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Comparative aneugenicity of doxorubicin and its derivative idarubicin using fluorescence *in situ* hybridization techniques

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ABSTRACT

The present study was designed to evaluate and compare the aneugenicity of idarubicin and doxorubicin, topoisomerase-targeting anticancer anthracyclines, using fluorescence *in situ* hybridization techniques. It was found that idarubicin and doxorubicin treatment (12 mg/kg) induced sperm meiotic delay of 24 h. To determine the frequencies of disomic and diploid sperm, groups of 5 male Swiss albino mice were treated with 3, 6 and 12 mg/kg idarubicin or doxorubicin. Significant increases in the frequencies of disomic and diploid sperm were caused by treatment with all doses of idarubicin and the two highest doses of doxorubicin compared with the controls. Moreover, both compounds significantly increased the frequency of diploid sperm, indicating that complete meiotic arrest occurred. The observation that XX- and YY-sperm significantly prevailed XY-sperm indicates missegregation during the second meiotic division. The results suggest also that earlier prophase stages contribute relatively less to idarubicin and doxorubicin-induced aneuploidy. Effects of the same doses were investigated by the bone-marrow micronucleus test. Significant increases in the frequencies of micronuclei were found after treatment with all doses of both compounds. The responses were also directly correlated with bone marrow suppression. Idarubicin was more toxic than doxorubicin. Exposure to 12 mg/kg of idarubicin and doxorubicin yielded 3.82 and 2.64% micronuclei, respectively, and of these an average of 58.3 and 62.8%, respectively, showed centromeric signals, indicating their formation by whole chromosomes and reflecting the aneugenic activity of both compounds. Correspondingly, about 41.7 and 37.2% of the induced micronuclei, respectively, were centromere-negative, demonstrating that both compounds not only induce chromosome loss but also DNA strand breaks. Based on our data, aneuploidy assays such as sperm-fluorescence *in situ* hybridization assay and micronucleus test complemented by fluorescence *in situ* hybridization with centromeric DNA probes have been to some extent validated to be recommended for the assessment of aneuploidogenic effects of chemicals.

1. Introduction

The anthracycline group of antibiotics includes the most effective classes of antineoplastic agents available for the treatment of human cancers [1]. Among the anthracyclines, idarubicin is an important anticancer agent. Idarubicin (4 demethoxydaunorubicin) is a synthetic analog of daunorubicin and doxorubicin. Deletion of the methoxy group increases lipophilicity of idarubicin compared with that of daunorubicin and doxorubicin, enhances its uptake into tumor cells and may increase its binding to DNA. Idarubicin has demonstrated a 10-fold higher cytotoxic activity than daunorubicin in cultured human cancer cells [2] which has been attributed to its greater hydrophobicity, greater induction of DNA strand breaks [3], and generation of free radicals and G_2 cell cycle arrest. The primary mechanism of tumor cytotoxicity appears to be the inhibition of topoisomerase II [2,4]. Idarubicin and doxorubicin stabilize the topoisomerase II-DNA complexes, preventing the rapid turnover of the protein-cross-linked DNA strand breaks and interfere with processes that require changes in DNA topology, such as DNA replication, repair and transcription. Other mechanisms of these agents include DNA intercalation, nuclear helicases inhibition and free radical formation [2,4]. As a consequence of these multiple effects, single- and double-strand breaks are introduced into the DNA [2,4]. DNA damage can have a variety of biological ramifications including inhibition of transcription and/or replication and ultimately leads to cell death [5].

As with many other anticancer drugs, high doses of anthracyclines, as doxorubicin induce apoptosis and disruption of inner mitochondrial membrane potential [6]. However, several lines of evidence indicate that low doses of various chemotherapeutic drugs are capable of inducing mitotic catastrophe, resulting from abnormal mitotic events that produce improper chromosomal segregation and cell division, leading to the formation of mutant cells [7]. Chromosomal imbalance (aneuploidy) induced by aneugens

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is a phenomenon observed in over 90% of all solid tumors. Even haematological cancers, known to have a rather stable chromosome number, frequently display loss or gain of few chromosomes [8]. Diploidy and aneuploidy in germ cells may cause abortions or children with congenital abnormalities such as trisomy 21, Klinefelter's or Turner syndromes, respectively [9]. Despite anthracycline's increasing use in malignancies, scarce data are available in literature on its potential aneugenicity *in vivo*.

