostáticos; prueba de micronúcleos; salud ocupacional.

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INTRODUCTION

Biosecurity is an important issue to be considered in hospitals, where the personal is occupationally exposed to physical and chemical agents such as cytostatics used in the treatment of cancer (INFOSAN, 2010; Alados Arboledas et al., 2014; Sandoval-Flores, 2014). Those who handle these drugs give rise to concern in the health sector due to the nonspecific cytotoxic drug (NIOSH, 2014). The physicochemical properties of these agents, the ways and magnitude (concentration and duration) of exposure may cause acute or chronic effects in humans.

Researchers have reported effects such as: mutagenicity, immunotoxicity, teratogenicity and carcinogenicity associated with repeated exposure to cytostatics (Falk et al., 1979; De la Peña et al., 2012). Due to the above-mentioned effects, the staff responsible for handling cytostatics must be aware of the risk associated with their work and comply with the Standardized Operating Procedures and Good Laboratory Practices. INOR is the leading institution in Cuba for the diagnosis, treatment, monitoring and research on the topic of cancer, pathology among the top three causes of death in Cuba (Anuario Estadístico de Salud, 2014). There are three methods to treat cancer: by surgery, radiotherapy, and drug treatment. The latter involves the use of hazardous substances such as cytostatics, risking the health of those occupationally exposed when handle them. By Resolution No. 07-97, the Center for State Control of the Quality of Medicines (CECMED) under the Ministry of Public Health, issued a set of methodological guidelines and measures to reduce the risks that personnel handling antineoplastic drugs (Regulación 7-97).

There are extrinsic and intrinsic factors that jeopardize compliance with standard biosafety procedures for handling cytostatics at INOR. Among them: i) the increase both in of the number of cytostatics used today and of treatments based in their combinations; ii) the yearly increase in the number of new cases of cancer (Galán et al., 2009; Romero-Pérez et al., 2012); iii) improved survival rates of patients, which implies an increase in the number of follow-ups (Gracia-Medina et al., 2007); iv) admission of very young staff in the area of cytostatics preparation; v) the high turnover of personnel in preparation areas and the lack of a pre-employment training system.

Biomarkers are used to detect the adverse effects caused in the body by physical, chemical and biological agents. Biomarkers provide quantitative information about exposure and corroborate the entry of toxic agents into the body (Bonassi et al., 2011). The biomarker must be objectively measured and evaluated as an indicator of a normal biological process, pathological state or response to drug therapy, occurring at cellular or molecular level. The interaction depends on the inherited and acquired characteristics of the individual and on the circumstances of exposure (Biomarkers Definition Working Group, 2001; Tambor et al., 2010).

Biomarkers of genotoxicity are measured both, in natural populations from contaminated habitats and in organisms experimentally exposed to pollutants (Faust et al., 2004).

In the field of genotoxicology, bacterial assays are widely used due to speed, low cost and relative simplicity, which enable them to explore experimental details and to repeat tests several times. In prokaryotes, SOS response is the name given to the emergency cellular system that allows bacterial survival when faced to the halt of DNA replication that has been damaged by genotoxic agents (Janion et al., 2003). The SOS induction factor has been used as a biomarker of exposure. The protocol used was that described by Cuétara et al. (2012), which is a variant of the one described by Quillardet et al. (1982). The test involves the measurement of constitutive and inducible enzyme activities using substrates, which develop fluorescence. In parallel, alkaline phosphatase activity is determined. This enzyme is expressed constitutively and allows quantifying the progress of cellular protein synthesis.

In this study, the micronucleus test was used as biomarker of effect. During cell division, genetic material (DNA) contained in the cell nucleus is equally replicated and divided resulting in two identical daughter cells. Sometimes, this process can go wrong spontaneously or induced by physical or chemical agents. The genetic material that separates and remains excluded is not properly incorporated into the nucleus of the daughter cell but creates a new core smaller than the primary one taking

the name of micronucleus (MN) (Matheus and Bolaños, 2014). The MN technique has been validated worldwide by the international human micronucleus program (HUMN: Human MicroNucleus Project), involving 42 laboratories and evaluating approximately 16,500 persons from different populations around the world (Zalacain et al., 2005).

