Occupational risk assessment of genotoxicity and oxidative stress in workers handling anti-neoplastic drugs during a working week

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Twenty pharmacists and nurses handling anti-neoplastic drugs in a hospital were monitored during a working week, from Monday to Friday, in the morning (only on Monday) and afternoon (all days). Genotoxicity was analysed by the comet assay and the micronucleus (MN) test, while oxidative stress was analysed in serum by thiobarbituric acid reactive substances (TBARS) and by measurements of the antioxidant enzymes superoxide dismutase (Sod) and catalase (Cat). The exposed workers presented increased DNA damage levels by the comet assay as compared to the controls. The comet assay results have also shown significant positive correlation with the day of the week and with alcohol consumption. MN frequency was significantly higher in the exposed workers and presented noteworthy correlation with age and working time. In the oxidative stress parameters, only Cat presented a significant increase in the exposed group, considering all the samplings. However, TBARS data showed interesting results, considering the different sampling times; the exposed group presented a significant correlation with the working days and significantly higher results on Friday as compared to the controls and Monday morning. Monitoring occupational risk during a longer time, e.g. during a working week as done in this study, introduces additional aspects of risk behaviour, which can improve risk management. This study demonstrates the usefulness of evaluating oxidative stress also in genotoxic risk assessment since both events often result from the same factors.

Introduction

It is well known that hospital workers are subject to many health hazards, in situations in which several genotoxic agents are used, either due to maintenance or for patient diagnosis and treatment. The professionals who are continuously exposed to these agents need to be monitored for risk behaviour, so that such hazardous compounds are properly managed by hospital staff. Many studies have assessed the risk that workers who occupationally handle anti-neoplastic drugs (1–13) are exposed to. The following end points have been detected: an increase in chromosome aberrations (1,3,4), sister chromatid exchanges (1,4), micronucleus (MN) frequency in lymphocytes (5,7,8,12,13), DNA damage by comet assay (2,6,8,9,11–13), high rate of gene mutations (12, 13) and increased levels of anti-neoplastic drugs in urine (10). In general, the studies published detected an increase in some end point in the workers, but the risk may be reduced by better management (8). Therefore, it is important to establish all the aspects surrounding the risks in order to enable better hazard management. Although the risk to workers handling anti-neoplastic drugs is well known, it is useful to monitor risk behaviour during the working week and after the weekend rest period.

Anti-neoplastic drugs can also induce reactive oxygen species (ROS). Anthracyclines, cyclophosphamide and cisplatin produce hydrogen peroxide, which supposedly induces DNA damage (14–17) that may lead to mutations. ROS can affect cell function by acting directly on cell components, including lipids, protein and DNA, therefore destroying the structure (18). In view of the cytostatic drugs can induce ROS (14–17), it was considered interesting to evaluate end points of oxidative stress such as superoxide dismutase (Sod), catalase (Cat) and thiobarbituric acid reactive substances (TBARS) since all these end points are expected to be increased. Until now, no study of occupational risk for hospital staff handling anti-neoplastic drugs and oxidative stress evaluation has been reported in the literature. Cytogenetic methods have been extensively used for the biological monitoring of populations exposed to mutagenic and carcinogenic agents, and the comet assay is increasingly being used in genotoxicity testing. The advantages of the comet assay include its demonstrated sensitivity in detecting low levels of DNA damage, the requirement for small numbers of cells per sample, flexibility, low costs, ease of application and the short time needed to complete a study (19). Another method equally indicated to assess occupational exposure is the MN test. Micronuclei areacentric fragments or complete chromosomes which fail to attach to the mitotic spindle during cytokinesis and are excluded from the nuclei. The advantages of this test over other cytogenetic mutational tests include the speed and ease of analysis, and the fact that it does not require metaphase cells (20) and detects historical accumulation of mutagenic events in lymphocytes of the last 3 months. In our previous unpublished evaluation of hospital workers handling anti-neoplastic drugs, more DNA damage was detected on Monday evening (after the working day) and on Friday morning, and at both evaluation times, the workers presented more damage than the controls. Therefore, in this study, the genotoxicity and oxidative stress parameters were evaluated in hospital workers handling anti-neoplastic drugs, throughout the working week. The workers were sampled on Monday before working and from Monday to Friday when the work finished. Genotoxicity was evaluated by