Considering the deleterious consequences of aneuploidy in germinal and somatic cells, it is important that validated assays for the detection of chemically induced aneuploidy *in vitro* and *in vivo* are available. An important advancement in sperm cytogenetics for detecting the consequences of chemically induced missegregation during meiosis was the adaptation of fluorescence *in situ* hybridization (FISH) with chromosome-specific DNA probes [10,11]. Using FISH to score the numbers of signals in sperm, large numbers of sperm can be scored quickly. Furthermore, the use of multicolor FISH techniques made it possible to study several chromosomes simultaneously for disomy and to distinguish between diploid and disomic sperm in rodents and humans after exposure to chemical aneugens [10–13]. The micronucleus test is used extensively to evaluate irreversible impacts on chromosome stability, and increased micronuclei (MN) frequency predicts the risk of cancer in humans [14]. Since MN can result from lagging chromosomes (aneugenicity) or chromosome breakage (clastogenicity), the detection of MN has the potential to be used as a screen for numerical chromosomal aberrations induction if methods are included to allow the identification of whole chromosomes inside of MN. Various techniques have been developed to distinguish MN induced by aneugens or by clastogens. However, the most reliable methods are the ones that mark centromeres (i.e. Immunofluorescent CREST-staining and FISH techniques).

In an effort to predict patients' reaction to tumor therapy, the use of *in vivo* tests has gained increasing importance. In order to test the suitability of the FISH assay for predictive purposes, the present study was designed to evaluate and compare the aneugenicity of idarubicin and doxorubicin in germinal and somatic cells of male mice. Three types of experimental studies were applied: the BrdU-incorporation assay to test if the chemical treatment altered the duration of the meiotic divisions, the sperm-FISH assay for aneugenicity induction during male meiosis and the bone marrow MN test complemented by FISH assay to determine the aneugenic or clastogenic origin of MN. In order to determine the reliability of the methods, two-model mutagens, colchicine and mitomycin C, known to be predominantly aneugenic and clastogenic, respectively, were used as positive control substances.

2. Materials and methods

2.1. Animals

Adult male white Swiss albino mice weighing 23–27 g (10–12 weeks old) were obtained from Experimental Animal Care Center at our university. The animals were housed in groups of five mice in polypropylene cages at an ambient temperature of 25–28 °C, and 45–55% relative humidity, with a 12:12 h light/dark cycle. Animals were provided with commercial food pellets and water *ad libitum*. All the animals were acclimatized to laboratory conditions for at least one week before using them for the experiments. All experiments were carried out according to the Guidelines of the Animal Care and Use Committee at our university.

2.2. Drugs and treatment

Idarubicin and doxorubicin were supplied by Farmitalia Carlo Erba, Milano (Italy). The drugs were reconstituted in sterile water prior to use. Colchicine and mitomycin C (Sigma Chemical St. Louis, MO) were dissolved in sterile water and were used as a positive control aneugen and clastogen, respectively. The working solutions of all compounds were prepared no longer than 30 min prior to intraperitoneal injection and the volume of each injection was 0.1 ml/10 g body weight of mice. Control mice receiving sterile water was included in order to code the slides and avoid scoring biases. All other chemicals were of the finest analytical grade.

