Mutagenesis vol. 26 no. 3 pp. 359–369, 2011
Advance Access Publication 26 November 2010
doi:10.1093/mutage/geq102

Assessment of primary, oxidative and excision repaired DNA damage in hospital personnel handling antineoplastic drugs

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Received on March 10, 2010; revised on October 20, 2010; accepted on October 22, 2010

The International Agency for Research on Cancer has classified several antineoplastic drugs in Group 1 (human carcinogens), among which chlorambucil, cyclophosphamide (CP) and tamoxifen, Group 2A (probable human carcinogens), among which cisplatin, etoposide, *N*-ethyl- and *N*-methyl-*N*-nitrosourea, and Group 2B (possible human carcinogens), among which bleomycins, merphalan and mitomycin C. The widespread use of these mutagenic/carcinogenic drugs in the treatment of cancer has led to anxiety about possible genotoxic hazards to medical personnel handling these drugs. The aim of the present study was to evaluate work environment contamination by antineoplastic drugs in a hospital in Central Italy and to assess the genotoxic risks associated with antineoplastic drug handling. The study group comprised 52 exposed subjects and 52 controls. Environmental contamination was assessed by taking wipe samples from different surfaces in preparation and administration rooms and nonwoven swabs were used as pads for the surrogate evaluation of dermal exposure, 5-fluorouracil and cytarabine were chosen as markers of exposure to antineoplastic drugs in the working environment. The actual exposure to antineoplastic drugs was evaluated by determining the urinary excretion of CP. The extent of primary, oxidative and excision repaired DNA damage was measured in peripheral blood leukocytes with the alkaline comet assay. To evaluate the role, if any, of genetic variants in the extent of genotoxic effects related to antineoplastic drug occupational exposure, the study subjects were genotyped for *GSTM1, GSTT1, GSTP1* and *TP53* polymorphisms. Primary DNA damage significantly increased in leukocytes of exposed nurses compared to controls. The use of personal protective equipment (i.e. gloves and/mask) was associated with a decrease in the extent of primary DNA damage.

Introduction

During the past decades, the marked increase in neoplastic pathologies has led to an increase in the use of antineoplastic drugs, which involves chemicals that have different structures, origins and activities as well as different effects at the cellular level (1,2). Thus, antineoplastic drugs constitute a vast heterogeneous class of compounds (e.g. alkylating drugs, antibiotics, metabolic antagonists, plant alkaloids, DNA chelators, hormones and enzymes) able to inhibit tumour growth by disrupting cell division and killing actively growing cells. However, their action on tumour cells is only partially selective (3), and normal cells may also suffer DNA changes as a result, leading to significant toxic side effects, including the induction of secondary tumours in individuals undergoing chemotherapy, most notably acute non-lymphocytic leukaemia (4). At present, a number of antineoplastic drugs commonly used in the treatment of cancer have been associated with secondary cancers in treated patients and several have been identified as rodent carcinogens or have shown genotoxic properties in *in vitro* test systems (5). On the basis of epidemiological findings, animal carcinogenicity data, as well as the outcomes of *in vitro* studies, the International Agency for Research on Cancer has classified several antineoplastic drugs in clinical use in Group 1 (human carcinogens), among which chlorambucil, cyclophosphamide (CP), etoposide and tamoxifen, Group 2A (probable human carcinogens), among which adriamycin, cisplatin, *N*-ethyl- and *N*-methyl-*N*-nitrosourea, and Group 2B (possible human carcinogens), among which bleomycins, merphalan and mitomycin C (6–12).

The widespread use of these mutagenic and carcinogenic drugs in the treatment of cancer has led to anxiety about possible genotoxic hazards to medical personnel handling these drugs, such as pharmacists (preparation), nurses (administration) and physicians and nurses (patient care) (13,14). Following the first evidence documenting occupational exposure to genotoxic/mutagenic compounds in health care workers involved in preparation and administration of antineoplastic drugs (15), several studies of occupational exposures have shown detectable levels of genototoxic agents in the air of hospital units (16–26). Cytogenetic methods have been used in the monitoring of occupational exposure to antineoplastic drugs. With regard to these methods, sister-chromatid exchanges, chromosomal aberrations and micronuclei (MN) are the most frequently used (27–35), with studies showing both positive and negative results.

More recently, the single-cell microgel electrophoresis (comet) assay (36,37) has also been applied to evaluate primary DNA damage in workers occupationally exposed to antineoplastic drugs (28,29,31–33,35,38–47).

The aim of the present study was to evaluate work environment contamination by antineoplastic drugs in a hospital in Central Italy and to assess the genotoxic risks associated with antineoplastic drug handling. The study group comprised 52 exposed and 52 non-exposed nurses. Environmental contamination was assessed by taking wipe samples from different surfaces in preparation and administration rooms and nonwoven swabs were used as pads for the surrogate evaluation of dermal exposure, 5-fluorouracil (5FU) and cytarabine (CYT) were chosen as markers of exposure to antineoplastic drugs in the

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working environment. The actual exposure to antineoplastic drugs was evaluated by determining the urinary excretion of CP.

The extent of primary DNA damage was measured in peripheral blood leukocytes with the standard procedure of the alkaline comet assay. Oxidised bases were determined by converting them to breaks using repair endonucleases such as endonuclease III (EndoIII), which recognises oxidised pyrimidines and formamidopyrimidine DNA-glycosylase (FPG), specific for altered purines as well as formamidopyrimidines and 8-oxo-7,8-dihydroguanine (48–50). Moreover, in order to increase the sensitivity of the assay, a modified protocol was used by incubating the lymphocytes with cytosine arabinoside (Ara-C). Ara-C is phosphorylated in the cell to its active metabolite, 1-β-D-arabinofuranosylcytosine 5′-triphosphate, which is in part incorporated into DNA thereby terminating DNA elongation, mostly by inhibiting the DNA polymerases (51,52). Breaks that occur as intermediates in nucleotide excision repair of DNA damage [e.g. induced by methylating agents or ultraviolet (UV)] or bulky adducts are normally short lived. Incubation of cells with DNA synthesis inhibitors, such as Ara-C, blocks repair patch synthesis and causes incision breaks to accumulate. Single-strand breaks accumulated at sites of incomplete repair can be thus detected by the alkaline comet assay (53). The usefulness of using DNA repair inhibitors in genotoxicity testing to increase the sensitivity of the assays has been highlighted in an *in vitro* study showing that several of the compounds that induced MN formation only produced increments in the comet assay in the presence of DNA repair inhibitors (54).