The oral cavity reflects the health of an individual, as changes indicative of disease are observed in the lining of the mouth, revealing systemic conditions, or showing side effects caused by chemotherapy and radiotherapy treatments since they limit the proliferative capacity of the epithelial cells. Such capacity makes it more vulnerable to lesions in DNA, which is very relevant since it is estimated that 90% of all cancers has an epithelial origin. Additionally, about 60% of the oral mucosa surface is non-keratinized epithelium, favoring the absorption of dyes and the identification of morphological characteristics through the microscope (Bonassi et al., 2011). Oral mucosa exfoliated cells can be collected by well accepted minimally invasive and relatively painless techniques facilitating the technique and lowering its cost (Torres-Bugarín et al., 2009).

Based on the foregoing, this study was aimed at evaluating biomarkers of genotoxicity, in the areas where the bulk of cytostatics is handled at the INOR; the Central Unit of Cytostatic Mixes (CUCM) and the Ambulatory Chemotherapy Room (ACR).

MATERIAL AND METHODS

The research was conducted at the INOR, a tertiary care institution under the Ministry of Public Health of Cuba. Specifically, two areas related to handling cytostatics were evaluated, the Central Unit of Cytostatic Mixes belonging to Pharmacy Services and the Central Unit of Ambulatory Chemotherapy Room, which is part of the Medical Oncology Service. INOR Ethics Committee approval was obtained in September 2013 and written informed consent was obtained from all study participants.

General description of the sample

The research was conducted on a sample of 14 test subjects who work daily in a range of six to nine hours with cytostatic agents. The gender composition was eleven women (80%) and three men (20%). Education level was thirteen university graduates subjects (86.6%) and two technicians (13.3%).

Determination of a biomarker of exposure, SOS induction factor

For this study, it was used the strain of *Escherichia coli* PQ-37 genotype: F thrleu-his-four pyrDthilacgalTgalE or galK ΔU169 srl300 :: Tn10 rpoBrpsLuvrA RFA Muc + sfiAtrp :: Mud (Ap, lac) cts (Huisman and D'ari, 1981). The culture medium used was Luria-Bertani (Maniatis et al., 1982) supplemented with 100 µg/mL ampicillin (LBA).

Cells in culture medium NB 1X was used as negative control and gamma radiation was used as positive control. For the irradiation of cells, it was used a source of Co60 (model PX-γ-30M, Russia) and conducted at a temperature of 2 ± 0.5°C. The initial value of the dose rate of the source was 2.7 kGy/h; the activity decreased at a monthly rate of 1%. The dose used was 150 Gy and was calculated using the Fricke dosimeter (Prieto and Cañet, 1990).

The two biological safety cabinets for daily work in the CUCM were utilized for this test. They were identified as Cabinet I and Cabinet II. Samples were taken at three points (center, right and left ends) and twice at the start of the working day and at the conclusion thereof, working time in the cabinets ranged from 6 to 8 hours. A sterile swab soaked in a sterile solution of 0.9% sodium chloride was used. Each point chosen was rubbed and stored at 4°C in an eppendorf vial until conduction of the test. Four independent samples were conducted.

Methodology of the SOS Chromotest Assay microanalytical-fluorescent variant

It was started with a pre-culture (2 mL) of the PQ 37 strain of Escherichia coli grown overnight in 1X Nutrient Broth (NB) medium supplemented with ampicillin. They were inoculated into a 50 mL Erlenmeyer with 1X NB medium (Siagma-Aldrich, Germany) and incubated at 37°C with circular shaking (100 rpm). The optical density of the culture was monitored at 600 nm when the culture reached the exponential phase (OD 600 nm = 0.4), 3 mL of a bacterial culture were taken and diluted 1:10 in fresh NB 2X medium also supplemented with ampicillin.

Test tubes were prepared with 250 µL of sterile water (C - and C +) and 250 µL of the samples to be tested. The cells were exposed to the samples for 30 minutes to allow entry of the compounds into them. The mixtures were incubated for 2 hours at 37°C.