2.3. Sperm BrdU-incorporation assay

The time of development from meiotic divisions in spermatocytes to epididymal sperm was assessed by labelling cells with 5-bromo-2′-deoxyuridine (BrdU). Mice were intraperitoneally injected with 100 mg/kg BrdU in order to label spermatocytes at S-phase during preleptotene of meiosis. During meiosis I and II, 13 days later, the mice were intraperitoneally injected with 12 mg/kg idarubicin or doxorubicin and the selected doses are within the dose range used for human chemotherapy. Five treated and 5 solvent control mice were sacrificed per day 33–37 after BrdU-injection (20–24 days after drugs treatment). Sperm were sampled and smears were prepared as described previously [15]. Decondensation of the sperm heads was performed by incubation of the slides in 10 mM dithiothreitol for 30 min on ice followed by incubation in 4 mM lithium 3,5-diiodosalicylic acid for 60 min at room temperature. Immunofluorescence staining with anti-BrdU antibody and washing conditions were as described by Schmid et al. [15]. Propidium iodide (2.0 μg/ml) was used as counterstaining. Averages of 10,000 BrdU-positive and BrdU-negative cells per animal were blind scored under an Axioplan Fluorescence Microscope.

2.4. Sperm multicolour FISH assay

Two experiments were performed with the sperm-FISH assay. In the first experiment, groups of 5 mice each were intraperitoneally injected with 3, 6 and 12 mg/kg idarubicin or doxorubicin. Colchicine was used as a positive control aneugen at the dose of 3 mg/kg [16]. After drug administration, the animals were maintained with food and water *ad libitum* until being sacrificed. Mice were sacrificed by cervical dislocation 23 days after drug treatment. To determine if subacute treatment would have an effect because prophase stages would be included in idarubicin or doxorubicin treatment, doses of 0.25, 0.5 and 1 mg/kg of each compound were intraperitoneally injected on 12 consecutive days in the second experiment and sperm were sampled 23 days after the last treatment. Immediately after sacrificing, the sperm were collected from the *Caudae epididymes*. The time of sampling was chosen on the basis of the BrdU-incorporation study.

The frequencies of disomic and diploid sperm were determined by FISH with DNA probes specific for mouse chromosomes 8, X and Y each labelled with a different colour. Hybridization, washing, and amplification of the signals were performed as described previously [16]. DAPI (4′,6-diamidin-2′-phenylindol-dihydrochloride, 0.05–0.1 μg/ml) was used as counterstaining. Slides were examined for aneuploid sperm under an Axioplan Fluorescence Microscope. Per animal, fluorescent signals of coded slides were counted in ~10,000 sperm and sperm were designated as normal (X8 and Y8), disomic (X88, Y88 and XY8) or diploid (XY88, XX88 and YY88).

2.5. Bone marrow conventional MN test

Animals were intraperitoneally injected with 3, 6 and 12 mg/kg idarubicin or doxorubicin and bone marrow was sampled 24 h after treatment. Colchicine and mitomycin C were used as a positive control aneugen and clastogen, respectively, at the dose of 2 mg/kg each [17]. Bone marrow smears were prepared and stained with May–Gruenwald–Giemsa as described previously [18]. At least four slides were made for each animal and allowed to dry overnight. One slide per animal was stained with May–Gruenwald/Giemsa solutions for conventional assessment of the MN frequencies. The remaining unstained slides were stored at −20 °C for the distinction between the clastogenic and aneugenic effects, by identifying the origin of MN. Per animal, 1000 polychromatic erythrocytes (PCE) of coded slides were scored for the presence of MN. The frequencies of PCE were determined in slide areas where 1000 normochromatic erythrocytes (NCE) could be counted to determine a shift in erythroblast proliferation. The values were expressed as %PCE of the total erythrocyte counts to determine a reduction of erythroblast proliferation. Micronucleated normochromatic erythrocytes (MNNCE) were also recorded.

2.6. Bone marrow FISH analysis of MN using centromeric DNA-probe

Preparation of mouse biotin-labelled centromeric DNA probe p^{MKB6} (*minor* satellite DNA probe), hybridization, washing, and amplification of the signals are described elsewhere [19–21]. The cells were counterstained with DAPI (0.1–0.5 μg/ml) for 10 min at room temperature and coverslipped in Vectashield mounting medium. The slides were scored immediately or following storage for several days at 4 °C in the dark. The presence of centromeric signals in the MN was analysed under an Axioplan Fluorescence Microscope. 50–150 MN per group of coded slides were examined for the presence or absence of centromeric DNA probe as previously described [17].