Attention has been recently focused on genetic polymorphisms that are thought to be involved in individual susceptibility to environmental genotoxins (55), and many genes encoding xenobiotics metabolising enzymes have been found to be polymorphic in humans, with relevance to the individual response to carcinogens, probably acting as modifiers of exposure biomarkers (susceptibility markers) (56–58). To evaluate the role, if any, of genetic variants in the extent of genotoxic effects related to antineoplastic drug occupational exposure, the study subjects were genotyped for three genes codifying for phase II enzymes involved in the inactivation of a variety of reactive chemical species: *GSTM1*, *GSTT1* and *GSTP1*. The major characteristic of *GSTM1* and *GSTT1* polymorphisms is the null genotype resulting from large deletions in coding sequences of the genes. The influence of polymorphism is more complex for *GSTP1*, in which single-nucleotide polymorphisms in two codons modify conjugating activity in a substrate-dependent manner (59). Genotyping was also performed for variants in the *TP53* gene, one of the most important tumour suppressor genes controlling DNA transcription and cell cycle regulation. The p53 protein induces apoptosis or cell cycle arrest in response to DNA damage, allowing the injured cells to be destroyed or repaired before reinitiating replicative DNA synthesis (60). The variant allele of a well-characterised polymorphisms, an arginine-to-proline nucleotide substitution at codon 72 (Arg72Pro), was been associated with lower apoptotic indices and DNA repair capacity (61). Moreover, some studies reported that the p53 Pro allele in homozygosity is associated with a higher risk for cancers in p53Pro homozygotes (62).

Materials and methods

Study subjects

A group of 52 health care workers involved in preparation, transportation, administration and disposal of anticancer agents was recruited on a voluntary basis in a hospital in Central Italy. A reference group of 52 healthy subjects working in public structures, not occupationally exposed to chemicals and comparable for age, sex and life style, was simultaneously examined. The non-exposed subjects were from the same localities as the exposed subjects to minimise the influence of other environmental factors on DNA damage. The subjects of both groups (exposed and non-exposed) were interviewed using a questionnaire to obtain details regarding age, nature of occupation, work experience, years of service, personal habits (e.g. smoking, health status, previous and present diseases, alcohol and drug consumption and the presence of other potential confounding factors). Subjects who had had radiation exposure, either for therapeutic or diagnostic purposes, were not included in the study.

Before the start of the study, approvals were obtained from the local Ethical Committee and Health Authorities. Exposed and non-exposed workers were informed about the aim and the experimental details of the study. Informed consent was obtained from all participating subjects.

Chemicals, media and enzymes

All reagents used were of analytical grade. Hydrochloric acid (HCl), dimethyl sulphoxide (DMSO), ethanol, ethylenediaminetetracetic acid disodium (Na_2EDTA) and ethylenediaminetetracetic acid tetrasodium (Na_4EDTA) salt, sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Carlo Erba Reagenti Srl, Milano, Italy. Ara-C, tris(hydroxymethyl)aminomethane (Tris) and Triton X-100 were obtained from Sigma–Aldrich Srl, Milano, Italy. Ethidium bromide (EB), normal-melting point agarose (NMPA), low-melting point agarose (LMPA) and agarose HR 3:1 were purchased from Euroclone SpA, Italy. Vacutainer® blood collection tubes were from Becton Dickinson Italia SpA, Milano, Italy. Polysucrose density gradient (Lympholyte® H) was from Cedarlane Laboratories Ltd, Burlington, Ontario, Canada. FLARE™ slides, EndoIII- and FPG–FLARE assay kits were purchased from Trevigen Inc., MD, USA. Gibco® culture medium RPMI-1640, L-glutamine, foetal calf serum, antibiotics (i.e. penicilin and streptomycin), phytohemagglutinin (PHA) and Dulbecco's phosphate-buffered saline (PBS) pH 7.4 were purchased from Invitrogen Srl, Milano, Italy. Primers (sequences were those reported in the works cited) were synthesised by MWG-Biotech AG, Ebersberg, Germany. Restriction enzymes Bsh1236I and Alw26I were purchased from Fermentas, Glen Burnie, MD, USA. *PfuTurbo*® DNA polymerase, 2′-deoxynucleoside-5′-triphosphates (dNTP) and magnesium chloride ($MgCl_2$) were obtained from Stratagene, Santa Clara, CA, USA. Conventional microscope slides and coverslips were supplied by Knittel-Glaser, Braunschweig, Germany. Distilled water was used throughout the experiments.

Evaluation of surface contamination and dermal exposure

Selected surfaces of the hospital pharmacy and oncological wards were analysed for antineoplastic drug contamination. Nonwoven swabs, wetted with 1 ml of 0.02 M ammonium acetate buffer (pH 4.7), were used to collect wipe samples. Wipe samples taken from work areas and floors were collected within distinct surface areas defined by plastic frames with internal sizes of either 10×10 cm or 20×20 cm (100 and 400 cm^2, respectively). Wiped objects such as handles, boxes and infusion bags were self-defined areas.

Nonwoven swabs (size of 7.5×7.5 cm) were also used as pads, for the surrogate evaluation of dermal exposure. Each exposed worker wore six pads, three outside and three inside their (protective) work-clothes: on the right and left forearms and on the chest. When the forearms of nurses and cleaners were not covered by clothing, the pads were attached directly to the skin at that body location. The sampling time covered the whole work-shift.

Wipe and pad samples were extracted with 0.02 M ammonium acetate buffer (pH 4.7) and subsequently analysed for 5FU and CYT. Model compounds (i.e. 5FU and CYT) were then determined by high-performance liquid chromatography with ultraviolet detection methods (63). The limits of detection (LoD) of the methods were 0.01 μg/ml.

Determination of CP in urine

Urine samples from exposed personnel were collected at the end of their work-shifts and 300 μl of 1 M Tris buffer (pH 8) were added to the urine samples (3 ml aliquots) which were then purified from the matrix by solid phase extraction using Extrelut NT3 glass columns (VWR International, Leicestershire, UK). The marker drug (i.e. CP) was eluted with 10 ml of dichloromethane. The eluates were evaporated to dryness under a nitrogen stream (40°C), the residues reconstituted with 100 μl of tetrahydrofurane and then derivatised by adding 50 μl of heptafluorobutyric anhydride (1 h, 70°C). After evaporation to dryness and redissolution with 100 μl of toluene, 1 μl aliquot was injected into a gas chromatograph. CP in urine samples was then determined by gas chromatography/mass spectrometry (64). The LoD of the method was 0.1 μg/l.