Ninety-six well plates were used for microanalytical scale enzyme assays, and the final volume was adjusted to 150 µL. For detection of enzyme activities, the plate was imaginarily divided into two. In the upper four rows 110 mL of Z Buffer were placed (60 mM Na_2HPO_4, 40 mM Na_2PO_4, 10 mMKCl, 1 mM $MgSO_4$, 0.1% SDS, 40 mM β-mercaptoethanol; pH 7.0) for the β- galactosidase (β–gal) assay. In the bottom, four rows 110 µL of buffer T were placed (1 M Tris pH adjusted to 8.8 with HCl) for Alkaline Phosphatase Assay (PASA). Subsequently, 20 cells were plated per well, each column corresponds to treatment (negative and positive controls and samples). The plate was incubated for 20 minutes at 25°C to lyse the cells. After this time in each case 26 µL per well of the corresponding substrate were added. In the upper four rows, for β-gal assay 4-methyl-umbelliferyl-β-D-galactopyranoside was used dissolved in buffer T at a concentration of 0.39 mg/mL. The mixture was homogenized and allowed to react for 40 minutes at 25°C in the dark. In the lower four rows, for the PASA assay, substrate 4-methylumbelliferyl phosphate was added dissolved in diethanolamine buffer (89 mM diethanolamine, 0.13 mM magnesium chloride, pH 9.8) at a concentration of 0.26 mg/mL.

In both trials fluorescence was measured in arbitrary units (AU) using a SUMA PR-531 fluorometer (TECNOSUMA International S.A., Cuba). The wavelength for excitation of the substrate was 365 nm and the emitted fluorescence product of enzymatic reactions was detected in the 420 - 500 nm range. These data were used to calculate the SOS induction factor (IF), as followed:

$$\mathrm{SOSIF} = \frac{\left[\frac{\beta - \text{galactosidase}}{\text{alkaline phosphatase}}\right]_{treatment}}{\left[\frac{\beta - \text{galactosidase}}{\text{alkaline phosphatase}}\right]_{negative\ \ control}}$$

Considering the criterion of Kevekordes et al. (1999), when the FI takes values from 1 to 1.5, the test sample is considered as non-genotoxic. If the values are between 1.5 and 2 it is considered a dubious sample and it is recommended to increase the pre-incubation time to 4 degrees or concentrate the sample depending on the study in question.

Determination of a biomarker of effect. The frequency of micronuclei in exfoliated cells of the oral mucosa

An observational analytical study involving the five workers of the Central Unit of Cytostatic Mixes preparation and the nine workers of the Ambulatory Chemotherapy Room was made.

INOR workers exposed to cytostatics and who voluntarily consented to participate were used as controls, matched by age, sex and lifestyle. The inclusion and exclusion criteria in the study are summarized in Table 1. Ethical and legal aspects related to research involving human beings were respected, and the subjects expressed their willingness to participate by signing the written consent form.

Sampling

For sampling, subjects were required to rinse their mouths thoroughly with water. Exfoliated epithelial cells of oral mucosa were obtained by scraping the lining of the inner cheek with a sterile wooden spatula, and the exfoliated cells were deposited in test tubes containing 3 mL of sterile saline solution (0.9% NaCl). Subsequently, they were washed twice with a sterile saline solution by centrifugation at 1500 rpm at 4°C. Supernatant was discarded and 3 mL of fixative solution (3 parts 99% ethanol and 1 part acetic acid) were added.

After 20 minutes, epithelial cells were smeared onto clean microscope glass slides. The slides were air-dried and fixed with 96% ethanol. The same researcher conducted both the staining of epithelial cells using the Papanicolaou staining kit (Merck, Germany) and the microscopic analysis of micronuclei. A minimum of 1000 consecutive cells per worker was observed; when the frequency of micronuclei was less than 3/1000, a maximum of 3000 cells were evaluated to decrease the likelihood that the absence of micronuclei was due to a random event. The criterion for selection of micronuclei considers that the cell is characterized by the presence of a core and one or more small nuclear struc-

tures called MN. A MN is round or almond shaped and measures between 1/3 and 1/16 of the core, presents the same intensity, texture, and focal plane as the core and is a fragment of or a complete chromosome that when mitosis takes place does not integrate into one of the nuclei of the daughter cells (Thomas et al., 2009).

Statistical analysis

The results of SOS Chromotest were analyzed using Graph Pad 5 (Software Inc, San Diego, CA, USA). The normality of distribution was checked with the Shapiro-Wilk test. Data did not meet the assumption of normality so Dunn's Multiple Comparison test was used to compare each sample with the negative control.

The results of Micronucleus test were analyzed using the R 2.14.1 program for Linux. The three numeric variables (age, time of exposure and number of MN cells) were transformed into categorical variables for analysis of independence (Fisher exact test) respect to the rest of variables. The normality of distribution was checked with the Shapiro-Wilk test. Nonparametric tests were applied when not meeting the assumption of normality, so the Mann-Whitney U-test was used to compare independent quantitative variables. Also an analysis of correlation between continuous variables was conducted.