2.7. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of the means. Significant differences between individual treatment groups and corresponding solvent controls were calculated on an animal to animal basis by the non-parametric Mann–Whitney *U*-test. Results were considered significantly different if the *P* value was ≤0.05.

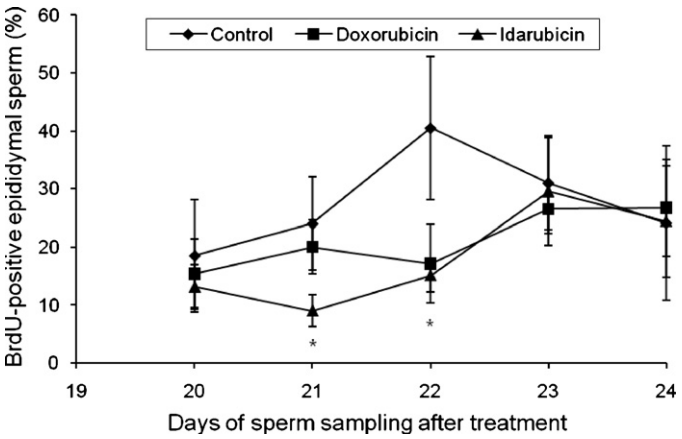

Fig. 1. Time course of appearance of BrdU-labelled sperm in the epididymes after treatment with idarubicin or doxorubicin. *$P < 0.05$ compared with the concurrent control (Mann–Whitney *U*-test).

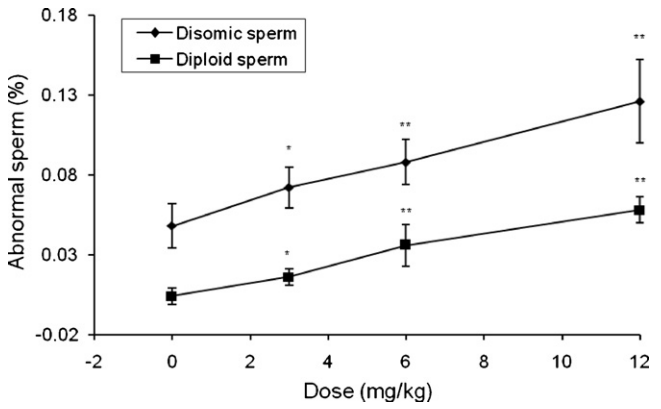

Fig. 2. Dose–response curves of the frequencies of abnormal sperm from mice after treatment with idarubicin. *$P < 0.05$, **$P < 0.01$ compared with the concurrent control (Mann–Whitney *U*-test).

3. Results

3.1. Sperm BrdU-incorporation assay

The results of the BrdU-incorporation assay are presented in Fig. 1. With 12 mg/kg of idarubicin and doxorubicin, prolongations of the duration of the meiotic divisions were observed. On days 21 and 22, the frequencies of BrdU-labelled sperm in the idarubicin groups were significantly below the control values ($P < 0.05$), while on days 23 and 24, there were no significant differences between the idarubicin-treated and control animals. For treatment with doxorubicin, the frequency of BrdU-labelled sperm was significantly below the control value only on day 22 and came up to control levels on day 23 ($P < 0.05$). According to Oakberg [22] the development from meiotic spermatocytes of mice to epididymal sperm takes 22 days. Thus, the meiotic delay caused by idarubicin and doxorubicin was about 48 and 24 h, respectively. Therefore, the optimum day for sperm sampling in the sperm-FISH assays was concluded to be 23 with idarubicin and doxorubicin.