Alkaline comet assay

Peripheral blood samples were collected by venipuncture into Vacutainer heparinized tubes (blood sampling of exposed and non-exposed subjects was

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carried out simultaneously). Samples from exposed subjects were collected at the end of the work-shift after at least four consecutive days at work. Blood samples were coded, cooled (4°C), protected from light and processed as quickly as possible (usually within 4 h following withdrawal).

For the evaluation of primary DNA damage, peripheral blood leukocytes were processed in the comet assay following the standard alkaline protocol (alkaline unwinding/alkaline electrophoresis; pH > 13) (36,37) with minor modifications (65,66). Aliquots (10 µl) of heparinised blood were mixed with 100 µl of LMPA (0.7% in Ca^{++}/Mg^{++}-free PBS) and layered onto pre-coated (1% NMPA in Ca^{++}/Mg^{++}-free PBS) conventional slides. The cellular and nuclear membranes of the cells embedded in agarose microgels were lysed (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, NaOH to pH 10; 1% Triton X-100 and 10% DMSO were added just before use) for at least 1 h at 4°C. The slides were then drained and placed in a horizontal electrophoresis box (HE99; Hoefer Scientific, Holliston, USA) filled with a freshly prepared electrophoresis solution (10 mM Na_4EDTA, 300 mM NaOH; pH > 13). After 20 min of pre-electrophoresis (DNA unwinding), electrophoresis was performed for 20 min by applying an electric field of 25V (1 V/cm) and adjusting the current to 300 mA (Power Supply E411; Consort, Turnhout, Belgium). Finally, the microgels were neutralised with 0.4 M Tris–HCl buffer (pH 7.5) and then dehydrated by placing the slides in 70% ethanol for 15 min.

A modification of the standard comet assay was used to evaluate oxidative DNA damage (50). Oxidised bases were determined by converting them to breaks using repair endonucleases (i.e. EndoIII and FPG) in the FLARE (fragment length analysis using repair enzymes) test (48,49). Freshly collected white blood cells were included in agarose microgels and spread over sample areas of FLARE slides. After the lysis step, the slides were washed three times, 10 min each, with the FLARE buffer (10 mM HEPES–KOH, 100 mM KCl; pH 7.4) and then the nucleoids in the microgels were incubated with EndoIII or FPG for 60 min at 37°C in a humidity chamber. EndoIII or FPG solutions were prepared in FLARE reaction buffer (10 mM HEPES–KOH, 100 mM KCl; pH 7.4 containing 0.1 mg/ml bovine serum albumin) and spotted on samples areas (1 IU/microgel in 100 µl buffer) of the FLARE slides. Control microgels were incubated with 100 µl buffer only. For each subject, the results obtained for each enzyme were normalised by subtracting the level of DNA migration observed in the microgels incubated with the buffer only. To minimise potential variation in EndoIII or FPG activities, enzymes from the same batches, stored at −80°C in aliquots, were used throughout the experiments. The slides were then subjected to alkaline pre-electrophoresis/electrophoresis as above described for the standard comet assay. As suggested by the kit manufacturer, the ability of the enzymes to recognise oxidised DNA bases in our experimental conditions was checked by exposing Jurkat (human lymphoblastoid) cells to 20 mM hydrogen peroxide for 10 min on ice; the cells were then lysed and post-treated with EndoIII or FPG.

For the evaluation of excision repaired DNA damage, peripheral blood lymphocytes were isolated from whole blood samples by density–gradient centrifugation (Eppendorf 5804R; Eppendorf AG, Hamburg, Germany) at 800×*g*, for 20 min, without brake, using Lympholyte-H. The lymphocytes were resuspended in RPMI-1640 medium supplemented with 2 mM L-glutamine, 20% heat-inactivated foetal calf serum, 2% PHA, 1% antibiotic solution (containing 5000 IU/ml of penicilin and 5000 mg/ml of streptomycin) and cultured for 16 h in a humidified atmosphere at 5% CO_2. The cells were cultured in the presence or the absence of Ara-C (1 µg/ml) (67). At the end of the culture time, the cells were washed and harvested by centrifugation. Each pellet was mixed with 0.7% LMPA, subjected to alkaline lysis and electrophoresis as for the standard comet assay.

Computerised evaluation of DNA damage

Immediately before scoring, the air-dried slides were stained with 50 µl of EB (20 µg/ml). The comets in each microgel were analysed (blind), at ×500 magnification, with an epifluorescent microscope (Olympus BX41; Olympus, Tokyo, Japan) under a 100 W high-pressure mercury lamp (HSH-1030-L; Ushio, Tokyo, Japan), using appropriate optical filters (excitation filter 510–550 nm and emission filter 590 nm). The microscope, equipped with a high-sensitivity black and white CCD camera (PE2020; Pulnix Europe Ltd, Basingstoke, UK), was connected to a computerised analysis system ('Comet Assay III'; Perceptive Instruments, Ltd., Haverhill, Suffolk, UK) that acquires images, computes the integrated intensity profile for each cell, estimates the comet cell components, head and tail and evaluates a range of derived parameters. These include: tail length (measured from the head centre, expressed in micrometer), tail intensity (percent of fluorescence in the comet tail) and tail moment, a composite parameter in which the migration distance and the amount of migrated DNA (by analogy with the mechanical term) are expressed as a single value. For each subject, the DNA migration extent was evaluated in a total of 150 comets (50 comets per slide, from at least three replicate slides) for each experimental set (i.e. primary, oxidative and excision repaired DNA damage).

DNA extraction and genotyping

Genomic DNA was extracted from whole blood with a robotic magnetic particle processor (KingFisher mL; Thermo Scientific, Vantaa, Finland) using a commercially available DNA extraction kit (NucleoMag 96 Blood; Macherey–Nagel, Düren, Germany). Polymerase chain reaction (PCR) amplifications were conducted with a PCR 'Sprint' thermocycler (Hybaid Ltd, Middlesex, UK).