In all cases, the level of statistical significance was set at values of $\alpha = 0.05$.

Table 1. Criteria for inclusion and exclusion of the subjects in the study.

Criteria	Exposed Subjects	Non-Exposed Subjects
Inclusion	• Involved in handling cytostatics during their work. • Belonging to the CUCM or ACR.	• INOR workers belonging to areas not related to the handling of cytostatics. • Matched by age and sex with the group of subjects exposed.
	Willingness expressed by signing the written consent form	
Exclusion	• Having recently suffered from viral or bacterial infections. • Having received radiation to the face and neck in the last six months before the study. • Having taken legal and/or illegal drugs or medication (antiparasitic, antibacterial, antibiotics) 25 days before sampling. • Possess a presumptive or confirmatory diagnosis of pregnancy. • To be a patient with a degenerative disease or other location cancer diagnosis.	

All workers that belong to the selected areas (CUCM and ACR) voluntarily agreed to participate in the study.

$N_{\text{exposed subjects}} = 14$ and $N_{\text{non-exposed subjects}} = 14$. Groups were matched by age, gender and toxic habits (smoking and alcohol consumption).

RESULTS AND DISCUSSION

Genotoxicity of samples in biological safety cabinets of UCMC (SOS Chromotest)

Fig. 1 shows the genotoxicity of samples taken in biological safety Cabinets I and II at the UCMC. As can be seen in Cabinet I all surface points checked before starting the workday had SOSIF values similar to the negative control, implying that the cleanup of the previous day had been successful. After the end of the workday points Nº 1 and Nº 2 showed differences statistically significant ($p < 0.0001$ and $p < 0.001$, respectively) indicating the presence of genotoxic substances. It implies that there have been spilled or that aerosols have been deposited in this area. This is justified because in point N° 1 the original vials of cytostatics used to prepare mixtures were accumulated and in point N° 2 was the area where manipulation occurs.

In Cabinet II, it was identified that there were hazardous substances in points N° 1 and N° 3 ($p < 0.05$), evidencing a deficient cleanup in the previous day. After workday all points were affected ($p < 0,0001$). It was known that staff members preferred to use this cabinet due to its location and that there was not differences in the chemical nature of prepared mixtures. The microanalytical variant of the SOS Chromotest was sensitive to detect the presence of hazardous substances in the work surfaces as can be seen in the data shown in Fig. 1.

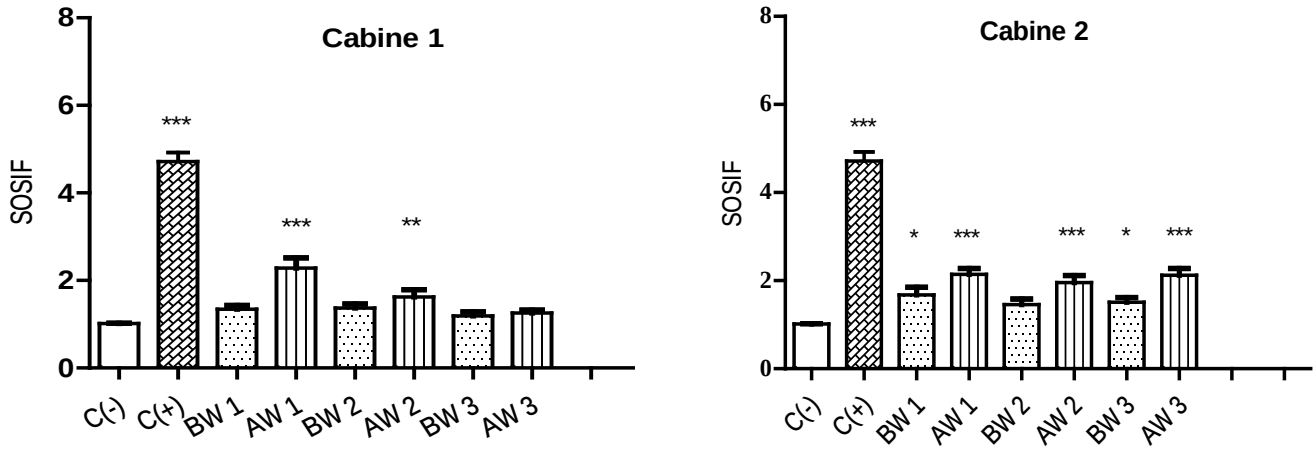

Figure 1. Identification of the presence of mutagenic substances in biological security cabinets by mean of SOS induction factor.