3.2. Multicolour sperm FISH assay

The results of the analysis of aneugenic effects in germ cells of male mice after single exposure to idarubicin and doxorubicin are presented in Tables 1 and 2, together with the negative and positive control data. Sex ratios were found to be in the same range as the theoretical ratio of 1:1 for X- versus Y-bearing sperm in all groups. Significant increases in the frequencies of disomic and diploid sperm were caused by treatment with all doses of idarubicin compared with the control values. Similarly, significant increases in the frequencies of disomic and diploid sperm were caused by treatment with 6 or 12 mg/kg of doxorubicin compared with the corresponding control values. Using linear regression analysis, the dose–response curves for idarubicin-induced disomic and diploid sperm can be described by the linear equations $y = 0.006x + 0.05$ ($r^2 = 0.996$) and $y = 0.004x + 0.004$ ($r^2 = 0.986$), respectively (Fig. 2). The dose–response curves for doxorubicin-induced disomic and diploid sperm can be described by the linear equations $y = 0.004x + 0.05$ ($r^2 = 0.979$) and $y = 0.003x + 0.003$ ($r^2 = 0.970$), respectively (Fig. 3). The frequency of disomic sperm induced by colchicine was significantly increased by a factor of 1.625 compared with the control value. However, in contrast to the idarubicin and doxorubicin, colchicine did not significantly increase the frequency of diploid sperm, indicating that no complete meiotic arrest occurred.

The results of the multiple exposures to idarubicin and doxorubicin are shown in Tables 3 and 4. Treatment of mice with 0.25, 0.5 and 1 mg/kg/day of idarubicin on 12 consecutive days induced significant increases in disomic and diploid sperm as compared to the control group. On the other hand, treatment of mice with 0.25 and 0.5 mg/kg of doxorubicin on 12 consecutive days

Table 1
Result of the multi-colour FISH with epididymal sperm of mice treated with colchicine and idarubicin.

	Control	Colchicine (3 mg/kg)	Idarubicin (3 mg/kg)	Idarubicin (6 mg/kg)	Idarubicin (12 mg/kg)
No. of animals	5	5	5	5	5
Sperm scored	50,027	50,009	50,111	50,021	50,014
X/Y bearing sperm	0.9924	0.9971	1.0715	1.0661	0.9680
Disomic sperm					
X-X-8	7	8	10	10	17
Y-Y-8	6	11	8	10	16
X-Y-8	2	5	3	3	6
X-8-8	4	10	8	12	12
Y-8–8	5	5	7	9	12
Total	24	39	36	44	63
% disomies ± SD	0.048 ± 0.014	0.078* ± 0.027	0.072 ± 0.013*	0.088* ± 0.014	0.126** ± 0.026
Diploid sperm					
X-Y-8-8	0	1	1	3	4
X-X-8-8	1	2	4	9	15
Y-Y-8-8	1	0	3	6	10
Total	2	3	8	18	29
% diploidies ± SD	0.004 ± 0.005	0.006 ± 0.008	0.016 ± 0.005*	0.036** ± 0.013	0.058** ± 0.008

* $P < 0.05$, compared with the concurrent control (Mann–Whitney *U*-test).
** $P < 0.01$, compared with the concurrent control (Mann–Whitney *U*-test).

Table 2
Result of the multi-colour FISH with epididymal sperm of mice treated with doxorubicin.

	Control	Doxorubicin (3 mg/kg)	Doxorubicin (6 mg/kg)	Doxorubicin (12 mg/kg)
No. of animals	5	5	5	5
Sperm scored	50,027	50,027	50,010	50,119
X/Y bearing sperm	0.9924	0.9951	0.9975	1.0240
Disomic sperm				
X-X-8	7	10	10	15
Y-Y-8	6	7	10	12
X-Y-8	2	3	3	5
X-8-8	4	7	11	10
Y-8-8	5	5	7	9
Total	24	32	41	51
% disomies ± SD	0.048 ± 0.014	0.064 ± 0.008	0.082** ± 0.008	0.102** ± 0.013
Diploid sperm				
X-Y-8-8	0	1	2	3
X-X-8-8	1	2	7	8
Y-Y-8-8	1	2	4	9
Total	2	5	10	20
% diploidies ± SD	0.004 ± 0.005	0.010 ± 0.007	0.026* ± 0.011	0.040** ± 0.012

* $P < 0.05$, compared with the concurrent control (Mann–Whitney U-test).
** $P < 0.01$, compared with the concurrent control (Mann–Whitney U-test).