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the comet assay and MN frequency in lymphocytes, while oxidative stress parameters were analysed in serum by TBARS and measurement of the antioxidant enzymes Sod and Cat.

Materials and methods

Subjects

The exposed group consisted of 20 professional, nurses and pharmacists, currently employed in the oncology departments of different hospitals and Oncology clinics in Caxias do Sul and Bento Gonçalves, towns in the state of Rio Grande do Sul, southern Brazil. Twenty non-exposed workers of comparable age, sex and lifestyle—such as smoking and alcohol habits at the same hospitals—were part of the control group.

All individuals in both groups answered a Portuguese version of the personal health questionnaire published by the International Commission for Protection against Environmental Mutagens and Carcinogens (21). Characteristics of workers in the exposed group, such as use of protective equipment (gloves, masks, gowns, goggles, protective clothes and vertical laminar flow safety hoods) were also investigated. The characteristics of the study groups are presented in Table I.

Sampling

Biological samples were obtained at six different times on five consecutive days (Monday through Friday) by venipuncture using vacutainers with heparin. The first and second samples were obtained on Monday, at two distinct times: the first at the beginning and the second at the end of the working day. The weekly resting time of the workers with anti-neoplastic drugs was always at Saturday and Sunday. The other samples were taken at the end of the working day, from Tuesday through Friday. For the control group, a single sample was collected at the end of each working day, i.e. from Monday to Friday.

Comet assay

Blood was obtained from each subject by venipuncture using vacutainers with heparin and refrigerated at between 10 and 20°C for a maximum of 4 h, upon processing. A standard protocol was adopted for comet assay preparation and analysis (19,22). Slides were prepared by mixing 5 µl whole blood with 95 µl normal whole blood with 13 µl methyl methanesulphonate (M4016/Sigma, St Louis, MO) to 8 × 10^{-3} M final concentration. This mix was incubated for 2 h at 37°C. The result of each electrophoresis was considered only if the negative and positive controls yielded negative and positive results, respectively. Images of 100 randomly selected cells (50 cells from each of two replicated slides) were analysed from each sample. Cells were also visually scored according to tail size into five classes, from no tails (0) to maximal (4), resulting in a single DNA damage score for each subject and consequently for each study group. Therefore, a group damage index (DI) could range from 0 (all cells with no tail, 100 cells × 0) to 400 (all cells with maximally long tails, 100 cells × 4) (23,24). All electrophoresis performed in this study showed the following results to positive and negative controls: DNA DI to positive control 0–400 and for the negative control 0–12.

It is important to remark that International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method. It has a high correlation with computer-based image analysis. Damage score is based on the length of migration and on the amount of DNA in the tail and is considered a sensitive measure of DNA damage (25).

MN test

Blood was obtained from each subject by venipuncture using vacutainers with heparin and refrigerated at between 10 and 20°C upon processing. Lymphocyte cultures were set up by adding 0.3 ml blood to 5 ml standard culture (Nutricell®, Campinas, São Paulo, Brazil). The flasks were cultured at 37°C. Two cultures per subject were established. At 44 h after initiation of the lymphocyte culture, cytotoxin B (Sigma) was added to the 5-µg/ml concentration (26). At the 72nd hour of incubation, the cultures were harvested by centrifugation at 800 r.p.m. for 5 min and fixed in 3:1 methanol:acetic acid without hypotonic treatment and dropped onto clean slides. Staining was done with Giemsa 5%. Two thousand binucleated lymphocytes per individual (1000 cells from each of two replicate slides) were scored for the presence of micronuclei, with ×200–1000 magnification. Slides were labelled for blind analysis. For the MN test, both control and exposed groups were submitted to only one collected sample, and the samples were taken at different days of the week.