SOS induction factor (SOSIF) was determined using SOS Chromotest. SOSIF ≥ 2 indicates the presence of hazardous substances in the workplace.

C(+) = ionizing radiation; C(-) = fresh nutrient broth; BW: before work; AW: after work.

The two cabinets of Cytostatic Mix Preparation Unit were sampled in three points, before and after work.

Values are expressed as mean and standard errors of mean. *$p < 0.05$; **$p < 0.001$ and ***$p < 0.0001$ represent statistically significant differences respect to control group [C(-)]

Four sampling days evaluated in two independent experiments, four replicas each time.

Each SOSIF value was compared with C(-) using Dunn's test. $p \leq 0.05$.

Several genotoxicity tests to evaluate this type of biological samples have been developed since 1979, when Falck et al. (1979) used the Ames test to demonstrate that the urine of nurses who handled cytostatic agents was mutagenic. In principle, occupational exposures can be regulated, minimized or eliminated and in this respect, occupational carcinogens have a high preventive potential (Domínguez Odio et al., 2004).

The SOS test has been used to detect genotoxic metabolites derived from the biotransformation of antineoplastic drugs in urine, such as: adriamycin, bleomycin, dacarbazine, cisplatin, vincristine and their mixtures (Kohn et al., 1988). Vojteková et al. (1990) proved that the SOS assay was able to detect the toxicity to levels of 0.09 mg/L in water and urine samples.

Giuliani et al. (1996) used the umuC Test (analog to SOS) for the analysis of wastewater from a hospital with 1400 beds in Zurich (Switzerland). The authors reported a considerable amount of genotoxic substances released into the environment. Another study showed that the effluent from a clinic in Porto Alegre (Brazil) was weakly genotoxic and came from patients' treatment areas (Silva and Zachia, 2007).

Destination and effects of cytostatic drugs are widely acknowledged and are known to be able to contaminate effluents and affect marine ecosystems. Zounková et al. (2007) evaluated the effect of cyclophosphamide, cisplatin, 5-fluorouracil, doxorubicin and etoposide with *Pseudomonas putida* and algae *Pseudokirchneriella subcapitata* growth inhibition tests and with *Daphaniamagna* immobilization test. They also used the SOS Chromotest (with and without metabolic activation) and the Green Screen trial using the *Saccharomyces cerevisiae* yeast. Poyen et al. (1988) showed that the LD50 (median lethal dose) parameter took values in the range of micrograms/L to milligrams/L, as part of a study conducted on human reproduction with a urine sample of 47 nurses in seven oncology units in Marseille (France). In such study, thirty-seven subjects from a cardiology area were used as a control and according to the Ames test, no significant differences were found between both groups, although some samples of exposed subjects were positive.

Genotoxicity of biological samples of subjects exposed (MN Test)

Biomonitoring of genotoxicological effects of occupationally exposed staff and polymorphism analysis of susceptibility to damage are key tools in public and occupational health assessment for prevention of tumor occurrence induced by environmental factors. Analytes regarded as cancer biomarkers are clustered into three general categories: nucleic acids, proteins and metabolic (Kohn et al., 1988). The present study was aimed at determining the frequency of micronucleus in oral mucosa exfoliated cells, considered as effect biomarker indicative of chromosomal damage.

Fig. 2 evidences the differences in the frequency of MN between controls and exposed subjects. Data were analyzed using Shapiro Wilk test. This test is ideal for determining normality in samples measuring less than or equal to 50. If the p-value is $p < 0.001$ it is classified as non-normal distribution (Fenech et al., 2013). Data for the majority of variables do not meet the assumption of normality (only age does) therefore; non-parametric tests were applied to analyze the differences.

Note that there were no significant differences ($p = 0.1483$) between the number of MN of the exposed subjects (5.78 ± 1.86) and those of the controls (1.38 ± 0.52), according to the Wilcoxon test (also known as Mann-Whitney) (Fig. 2). In the consulted literature is accepted as normal up to 4 MN per 1000 cells (Bonassi et al., 2011) and the results are variables, which will be discussed below. It is a small sample but represents the total number of subjects working in these areas. The standard error of the mean of exposed subjects was high since there was a significant variability in exposure times. Literature reports an association between exposure time and frequency of MN (Rosales-Rimache et al., 2013).