Table 3
Result of the multi-colour FISH with epididymal sperm of mice treated with idarubicin (daily exposure for 12 consecutive days).

	Control	Idarubicin (0.25 mg/kg)	Idarubicin (0.5 mg/kg)	Idarubicin (1 mg/kg)
No. of animals	5	5	5	5
Sperm scored	50,087	50,027	50,117	50,111
X/Y bearing sperm	0.9944	0.9921	0.9925	0.9949
Disomic sperm				
X-X-8	8	9	11	12
Y-Y-8	7	9	8	11
X-Y-8	2	3	3	5
X-8-8	7	5	9	9
Y-8-8	4	5	9	9
Total	28	31	40	46
% disomies ± SD	0.056 ± 0.008	0.062 ± 0.01	0.080* ± 0.015	0.092* ± 0.017
Diploid sperm				
X-Y-8–8	0	1	2	3
X-X-8–8	1	2	5	9
Y-Y-8–8	0	1	4	8
Total	1	4	11	20
% diploidies ± SD	0.002 ± 0.004	0.008 ± 0.004	0.022* ± 0.016	0.041** ± 0.015

* $P < 0.05$, compared with the concurrent control (Mann–Whitney U-test).
** $P < 0.01$, compared with the concurrent control (Mann–Whitney U-test).

did not induce any significant increases of disomic or diploid sperm. However, the highest dose group (1 mg/kg/day), which received a total of 12 mg/kg, induced significant increases in disomic and diploid sperm compared with the corresponding control values. Using linear regression analysis, the dose–response curves for idarubicin-induced disomic and diploid sperm can be described by the linear equations $y = 0.003x + 0.056$ ($r^2 = 0.949$) and $y = 0.003x + 0.0008$ ($r^2 = 0.987$), respectively (Fig. 4). The

Table 4
Result of the multi-colour FISH with epididymal sperm of mice treated with doxorubicin (daily exposure for 12 consecutive days).

	Control	Doxorubicin (0.25 mg/kg)	Doxorubicin (0.5 mg/kg)	Doxorubicin (1 mg/kg)
No. of animals	5	5	5	5
Sperm scored	50,087	50,001	50,014	50,071
X/Y bearing sperm	0.9944	0.9935	1.0481	0.9980
Disomic sperm				
X-X-8	8	9	9	9
Y-Y-8	7	6	7	9
X-Y-8	2	3	3	5
X-8–8	7	6	7	9
Y-8-8	4	6	8	7
Total	28	30	34	39
% disomies ± SD	0.056 ± 0.008	0.060 ± 0.01	0.068 ± 0.019	0.078* ± 0.01
Diploid sperm				
X-Y-8-8	0	1	1	3
X-X-8-8	1	2	2	6
Y-Y-8-8	0	1	2	6
Total	2	4	5	15
% diploidies ± SD	0.002 ± 0.004	0.008 ± 0.004	0.010 ± 0.007	0.03* ± 0.012

* $P < 0.05$, compared with the concurrent control (Mann–Whitney U-test).

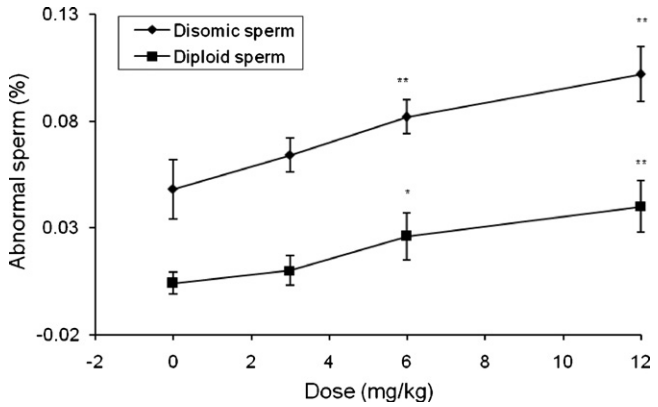

Fig. 3. Dose–response curves of the frequencies of abnormal sperm from mice after treatment with doxorubicin. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the concurrent control (Mann–Whitney *U*-test).