GSTM1 and *GSTT1* genotyping for gene deletions was carried out by determining whether the intact gene was present or the absent (68). PCRs were carried out, separately for each gene, in a 50 µl volume containing 100 ng of genomic DNA, 2 mM $MgCl_2$, 250 µM dNTP, 0.1 µM primers and 1.5 U *PfuTurbo* DNA polymerase. In the thermocycling procedure, initial denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 45 sec before a final extension at 72°C for 5 min. PCR products were separated by electrophoresis (1 h, 100 mA, 150 V) on a 2% agarose HR 3:1 gel stained with EB (0.5 µg/ml). The presence or absence of the *GSTM1* (215 bp) and *GSTT1* (480 bp) amplicons was determined in the presence of the control β-globin gene (268 bp). The positive genotypes for *GSTM1* and *GSTT1* were defined by the occurrence of the specific bands (i.e. 215 and 480 bp) present in wild-type homozygotes and heterozygotes for the deletion (not differentiated in the analysis and both expressing GSTM1 or GSTT1 enzymes), whereas the absence of the *GSTM1*- or *GSTT1*-specific PCR product indicated the corresponding null genotype (homozygous deletion of the *GSTM1* or *GSTT1* gene).

GSTP1 polymorphism was detected by PCR followed by restriction fragment length polymorphism (RFLP) analysis (68). Amplifications were performed in 50 µl total volume (200 ng of genomic DNA, 250 µM dNTP, 0.2 µM primers, 2 mM $MgCl_2$ and 1.5 U *PfuTurbo* DNA polymerase). In the thermocycling procedure, initial denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec and extension at 72°C for 45 sec before a final extension at 72°C for 5 min. PCR products were electrophoresed (1 h, 100 mA, 150 V) on a 2% agarose HR 3:1 gel stained with EB (0.5 µg/ml). The 176 bp *GSTP1* gene fragment was digested at 55°C for 16 hr with 5 U of Alw26I restriction enzyme in a 50 µl reaction volume containing 7.5 µl of PCR product, 100 mM NaCl, 50 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$ and 1 mM dithiothreitol. The digestion products were then analysed electrophoretically (1 h and 30 min, 100 mA, 150 V) on a 2.5% agarose HR 3:1 gel stained with EB (0.5 µg/ml) and the genotype was determined by analysing the bands on the gel: the wild-type genotype (lacking the Alw26I site) homozygous for isoleucine (Ile/Ile) formed an uncleaved 176 bp band, the heterozygous genotype (isoleucine/valine) (Ile/Val) showed three bands of 176, 91 and 85 bp, while the variant genotype homozygous for the allele carrying the mutation with the Alw26I site (Val/Val) generated two bands of 91 and 85 bp.

The *TP53* codon 72 polymorphism was determined using PCR–RFLP analysis as described by Omori *et al.* (69). A genomic DNA sample (200 ng) was added to the PCR mixture containing 2.0 mM $MgCl_2$, 250 µM dNTP, 0.2 µM of each *p53* primer and 1.5 U *PfuTurbo* DNA polymerase in a final volume of 50 µl. The PCR was conducted using the following thermal profile: an initial denaturation cycle of 96°C for 12 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and a final cycle of 72°C for 5 min. PCR products were subjected to electophoresis (1 h, 100 mA, 150 V) in a 2% agarose HR 3:1 gel containing EB (0.5 µg/ml). After confirmation of an amplified fragment of the expected size (199 bp), the PCR products were digested with 5 U of restriction enzyme Bsh1236I at 60°C for 20 h. DNA fragments were subsequently elecrophoresed (1 h and 30 min, 100 mA, 150 V) through a 2.5% agarose HR 3:1 gel and stained with EB (0.5 µg/ml). The Pro allele is not cleaved by Bsh1236I at codon 72 and runs as a single band with a fragment length of 199 bp, whereas the Arg allele is cleaved by Bsh1236I and runs as two small fragments of 113 and 86 bp. Digestion of the heterozygote yields three bands of 199, 113 and 86 bp.

Statistical analysis

The Hardy–Weinberg equilibrium test for *GSTP1* and *p53* genotype distributions was performed using a χ^2 test with 1 degree of freedom; the *GSTM1* and *GSTT1* genotypes were coded as positive (wild-type homozygotes and heterozygotes for the deletion) or null (homozygous deletion), making direct calculation of Hardy–Weinberg equilibrium impossible. Pearson's χ^2 test was used to evaluate differences in the distributions of allele frequencies between exposed and non-exposed subjects.

In this approach, in the comet assay, the statistical unit is the subject (70) and, therefore, the series of measures obtained for each individual in the different experimental sets (i.e. evaluation of primary, oxidative and excision repaired DNA damage) were reduced to summary statistics representative of the comet distributions. The mean (± standard error of the mean), median, 75th

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percentile and dispersion coefficient (H) were chosen as measures to summarise DNA migration (70–72); H was calculated as the ratio of the sample variance to the sample mean in order to determine the effect of exposure on the distribution of the migration patterns within each subject (73). DNA migration values were tested for normal distribution with the Shapiro–Wilk's test.

The presence of possible significant differences between exposed and non-exposed subjects were tested with the non-parametric Mann–Whitney U-test. Two-sided P values <0.05 were regarded as statistically significant. Possible differences between subgroups were investigated through Kruskal–Wallis H test. For significant results, to examine where the differences actually occurred, post-hoc analysis was performed by running separate Mann–Whitney U-tests on the different combinations of related groups (multiple pairwise comparisons) with Bonferroni correction of the α in order to maintain the overall probability of a type I error at 0.05.

Multivariate regression analysis was performed to examine the possible influence of exposure status, gender, age, smoking habits, occupational assignment, job seniority, personal protection and genetic polymorphism profiles as independent variables on the extent of primary, oxidative and excision repaired DNA damage.

Statistical analyses were carried out using the SPSS statistical package (SPSS Inc., IL, USA).