When comparing the number of MN between subjects working in the areas of CUCM (1.6 ± 1.4) and those operating in ACR (8.1 ± 2.5) it was found by the Wilcoxon test that $p = 0.059$ (Fig. 3). Although values lower than 0.05 are considered sig-

nificant, the value obtained is very close being evident that there was a rising trend in ACR compared to CUCM. There is an explanation for this rise because although all mixtures used in the INOR are pre-pared in the CUCM, primary protective barriers are correctly and permanently employed and good laboratory practice is employed, as visits to both areas revealed.

The time variable of subjects working in ACR was greater than of those working in the CUCM (129.6 ± 38.7 and 12.8 ± 2.9 months, respectively). The result of p = 0.0912 showed no significant differences between exposure times (Fig. 4). The correlation value between MN number and exposure time in this study was 0.48 and does not evidence a strong association among the variables analyzed; this behavior could be attributed to the heterogeneity and size of the sample.

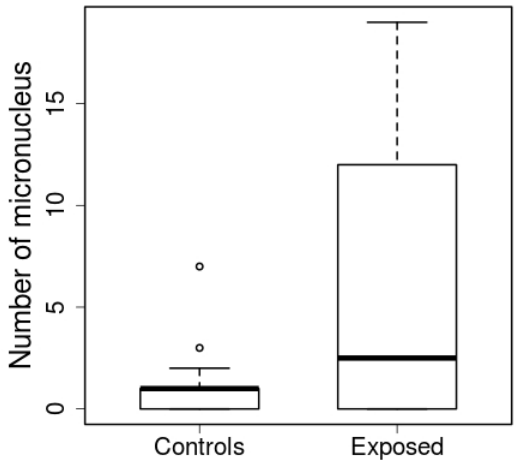

Figure 2. Genotoxicological effects of occupational exposure to cytostatic.

$N_{exposed\ subjects}$ = 14 and $N_{non\text{-}exposed\ subjects}$ = 14. Groups were matched by age, gender and toxic habits (smoking and alcohol consumption). Two slides were analyzed per subject and 1000 cells per slide were scored. Results are expressed in number of micronucleated cells per 1000. It is considered as normal up to 4 MN per 1000 cells. Although $MN_{exposed}$ = 5.78 ± 1.86 and $MN_{controls}$ 1.38 ± 0.52, not statistically significant differences were found between groups according Wilcoxon test (p = 0.1483).

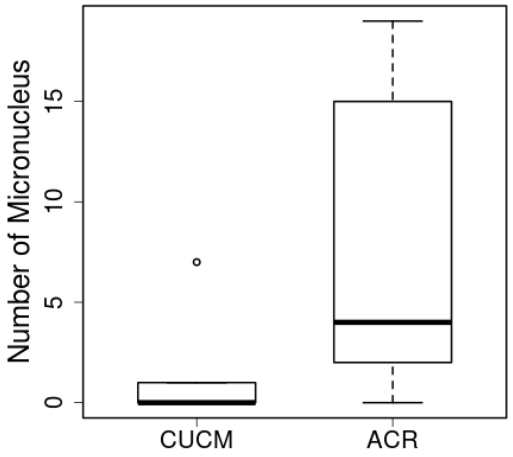

Figure 3. Comparison of genotoxicological effects of occupational exposure to cytostatic in Chemotherapy Ambulatory's Room (ACR) and the Cytostatic Mix Preparation Unit (CUCM).

Two slides were analyzed per subject and 1000 cells per slide were scored. Results are expressed in number of micronucleated cells per 1000. It is considered as normal up to 4 MN per 1000 cells. Although MN_{ACR} = 8.11 ± 2.51 (n = 4) and MN_{CUCM} = 1.6 ± 1.36 (n = 10), not statistically significant differences were found between groups according Wilcoxon test (p = 0.0588).

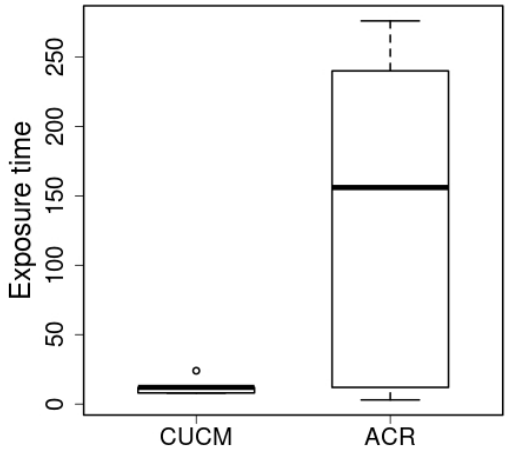

Figure 4. Influence of exposure time (ET) in the frequency of micronuclei. Comparison between subjects who work in Chemotherapy Ambulatory's Room (ACR) and the Cytostatic Mix Preparation Unit (CUCM).