Sod activity

Sod activity was determined by spectrophotometry in serum samples, by measuring the inhibition of the rate of auto-catalytic adrenochrome formation at 480 nm, in a reaction medium containing 1 mmol/l adrenaline (pH 2.0) and 50 mmol/l glycine (pH 10.2) (27), both from E. Merck. This reaction was conducted at a constant temperature of 30°C for 3 min. The enzymatic activity is expressed as Sod units per gram of protein. One unit is defined as the amount of enzyme that inhibits the rate of adrenochrome formation in 50%.

Cat activity

The assay was performed according to the method described by Aebi (28). The assay principle is based on the determination of the rate of hydrogen peroxide (E. Merck) decomposition at 240 nm. This reaction was conducted at a constant temperature (30°C) for 1 min. The enzymatic activity is expressed as Cat units per milligram of protein. One unit of Cat decomposed 1 µmol H₂O₂ per minute at pH 7.4 and 30°C.

Analysis of serum thiobarbituric acid reactive substances

Oxidative stress levels were measured spectrophotometrically by concentration of the thiobarbituric acid reactive substances (TBARS) (29). TBARS results were expressed as nmol/ml.

Total protein

The total protein levels were analysed by the Biuret method (Total Protein Kit—Labetst Diagnostica S.A., Brazil) for spectrophotometric determination in 545 nm.

Statistical analysis

Statistical evaluations were performed by using descriptive statistics and Kolmogorov–Smirnov test (verification of normality). The t-test was used to compare the mean of control and exposed groups and analysis of variance and Tukey post-test was used for multiple comparisons of mean values during the week. Statistical significance was accepted as P < 0.05. The Pearson correlation was used to verify the interrelation of the data. All tests were performed with the statistical program SPSS 12.0 for Windows.

Table I. Characterization of hospital workers exposed to anti-neoplastic drugs and controls

<table>
<thead>
<tr>
<th>Professionals</th>
<th>Exposed (n = 20)</th>
<th>Controls (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacists (drug mixing)</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>Nurses (infusion administration)</td>
<td>16</td>
<td>–</td>
</tr>
<tr>
<td>Admission office</td>
<td>–</td>
<td>9</td>
</tr>
<tr>
<td>Bureaucratic function</td>
<td>–</td>
<td>9</td>
</tr>
<tr>
<td>Telephone operator</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Women</td>
<td>18 (90%)</td>
<td>18 (90%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>31.50 ± 9.34</td>
<td>28.23 ± 6.30</td>
</tr>
<tr>
<td>Range</td>
<td>23–56</td>
<td>21–54</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of non-smokers</td>
<td>18 (90%)</td>
<td>18 (90%)</td>
</tr>
<tr>
<td>No. of smokers</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Alcohol drinking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of non-drinkers</td>
<td>6 (30%)</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>No. of habitual drinkers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Once a week</td>
<td>10 (50%)</td>
<td>14 (70%)</td>
</tr>
<tr>
<td>Twice a week</td>
<td>4 (20%)</td>
<td>2 (10%)</td>
</tr>
</tbody>
</table>
Results

The main characteristics of the workers exposed to anti-neoplastic drugs and controls are shown in Table I. No significant difference was detected between the two groups. All exposed workers had been involved continuously in the preparation or in the administration of the anti-neoplastic drugs for a period of 0.6–10 years, working 6 h/day. According to the information obtained in the informative questionnaire answered by the workers, all used protective equipment, such as protective clothes, eyes glasses, gloves, masks and all hospitals have vertical laminar flow safety hoods.