Results

Characteristics of the study population

Demographic characteristics of the study population, also grouped according to exposure status and smoking habits, are summarised in Table I. With respect to gender, age and smoking habits, exposed and non-exposed groups were comparable. The exposed workers and non-exposed subjects were stratified according to genotypes *GSTM1*, *GSTP1*, *GSTT1* and *TP53* (Table II). Similar frequency distributions (Pearson's χ^2 test) were observed in the groups for the genotypes considered. In the whole study population, the prevalence of *GSTM1*-null subjects was 63 (60.6%), of which 31 (59.6%) were exposed and 32 non-exposed (61.5%), whereas the prevalence of *GSTT1*-null subjects was 9 (8.7%), of which 4 were exposed (7.7%) and 5 non-exposed (9.6%). With regard to the *GSTP1* gene, 56 subjects (53.8%) resulted heterozygotes for the wild-type (Ile/Ile) allele, 26 exposed workers (50.0%) and 30 non-exposed subjects (57.7%); for statistical analysis, the subjects carrying the variant genotype in homozygosis (Val/Val) were combined with those having the mutation in heterozygosis (Ile/Val) and the 48 individuals (46.6%) carrying the variant genotype were 26 exposed workers (50.0%) and 22 non-exposed (42.3%). As regards the *TP53* gene polymorphism, in the group of 104 subjects examined, the Arg allele was carried in heterozygosis by 54 subjects (52.9%), 29 (55.8%) exposed workers and 25 non-exposed nurses; the Pro allele for *TP53* gene occurred in 50 individuals (48.1%), 23 exposed workers (44.2%) and 27 non-exposed subjects (51.9%) and subjects carrying the homozygous Pro/Pro variant were combined with heterozygous (Arg/Pro) individuals for statistical analysis. The above reported genotypes (i.e. *GSTP1* Ile/Val and *TP53*) among exposed and control subjects were in Hardy–Weinberg equilibrium (data not shown).

Surface and dermal contamination, cyclophosphamide in urine samples

Exposed subjects handled a multiplicity of antineoplastic drugs, often in mixtures of two or more drugs. Table III summarises the frequencies of antineoplastic drug handling reported as the percentage of subject who have handled each drug at least once over a period of 6 months before environmental and biological monitoring (data obtained by questionnaires).

Environmental monitoring of surface (wipes) and dermal (pads) contamination from the monitored model compounds (i.e. 5FU and CYT) showed the presence of detectable levels of antineoplastic drugs, with different amount rates, at the sites of preparation and administration.

A total of 22 wipes (29.3%) were positive for 5FU or CYT (LoD 0.01 μg/ml, for both chemicals), with concentrations ranging from 0.02 to 2 $\mu g/dm^2$. The highest contamination level was found in the preparation labs, with 36% positive wipe samples (concentration range: 0.13–2 $\mu g/dm^2$), with respect to the administration wards, with 21.2% positive samples (concentration range: 0.02–0.48 $\mu g/dm^2$).

For the evaluation of dermal exposure, pads were attached on the outer side of the clothing (potential exposure) and

Table I. Main characteristics of the study population (hospital personnel) grouped according to exposure status to antineoplastic drugs

	Exposed	Non-exposed
Subjects[a]	52	52
Gender[a]		
Males	7 (13.5%)	12 (23.1%)
Females	45 (86.5%)	40 (76.9%)
Age[b]	39.26 ± 9.59	36.21 ± 11.21
<40 years[c]	32 (61.5%)	35 (67.3%)
≥40 years[c]	20 (38.5%)	17 (32.7%)
Smoking habits[a]		
Non-smokers	32 (61.5%)	38 (73.1%)
Smokers	20 (38.5%)	14 (26.9%)
Occupational assignment[a]		
Pharmacy technicians	6 (11.5%)	—
Day hospital nurses	16 (30.8%)	—
Ward nurses	22 (42.3%)	—
Attendants	8 (15.4%)	—
Job seniority[a]		
<10 years	34 (65.4%)	—
11–20 years	14 (26.9%)	—
>20 years	4 (7.7%)	—
Personal protection[a]		
Gloves	3 (5.8%)	—
Gloves + mask	41 (78.8%)	
No protection	8 (15.4%)	—

[a]Data are reported as the number of subjects (% between brackets).
[b]Age and years employed are expressed in years and reported as the group mean ± standard deviation.
[c]Cut-off defined according to the mean value (i.e. 39.26 years) of the observed age distribution in the exposed subjects.

Table II. Frequency distribution of *GSTM1, GSTT1, GSTP1* and *TP53* genotypes among exposed and non-exposed subjects

Genotype	Exposed[a]	Non-exposed[a]
GSTM1		
Positive	21 (40.4%)	20 (38.5%)
Null	31 (59.6%)	32 (61.5%)
GSTM1		
Positive	48 (92.3%)	47 (90.4%)
Null	4 (7.7%)	5 (9.6%)
GSTM1[b]		
Ile/Ile	26 (50.0%)	30 (57.7%)
Ile/Val	23 (44.2%)	15 (28.8%)
Val/Val	3 (5.8%)	7 (13.5%)
TP53[c]		
Arg/Arg	29 (55.8%)	25 (48.1%)
Arg/Pro	17 (32.7%)	25 (48.1%)
Pro/Pro	6 (11.5%)	2 (3.8%)

[a]Data are reported as the number of subjects (% between brackets).
[b]Ile = common allele, Val = variant allele.
[c]Arg = common allele, Pro = variant allele.

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inside, directly to the uncovered skin (actual exposure). The analysis, at the end of the work-shift, of pads worn by the operators who handled 5FU ($n = 9$) or CYT ($n = 2$) showed that clothing exposure occurred in hospital personnel involved in the manipulation of antineoplastic drugs (Table IV).

Biological monitoring of exposed subjects ($n = 40$) showed detectable levels of CP in the post-shift urine samples of seven nurses (17.5%), with CP concentrations in the range 0.1–0.2 μg/l. One subject had a urinary CP concentration of 1.2 μg/l. The remaining samples had CP below the limit of detection.

DNA damage

The distributions of individual mean, median, 75th percentile and dispersion coefficient values (tail length and tail intensity) among exposed and control subjects are reported in Figure 1. Only tail length and tail intensity values have been considered as tail moment may be calculated differently among different image analysis systems, with quantitative differences which render this metric not comparable across studies. The distributions in Figure 1 clearly show that higher DNA migration extents are evident in the exposed subjects in terms of tail intensity but not in terms of tail length. By analogy with the process of validation of a bioanalytical method, an analytical parameter can effectively be considered to be sensitive if differences in the exposure levels cause clearly evident changes in the response function (74). For this reason, we have considered only tail intensity mean, 75th percentile and dispersion coefficient (H) individual values for subsequent statistical analyses. Moreover, the DNA migration extents outlined in Figure 1 showed distributions that differed from normality ($P < 0.05$, Shapiro–Wilk W-test).