Two slides were analyzed per subject and 1000 cells per slide were scored. Results are expressed in number of micronucleated cells per 1000. It is considered as normal up to 4 MN per 1000 cells. Although ET_{ACR} >> ET_{CUCM} (129.6 ± 38.7 *vs* 12.8 ± 2.9 months), not statistically significant differences were found between groups (p= 0.0912) due to the high variability among subjects work experience in ACR, thus R^2 = 0.48 meaning low association between MN and ET.

In Figs. 2, 3 and 4, whiskers represent upper value (largest observation that is less than or equal to the upper quartile plus 1.5 times the length of the interquartile range) and lower adjusted value (smallest observation that is greater than or equal to the lower quartile less 1.5 times the length of the interquartile range). Dark line inside the box represents de median. Circles represent values outside the range of the whiskers plotted individually.

Moreover, the non-lethal genetic damage is the core of carcinogenesis, which can be acquired by the action of environmental agents (Flores-Angulo and Lee, 2010). The MN assay in oral mucosa has proven to be a useful and widely used biomarker to measure DNA damage in human populations. MN formation in dividing cells may be the result of chromosomal breakage due to non-repaired or poorly repaired DNA injury or to segregation defects due to mitotic spindle malfunctioning. The sensitivity of the assay may be affected to some extent by polymorphisms associated with the repair, performance or deactivation of carcinogens/re-agents, drugs, alcohol, folate pathway, transport of micronutrients, exposure to environmental pollutants (pesticides, arsenic, formaldehyde) and exposure to medical procedures (radio and/or chemotherapy), as well as by inherited genetic defects in DNA repair (Fenech and Bonassi, 2011). XRCC1 polymorphisms (Arg280His), ERCC2 (Lys751Gln), CYP2E1 (c1/c2) and MTR (A2756G) associated with the formation of MN (Dhillon et al., 2011).

The sample size remains currently a critical point in the design of studies involving testing MN (Ceppi et al., 2011). Recent studies that consider gene-environment interaction often require hundreds of subjects making it difficult to achieve. Another challenge is the use of appropriate controls. The most common uncertainty factors include age, gender, and smoking. Particular attention has been paid to the inclusion of markers of susceptibility in the designs (Ceppi et al., 2011).

Data management is essential, starting with its collection and description followed by the verification of statistical requirements for subsequent more complex analysis. Approximately, in half of the reports in the literature on the subject multivariate comparisons are performed using non-parametric tests. To a lesser extent multivariate statistical models are used and when there are data settings to the normal distribution, logarithmic transformations or the Poisson model are used (Ceppi et al., 2011). The mean basal frequency of MN in controls was 1.1/1000 cells (95% CI: 0.70 - 1.72) and the increase thereof in subjects exposed to genotoxic agents or with genetic diseases showed a similar correlation to that observed in lymphocytes (R^2 = 0.74). The current recommendation is to analyze 4000 cells per subject, but the usual practice is 1000 (4 of 5 revised databases) (Fenech et al., 2010).

MN technique has been used in monitoring subjects occupationally exposed to chemicals. By using MN assay, Boughattas et al. (2010) evaluated 20 nurses of the Oncology Service at the Farhat Hached University Hospital in Tunisia and 20 controls matched for age, sex and toxic habits. The average age of the evaluated group was 36 years, with the prevalence of women, being 6.1 years the average exposure time. They found a significant increase of MN compared with controls.

A previous Cuban study evaluated a group of 11 nurses of the Chemotherapy Service of the Santiago de Cuba Provincial Hospital and 11 control subjects of the Administrative Section (Domínguez et al., 2004). The average age of both groups was 39 years. The authors found a significant increase in the frequency of MN (63%), results that do not match those of the present study due to the absence of biological safety cabinets in the Santiago de Cuba Hospital.

The results in the present study coincide with those reported by Lampurlanés et al. (2004). This study, carried out in Barcelona, included 13 nurses who worked in oncology and pharmacy, showed no differences between urine samples from exposed subjects and controls evaluated by the Ames test.