The results (mean ± standard deviation) of the analyses of all days (from Monday through Friday) of Cat, Sod, TBARS, comet assay, aside from the result for MN frequencies that was analysed using only one sample during the week, in both control and exposed groups, are shown in Table II.

A significant increase in the DNA DI, MN and Cat was observed in the exposed workers (P < 0.001, P < 0.001 and P < 0.01, respectively) as compared to the controls. The results of TBARS and Sod were similar in both groups. When the exposed group is divided into pharmacists (drug mixing) and nurses (infusions administration), it can be observed that the pharmacists presented an increase in the DNA DI when compared with the nurses and the control group. Nurses have also presented an increase in DNA DI in relation to the control group. The nurses presented higher MN frequency when compared to the pharmacist and control groups. In relation to oxidative stress, only the Cat level of exposed nurses is higher than the controls.

Analysis of genotoxicity

The comet assay results showed that the mean DI for the exposed group than the controls.

The comet assay results showed that the mean DI for the exposed group showed a significant difference between exposed (4.94 ± 1.95) and control groups (2.88 ± 0.78; P = 0.01) (Figure 1B). A positive correlation was found between MN frequency and age (r = 0.559, P < 0.001) and between MN and working time (r = 0.319, P = 0.001) and both (age and working time) are also highly correlated (r = 0.663, P < 0.001) for exposed group. Through an adjusted correlation, the working time lost significance, suggesting that age was the main correlation factor with MN frequency. In the control group, a positive correlation was only found between age and working time (r = 0.687, P = 0.001). MN and age, as well as MN and working time did not show a positive correlation (r = 0.130, P = 0.620 and r = 0.303, P = 0.236, respectively).

Oxidative parameter behaviour during the working week

The oxidative stress parameters of the exposed group, Cat, Sod and TBARS, were analysed at six different moments along five consecutive days from Monday morning, Monday afternoon to Friday afternoon. Considering the different sampling times, the results are shown in Figures 1C–E. No significant difference between the control and exposed group was detected in Cat and Sod activities for any sampling during the week. In the exposed group, no significant difference was detected among samplings on different working days. In contrast, the TBARS of the exposed group presented an increase of this oxidative parameter between Friday afternoon and the control group and also the intra-exposed group between Friday and Monday morning. A positive correlation can also be seen between the TBARS and the days (r = 0.369, P < 0.001), and this significance is maintained (r = 0.389, P < 0.01) when the influence of age, alcohol consumption and smoking is considered as confounding factors in a multiple regression. No significant correlations were observed between TBARS and Sod, Cat, DI and MN.

Discussion

Anti-neoplastic agents inhibit cell growth or kill the growing cells, generally by apoptosis. They can also induce a secondary cancer, but the benefit at the critical moment is unequivocal for the patient. Most studies published about professionals working with anti-neoplastic drugs assessed some degree of increasing genotoxicity (1–13). The data may be an over-estimation because reporting negative results is not encouraged. It is interesting to note that in three studies which included workers without recommended safety equipment (2,6,9), these people presented significantly higher

Table II. An overview of all data of oxidative stress and cytogenetic parameters of workers exposed to anti-neoplastic drugs and their controls, during one working week (weekly means)