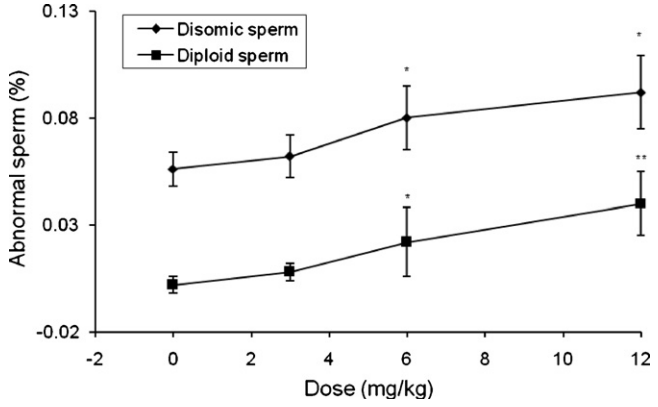

Fig. 4. Dose–response curves of the frequencies of abnormal sperm from mice 23 days after the last treatment with idarubicin (daily exposure for 12 consecutive days). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the concurrent control (Mann–Whitney *U*-test).

dose–response curves for doxorubicin-induced disomic and diploid sperm can be described by the linear equations $y = 0.001x + 0.055$ ($r^2 = 0.989$) and $y = 0.002x + 0.003$ ($r^2 = 0.944$), respectively (Fig. 5).

3.3. Bone marrow micronucleus studies

The positive controls colchicine and mitomycin C significantly increase the incidence of micronucleated polychromatic

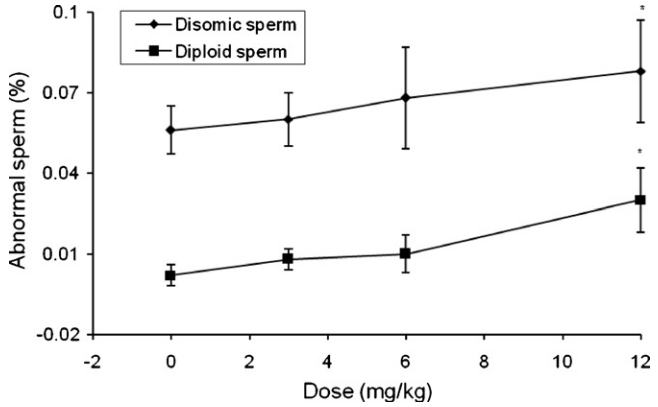

Fig. 5. Dose–response curves of the frequencies of abnormal sperm from mice 23 days after the last treatment with doxorubicin (daily exposure for 12 consecutive days). $^{*}P < 0.05$ compared with the concurrent control (Mann–Whitney *U*-test).

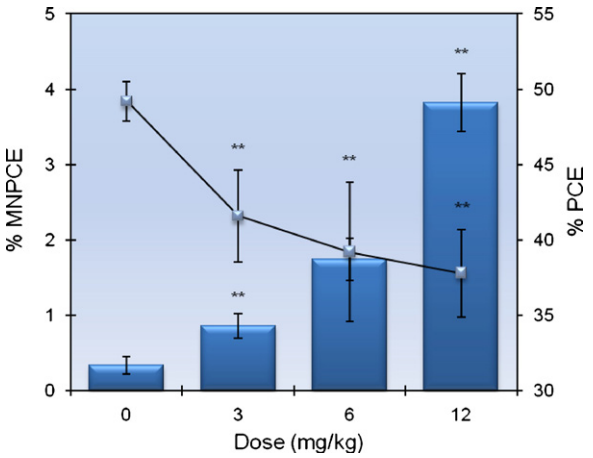

Fig. 6. Illustration of the dose–response obtained in the conventional micronucleus test with idarubicin. PCE = polychromatic erythrocytes, MNPCE = micronucleated polychromatic erythrocytes, shown as blue bars (mean percent ± SD). Reduction of %PCE [(PCE/NCE + PCE) × 100] is shown as solid line (mean percent ± SD). $^{**}P < 0.01$, compared with the concurrent control (Mann–Whitney *U*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