The effects of aging and gender in the frequency of MN have been reported by several international laboratories. MN levels increase with age in both sexes and it is higher in women than in men. The meta-analysis of the HUMN project data (www.humn.org) confirmed the statistical significance and the consistency of the previous approaches. Increase in the amount of MN with age is due to the combination of several factors: (i) accumulation of mutations in genes encoding proteins involved in DNA repair, chromosome segregation and checkpoints of the cell cycle; (ii) numerical and structural aberrations caused by exposure to endogenous Genotoxins, inadequate nutrition, environmental and occupational pollutants and unhealthy lifestyles. Literature reports

that, women exhibit a 19% MN frequency (95% CI: 14-24%) higher as average (Fenech et al., 2010). Such increase can be explained by the strong tendency of the X chromosome to "get lost" due to the presence of two copies (Fenech and Bonassi, 2011).

In 2013, it was evidenced the possibility of MN automated counting by cytometry using commercial platforms like Metafer (MetaSystems), Pathfinder™ (IMSTAR), iCyte® (Compucyte), particularly for lymphocytes. Additionally, it was demonstrated that there is a correspondence between visual analysis and the one carried out by automated systems with correlation factors between 0.58 and 0.99. The validation and calibration of these systems are still required to perform more reliable comparisons between laboratories and platforms (Fenech et al., 2013). In the INOR, it is not yet possible to apply this technological advantage due to insufficient equipment and reagents.

A study by Villarini et al. (2012) showed no significant differences in the frequency of MN in peripheral blood lymphocytes between exposed subjects and their controls, despite contamination with 5-fluorouracil and cytarabine on working surfaces and workers' uniforms (detected by Comet assay). The authors defend the hypothesis that these results could be due to the induction of repairable primary damage and therefore not detectable at the chro-mosome level, in the case of chronic exposure to low levels of cytostatics. In this study conducted in Italy, gender and age showed no association with the increased frequency of MN. There was also a predominance of female staff and, therefore, it was impossible to make comparisons between genders. In controls, frequency increased slightly with age. In literature, there are contradictory reports. Some authors do not obtain significant differences between exposed subjects and controls (Cavallo et al., 2007; Villarini et al., 2012). Such is the case of the study here presented. Other authors have indeed found such differences in both peripheral blood lymphocytes and exfoliated cells from buccal mucosa (Kopjar et al., 2009; Rombaldi et al., 2009). These differences are mainly due to disparities in exposure levels determined by the nature of the chemicals arisen, their relative proportions, the duration of daily routines and staff turnover, as the use of individual and collective protection means.

It has been proved that there are factors that increase or decrease the frequency of MN. Among those that increase it are aging (women have higher proportions of MN), deficiency of folate and vitamin B12, physiological processes (menopause, osteoporosis), cytostatic drugs, alcohol and exposure to toxic agents routinely. Antioxidants, vitamins C and E and beta-carotene have proven to reduce the frequency of MN (Fenech et al., 2013).

Genotoxicity biomonitoring of personnel exposed to mutagenic agents is justified by Boveri's mechanistic hypothesis (formulated in 1902) of the association of the occurrence of chromosomal abnormalities and cancer pathogenesis. The author argues that cancer can be considered a process of Darwinian evolution based on two events: continuous acquisition of heritable mutations and natural selection of the resulting phenotype. It represents the early stage of carcinogenesis (Bonassi, 2010).

In working environments where hazardous substances are handled, it is recommended to conduct environmental and biological monitoring to make sure biosafety standards are respected. Samples of working surfaces and biological samples of staff of areas of chemical risk are usually taken. Such samples are evaluated directly (using physicochemical methods to identify the presence of analytes of interest) or indirectly using biosensors emitting a signal indicative of the presence of hazardous substances. The current state of knowledge shows that the potential risk to the health of workers exposed can be controlled effectively by combining typical actions and controls in terms of hygiene, employee training, operating procedures, techniques and equipment for personal protection, and specific medical surveillance (Bonassi, 2010). Despite the obligation to comply with the actions described in the guidelines for handling cytostatics and their widespread use in hospitals, there are detectable amounts of these substances in clothing, working surfaces and in biological samples of pharmacy staff and nurses who apply chemotherapy.

CONCLUSIONS

The results obtained underline the efficiency of genotoxicological biomarkers in detecting the exposure levels and the deleterious effect of cytostatics

on occupationally exposed personal due to the inadequate use of primary barriers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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