<table>
<thead>
<tr>
<th></th>
<th>Comet assay DI</th>
<th>Micronuclei frequency</th>
<th>TBARS (nmol/ml)</th>
<th>Sod (USod/g protein)</th>
<th>Cat (UCat/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>18.86 ± 8.62**</td>
<td>4.94 ± 1.95*</td>
<td>5.72 ± 1.93</td>
<td>3.17 ± 3.55</td>
<td>1.38 ± 1.71**</td>
</tr>
<tr>
<td>Maximum–minimum</td>
<td>2–52</td>
<td>2–9</td>
<td>2.14–10.83</td>
<td>0.05–18.52</td>
<td>0.08–10.87</td>
</tr>
<tr>
<td>Median</td>
<td>18</td>
<td>5</td>
<td>5.93</td>
<td>2.31</td>
<td>0.88</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.21 ± 2.78</td>
<td>2.88 ± 0.78</td>
<td>5.12 ± 1.38</td>
<td>2.76 ± 2.40</td>
<td>0.77 ± 0.47</td>
</tr>
<tr>
<td>Maximum–minimum</td>
<td>2–12</td>
<td>1–4</td>
<td>1.72–6.88</td>
<td>0.17–8.80</td>
<td>0.16–2.06</td>
</tr>
<tr>
<td>Median</td>
<td>6</td>
<td>3</td>
<td>5.35</td>
<td>1.96</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Significance by t-test in relation to the control group: *P = 0.01, **P < 0.01; SD, standard deviation.
The use of appropriate protection or improving management did reduce or even eliminate genotoxicity detected in some end points analysed in workers handling anti-neoplastic drugs (2,8). In this study, as in others previously mentioned, all the workers used the individual safety protection equipment, but even so genotoxicity is important to be evaluated since it has been detected in most studies. In the studies where different end points were used, results are not easily compared. The conventional studies of chromosomal aberrations (CA) seem to be the best genotoxicity detector, showing an increase in the risk groups from 70 to 260% (1,3,4). Besides, by improving the CA analysis using fluorescent in situ hybridization labelling, only on two chromosomes (pair 1 and 4), a 286% increase in aberrations was detected in workers, as compared to controls (3). Thus, CA analysis seems to be the most sensitive end point to evaluate genotoxic risk, but it is also very laborious. Widely used end points in risk analysis in these professionals are the MN test and the comet assay, which were also performed in this study. The results reported in the literature obtained with the MN test vary from non-significant (14%) (8) to highly significant (377%) (13) and are higher in workers as compared to controls. In the comet assay, the results also vary from non-significant increase of DNA damage in some parameter (tail moment) (12,13) to highly significant (160%) (8). It must be noted that in studies that did not detect a higher risk for the workers by one given end point, a major risk was found by another. In this study, the occupational risk was evaluated within a week, so a lot of data were collected but, if the average of all analyses performed during the week is taken into

![Graphs showing DNA damage index, MN frequency, TBARS, U cat/mg protein, and U sod/g protein](https://example.com/graphs.png)

**Fig. 1.** The individual data of exposed and control group of the DNA damage, oxidative stress parameters and MN frequency on different days in 1 week: (A) DNA DI presenting significant increases in relation to the control group at *P* < 0.001 and in relation to Monday morning at *P* < 0.05. (B) MN frequency presenting significant increases in relation to the control group at *P* < 0.05 and also to the Monday morning at *P* < 0.001. (D) Serum Cat activity without significant difference between days as compared to the control. (E) Serum Sod activity without significant difference between days as compared to the control.
shown a positive correlation, possibly because the sum of both evaluation of this aspect. Nevertheless, the exposed group has sample is too small and does not include alcoholics to afford an controlled pathway with mitochondrial membrane changes production, which mediates apoptosis by a mitochondrial- as methotrexate and vincristine, commonly induce ROS literature. However, there have been many studies which workers handling anti-neoplastic drugs is reported in the between age and CA (30,32,33).

The result of the comet assay was also positively associated with alcohol ingestion in workers (r = 0.23, P = 0.01), but in the controls, this correlation was not significant (r = −0.19, P = 0.45). Alcohol consumption can induce CA (30), but our sample is too small and does not include alcoholics to afford an evaluation of this aspect. Nevertheless, the exposed group has shown a positive correlation, possibly because the sum of both risk agents (anti-neoplastic + alcohol) exceeds the limits of the organic capability needed to repair the damages.