DNA migration values evaluated by comet assay in the peripheral blood leukocytes of exposed and non-exposed subjects and referred to primary DNA damage are summarised in Table V. The results are reported as the group averages (± standard error of the mean) of individual mean, 75th percentile and dispersion coefficient (H) values. Data are also reported considering gender, age, smoking habits (whole population) and occupational assignment, job seniority, personal protection (exposed subjects).

Primary DNA damage significantly increased in leukocytes of exposed nurses, with group mean values (± standard error of the mean) of individual tail intensity averages being 2.73 ± 0.28, with non-exposed nurses being 1.67 ± 0.14 ($P < 0.0001$). Statistically significant differences between exposed and controls were observed also for group averaged 75th percentile (3.02 and 1.76 for exposed and controls respectively; $P < 0.015$) and H (10.05 and 6.90 for exposed and controls respectively; $P < 0.0001$) group values.

Gender, age and smoking habits were not associated with any increase in the extent of DNA migration, either in the exposed or in the reference group. Similarly, genetic polymorphisms did not have any influence on the extent of DNA migration, either in the exposed or in the controls. Among the exposed subjects, no statistically significant differences were observed for the extent of DNA migration in relation to occupational assignment and job seniority, whereas the use of personal protective equipments (i.e. gloves and/or mask) has been associated with a statistically significant decrease in the extent of primary DNA damage with subgroup mean values (± standard error of the mean) of individual tail intensity averages being 2.60 ± 0.31 for subjects wearing gloves and masks and 3.40 ± 0.47 for those who usually did not wear masks and gloves ($P = 0.045$).

Statistically significant differences were never observed for oxidative DNA damage evaluated as EndoIII or FPG sites (data not shown). The only statistically significant difference observed for excision repaired DNA damage was the higher DNA migration value in exposed nurses, with group mean values (± standard error of the mean) of individual tail intensity averages being 9.58 ± 0.62 ($P = 0.016$) as compared to control subjects (8.27 ± 0.46).

Table III. Frequencies of antineoplastic drug handling. Data reported as the percentage of subject who have handled each drug at least once over a period of 6 months

Drug	%
Cyclophosphamide	65.4
Cisplatin	63.5
Cytarabine	55.8
Doxorubicin	55.8
Vincristine	53.8
Carboplatin	51.9
Etoposide	48.1
Fludarabine	48.1
Epirubicin	44.2
Methotrexate	40.4
Gemcitabine	38.5
5-Fluorouracil	36.5
Taxanes	32.7
Mitomycin C	15.4
Ifosfamide	15.4
Raltitrexed	3.8

Table IV. 5FU and CYT dermal exposure: total pads number, percent of detectable samples (>LoD = 0.01 μg/pad) and concentration range (μg/pad) at each body location

Pads[a]	5FU			CYT		
	n	Detectable samples *n* (%)	Range (μg/pad)	*n*	Detectable samples *n* (%)	Range (μg/pad)
Torso outside	9	2 (22.2)	n.d. to 8.69	2	1 (50)	n.d. to 0.15
Torso inside	9	0	n.d.	2	0	n.d.
Right forearm outside	9	2 (22.2)	n.d. to 1.26	2	1 (50)	n.d. to 0.06
Right forearm inside	5	0	n.d.	2	0	n.d
Left forearm outside	9	2 (22.2)	n.d. to 0.04	2	0	n.d
Left forearm inside	5	0	n.d.	2	0	n.d
Total	46	6 (13.0)	n.d. to 8.69	12	2 (16.7)	n.d. to 0.15

n.d. = concentration below the LoD (i.e. 0.01 μg/pad).
[a]Exposed workers wore pads outside and inside (attached directly to the skin) their work-clothes.

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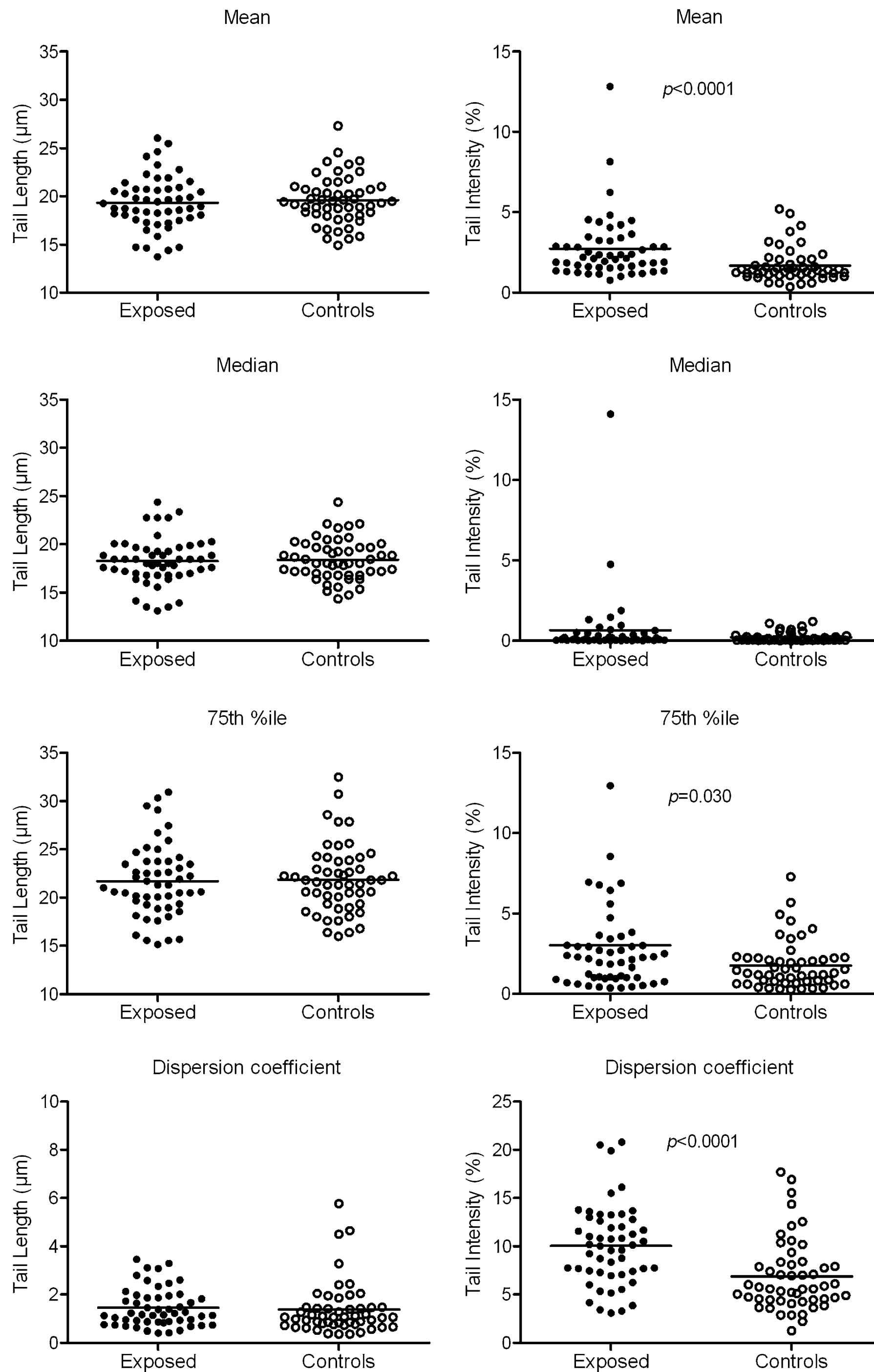

Fig. 1. Tail length and tail intensity of comets in peripheral blood leukocytes of 52 health care workers involved in preparation, transportation, administration and disposal of antineoplastic drugs and 52 unexposed nurses. Distributions of individual mean, median, 75th percentile and dispersion coefficient (*H*) values (transverse lines represent the mean values of each group). Differences between exposed and non-exposed subjects were tested with the non-parametric Mann–Whitney *U*-test (two-sided *P* values <0.05 were regarded as statistically significant).

Multivariate regression analysis (back-wise procedure; $P = 0.05$ for entry into the model) was performed using exposure status, gender, age, smoking habits and genetic polymorphism profiles (whole population), as well as occupational assignment, job seniority and personal protection (exposed subjects) as independent variables (data not shown). This statistical approach indicated that variance in the extent of DNA migration in the study population could be explained fundamentally by the subjects' occupation ($\beta = 0.316$, $P = 0.001$), thus confirming a statistically significant positive association of primary DNA damage with exposure to antineoplastic drugs.

Discussion

In the present study, contamination of working environments by antineoplastic drugs was assessed by monitoring surface (wipes) and dermal (pads) contamination. The standard

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Table V. Extent of primary, oxidative and excision repaired DNA damage (expressed as tail intensity) in peripheral blood leukocytes in exposed workers and non-exposed subjects with respect of gender, age, smoking habits (whole population) and occupational assignment, job seniority and personal protection (exposed subjects)

	Exposed				Controls			
	n	Mean ± SEM	75th percentile	*H*	*n*	Mean ± SEM	75th percentile	*H*
Total	52	2.73 ± 0.28*	3.02*	10.05*	52	1.67 ± 0.14	1.76	6.90
Gender								
Males	7	1.82 ± 0.28	1.76	7.01	12	1.76 ± 0.41	1.81	7.24
Females	45	2.86 ± 0.31#	3.22	10.52#	40	1.64 ± 0.15	1.74	6.79
Age								
<40 years	32	2.76 ± 0.38	3.16	9.76	35	1.70 ± 0.16	1.88	6.75
>40 years	20	2.66 ± 0.39#	2.79	10.53#	17	1.61 ± 0.29	1.51	7.20
Smoking habits								
Non-smokers	32	2.81 ± 0.41	3.17	10.56#	38	1.82 ± 0.19	1.96	7.36
Smokers	20	2.59 ± 0.31#	2.77	9.24#	14	1.27 ± 0.08	1.20	5.63
GSTM1								
Positive	21	2.43 ± 0.27	2.62	9.41	20	1.72 ± 0.24	1.96	6.79
Null	31	2.93 ± 0.43#	3.29	10.49#	32	1.65 ± 0.18	1.63	6.96
GSTT1								
Positive	48	2.65 ± 0.28#	2.86	10.07#	47	1.71 ± 0.16	1.82	6.94
Null	4	3.67 ± 1.57	4.94	9.86	5	1.30 ± 0.21	1.14	6.46
GSTP1								
Ile/Ile	26	3.06 ± 0.43#	3.49	10.39#	30	1.77 ± 0.21	1.83	7.59
Ile/Val + Val/Val	26	2.39 ± 0.34	2.55	9.71	22	1.54 ± 0.19	1.66	5.95
TP53								
Arg/Arg	29	3.05 ± 0.46#	3.61	9.89	25	1.59 ± 0.19	1.54	7.15
Arg/Pro + Pro/Pro	23	2.31 ± 0.23	2.28	10.26#	27	1.75 ± 0.21	1.96	6.66
Occupational assignment								
Pharmacy technicians	6	1.76 ± 0.34	1.39	11.22		—		—
Day hospital nurses	16	3.19 ± 0.69	3.42	11.20		—		—
Ward nurses	22	2.89 ± 0.39	3.67	8.73		—		—
Attendants	8	2.06 ± 1.89	1.63	10.50		—		—
Job seniority								
<10 years	34	2.39 ± 0.24	2.57	9.30		—		—
>10 years	18	3.34 ± 0.65	3.87	11.47		—		—
Personal protection								
No protection	8	3.40 ± 0.47§	3.91	10.16		—		—
Gloves/mask	44	2.60 ± 0.31	2.86	10.03		—		—

Data reported as the group averages (± standard error of the mean) of individual mean, 75th percentile and dispersion coefficient (*H*) values. Statistical significance: * versus corresponding non-exposed nurses (Mann–Whitney *U*-test); # versus corresponding non-exposed nurses (post-hoc analysis, Mann–Whitney *U*-test multiple pairwise comparisons with Bonferroni correction for positive Kruskal–Wallis *H* tests); § versus gloves/mask (Mann–Whitney *U*-test).

regulation in force regarding this topic in Italy is the Guideline Document from the State-Regions Conference (75) incorporating many of the measures included in the international warnings and guidelines, as well as the rules for safe and appropriate organisation of services for antineoplastic preparations (76–84). Wipe samples showed the presence of antineoplastic drugs, with different amount rates, at the sites of preparation and administration. The monitored model compounds (i.e. 5FU and CYT) showed detectable levels in a total of 22 wipes, with concentrations ranging from 0.02 to 2 μg/dm^2. The highest contamination level was found in the preparation labs, with 36% positive wipe samples (concentration range: 0.13–2 μg/dm^2), with respect to the administration wards, with 21.2% positive samples (concentration range: 0.02–0.48 μg/dm^2). Dermal exposure as evaluated with pads also occurred in hospital personnel involved in the handling of antineoplastic drugs and biological monitoring of exposed subjects showed detectable levels of CP in the post-shift urine samples of seven nurses (17.5%), with CP concentrations in the range 0.1–0.2 μg/l. One subject had a urinary CP concentration of 1.2 μg/l.