Micronuclei are defined as small and acentric fragments or complete chromosomes that failed to attach to the mitotic spindle during a mitotic cycle and were excluded from the nucleus (20). As is well known, anti-neoplastic drugs can damage DNA by a direct action, for example alkylating agents, or by an indirect action, for example antimetabolites, free radical generators, mitotic inhibitors and topoisomerase II inhibitors. Therefore, different mechanisms can be involved in the formation of micronuclei, and among the most commonly used anti-neoplastic drugs, there are clastogenic and aneugenic drugs. So it is expected to find a correlation between increased MN frequencies of micronuclei, more oxidative stress and DNA damage detected by comet assay.

The MN frequency was evaluated only once to see whether the DNA damage detected in the comet assay also resulted in CA since the comet assay detects repairable damage. The increasing chromosome mutations in the workers were confirmed, and it can thus be concluded that the workers are under actual risks, one of which is cancer, as the correlation between chromosome mutation and cancer is well established (31). However, we could not correlate the MN frequency in the exposed group with the working time since the initially estimated positive correlation lost significance when verified through the adjusted correlation test, suggesting that age is the main correlation factor with MN frequency—a finding clearly established in many studies that have shown the correlation between age and CA (30,32,33).

No study concerning the monitoring of oxidative stress in workers handling anti-neoplastic drugs is reported in the literature. However, there have been many studies which evaluated oxidative stress induced by anti-neoplastic both in vitro and in vivo (15,17,34–42). Anti-neoplastic drugs, such as methotrexate and vincristine, commonly induce ROS production, which mediates apoptosis by a mitochondrial-controlled pathway with mitochondrial membrane changes (36–40). In view of what was mentioned above, it was expected that some of the main consequences of the oxidative stress responses, such as genotoxicity, would be found in workers handling anti-neoplastic drugs. A study on health professionals exposed to anaesthetic gases has shown increasing lipid peroxidation in relation to the controls, but the antioxidant capacity remained normal (43).

In this study, three parameters of oxidative stress (TBARS, Sod and Cat) were evaluated during the week, on Monday morning after the weekend rest and from Monday through Friday at the end of the working day. Mean antioxidant enzyme activities showed an increase in Sod and Cat, but only mean Cat activity presented a significant increase in relation to the control. No significant difference was observed in TBARS mean, considering the data of all days, between the exposed and control groups. The mean results of exposed and control of this study remain in the range of normal healthy adults evaluated in our laboratory for TBARS 1.25–5.95 (minimum and maximum, respectively), for Sod 0.20–10.96 and for Cat 0.32–11.50 (44). Thus, it can be noted that the controls and exposed groups presented nearly the highest position in the normal range for TBARS, while for Sod and Cat enzymes both groups are in the normal range. This study suggests that anti-neoplastic drugs induce some modulation in the activity of the end points evaluated, but without changes in oxidative stress levels.

TBARS presented interesting results, with a clear correlation from Monday morning to Friday (r = 0.37, P < 0.001), similar to the results obtained in the comet assay (r = 0.27, P < 0.01). However, there is no correlation between these two parameters (r = 0.07, P = 0.48), suggesting that the observed genotoxicity could be induced by other mechanisms not related to oxidative stress.

The main anti-neoplastic drugs (cyclophosphamide, iphosphamide, cisplatin, oxalipaltin and doxorubicin) handled by the pharmacists and nurses studied in this work can interact with DNA, inducing double- and single-strand breaks, cross-links, alkylations and DNA intercalations, which could be related, at least partially, to the observed genotoxicity.

Although hospital staff handling anti-neoplastic drugs wear individual protective equipment, which certainly reduces the risks (8), protection is not complete. Perhaps this problem should be managed differently, by automating some of the high-risk activities, for instance. In cases where this is not possible, besides the use of protective equipment, a proper natural diet rich in antioxidants and antimutagens should also be considered. The results presented in this work suggest that in order to improve occupational risk monitoring, some parameters of oxidative stress, like TBARS, as well as Sod and Cat activities, could also be performed together with the genotoxicity tests.

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