However, nurses commonly handle many different antineoplastic drugs, in most cases in combination. Thus, the use of a limited number of marker compounds to evaluate exposure to complex mixtures of the various incorporated drugs by chemical determinations in urine, wipes and pads could be limitative. Therefore, we have integrated the above-mentioned approach by using biotoxicological biomarkers and nurses handling antineoplastic drugs were evaluated for genotoxic damage in peripheral blood leukocytes (primary and oxidative DNA damage) or lymphocytes (excision repaired DNA damage). DNA damage was evaluated by applying the comet assay for the detection of early biological effects of DNA-damaging agents (i.e. chemotherapeutic agents) (37,85).

In this work, the higher DNA migration extents were observed in the exposed subjects in terms of tail intensity rather than tail length. This behaviour for DNA migration could be, at least in part, explained with handling of cross-linking drugs by exposed nurses. The most commonly handled antineoplastic drugs by the hospital personnel monitored in this study were CP and cisplatin. Both CP and cisplatin are efficient producer of DNA–DNA interstrand and intrastrand cross-links (86,87) and cross-linking agents, for increasing the effective molecular weight of DNA, are thereby known to reduce the ability of DNA containing strand breaks to migrate in an electric field (88). The presence of a cross-linking agent could have hidden an increase in DNA migration associated with the induction of DNA strand breaks by other genotoxic agents, with an higher effect in terms of DNA tail mobility (i.e. tail length) and a less

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effect with regard of the amount of migrated DNA (i.e. tail intensity).

Our positive outcomes are in line with the findings reported in several studies indicating that individuals handling antineoplastic drugs have a significantly higher extent of primary DNA damage, as evaluated with the comet assay, compared to non-exposed subjects (28,29,31–33,35,38–43,45,46). Furthermore, very few studies have indicated that there is no correlation between exposure to antineoplastic drugs and DNA damage (44,47). On the basis of obtained results, we have planned a further biomonitoring approach aimed at evaluating whether occupational exposure to antineoplastic drugs could result in genetic damage indicative of long term adverse health effects (i.e. MN in peripheral blood lymphocytes).

Among the exposed subjects, there were no significant differences in terms of primary DNA damage between day hospital nurses, ward nurses, attendants and pharmacy technicians, although pharmacy technicians showed an extent of DNA damage similar to that observed in the control (non-exposed) subjects and day hospital nurses showed the highest observed extent of DNA damage among the considered subgroups. The use of personal protective equipment (i.e. masks and/or gloves) has been associated with a statistically significant decrease in the extent of primary DNA damage. The differences observed with regard to occupational assignment and personal protection confirm the weight of workplace conditions and training in the individual risk of exposure to genotoxic agents.

These findings are in agreement with published reports indicating that the use of appropriate protection and devices can reduce the genotoxic burden in the workplace (34,38,40,45). Moreover, our data confirm that sporadic exposure, a problem to be faced in daily practice, affects nurses more than pharmacists. In accordance with data retrieved in the literature, no correlations were found between age, job seniority or smoking habits and the levels of genotoxic damage (40,41,89).

Even though several guidelines for the handling of antineoplastic drugs, as well as safety recommendations, were issued to minimise the risk of occupational exposure (76–84), reports in the current literature indicate that significant incorporation of trace amounts of these agents still occurs in hospital personnel with a detectable residual genotoxic risks, also revealed by surfaces or urine contamination with agents such as CP or methotrexate (90–95).

The positive results obtained in this study, together with the findings of other researches aimed at evaluating DNA damage in health care workers handling antineoplastic drugs (28,29,31–33,35,38–43,45,46), suggest that the evaluation of DNA damage (i.e. comet assay) in peripheral blood leukocytes could be a useful tool to detect early genotoxic effects induced by antineoplastic drugs.

In conclusion, the implication of our study is that the handling practices of antineoplastic drugs adopted by the monitored nurses are not sufficient to prevent occupational exposure to genotoxic xenobiotics. The main routes of exposure to antineoplastic drugs are supposed to be inhalation of aerosolized drugs, percutaneous absorption of drugs contaminating the working environment (e.g. as the result of vial leakage or accidental spill) and accidental ingestion during drug preparation/administration, disposal of equipments or when human excreta are handled (96,97). To minimise the genotoxic risk arising from occupational exposure to antineoplastic drugs, our findings emphasise the importance of proper use of protective equipment and implementation of work-practices to avoid health hazards.

According to the standard regulation in force in Italy, as well as to several international guidelines, to ascertain the relationship between exposure to antineoplastic drugs and genotoxic hazard, environmental (i.e. surface and dermal contamination) and biological (i.e. antineoplastic drugs and/or metabolites in urine) monitoring are proposed to be carried out. Analytical methods for routine monitoring of occupational exposure to antineoplastic drugs (e.g. high-performance liquid chromatography, gas chromatography/mass spectrometry) are very sensitive and specific. Nevertheless, testing is generally limited to one or two agents that are considered as model compounds. However, exposed nurses are occupationally exposed to mixtures of antineoplastic drugs. Therefore, it should be important to modify guidelines for the evaluation of mutagenic/carcinogenic hazards in occupationally exposed subjects by considering an integrated chemical/biotoxicological approach. The use of biomarkers which measure changes in cellular or molecular endpoints (e.g. DNA damage) will allow to apply a more complete approach following not only environmental and biological monitoring but also biological effect monitoring using genotoxicity biomarkers. In this context, the comet assay represents a high sensitivity technique for detecting low levels of DNA damage in individual cells (98–100) and could be proposed to be used to perform accurate health surveillance of workers occupationally exposed to antineoplastic drugs.

Funding

Italian National Institute for Occupational Safety and Prevention, Grant No. B88/MDL/03.

Acknowledgements

The Authors wish to thank Dr Valentina Ortica and Dr Italo Lombardi for their valuable technical support and Prof. Giuseppina Scassellati-Sforzolini, Emeritus Professor of Hygiene, for her helpful assistance during this research and constructive comments on the manuscript.

Conflict of interest statement: None declared.

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