erythrocytes (MNPCE) compared to the control group ($P < 0.001$). Moreover, mitomycin C significantly decreased the %PCE from 49.2 ± 1.3 to 43.6 ± 4.3 ($P < 0.05$), indicating a reduction in erythroblast proliferation most likely by mitotic arrest. As shown in Fig. 6, idarubicin (3–12 mg/kg) significantly increases the MNPCE frequencies in a dose-dependent manner. Using linear regression analysis, the dose–response can be best described by the linear equation $y = 1.132x - 1.14$ ($r^2 = 0.908$). Furthermore, idarubicin caused a significant dose-dependent decrease of the PCE/NCE + PCE ratios which can be described best by the linear equation of $y = -3.66x + 51.1$ ($r^2 = 0.8646$). This indicates a suppression of bone marrow proliferation at all doses tested.

As seen in Fig. 7, doxorubicin at doses of 3, 6 and 12 mg/kg significantly increased the frequency of MNPCE in a dose-dependent manner. The dose–response can be best described by the linear equation $y = 0.742x - 0.72$ ($r^2 = 0.838$). Furthermore, doxorubicin at doses of 6 and 12 mg/kg significantly decreased the PCE/NCE + PCE ratio, this indicates a suppression of bone marrow proliferation. Using linear regression analysis, the dose–response can be best

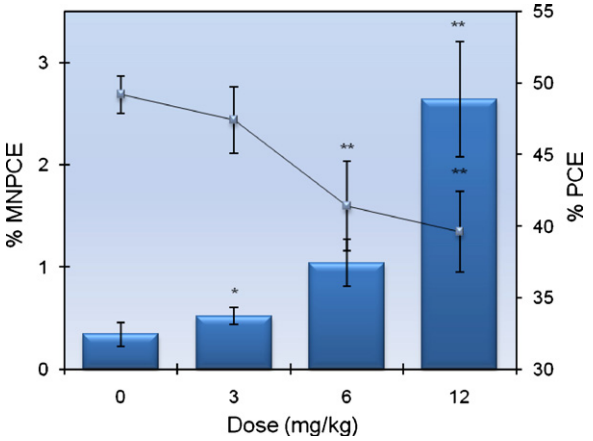

Fig. 7. Illustration of the dose–response obtained in the conventional micronucleus test with doxorubicin. PCE = polychromatic erythrocytes, MNPCE = micronucleated PCE, shown as blue bars (mean percent ± SD). Reduction of %PCE [(PCE/NCE+PCE) × 100] is shown as solid line (mean percent ± SD). $^{**}P < 0.01$, compared with the concurrent control (Mann–Whitney *U*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 5
Results of FISH analysis with the mouse centromeric DNA probe in micronuclei (MN) induced with colchicine (2 mg/kg), mitomycin C (2 mg/kg), idarubicin (12 mg/kg) and doxorubicin (12 mg/kg).

Chemicals	No. of MN scored	No. of signal-positive MN		Distribution of centromeric-signals per MN		
		Total	%	1 signal	2 signals	≥3 signals
Control	51	23	45.0	11 (47.9%)	8 (34.8%)	4 (17.3%)
Colchicine	94	71	75.5	25 (35.3%)	34 (47.8%)	12 (16.9%)
Mitomycin C	89	26	29.2	14 (53.8%)	9 (34.6%)	3 (11.6%)
Idarubicin	149	87	58.3	45 (51.7%)	29 (33.3%)	13 (15.0%)
Doxorubicin	132	83	62.8	41 (49.3%)	33 (39.7%)	9 (11.0%)

described by the linear equation $y = -3.48x + 53.1$ ($r^2 = 0.944$). On the other hand, doxorubicin treatment at a dose of 3 mg/kg did not exhibit any significant difference in the PCE/NCE + PCE ratio compared to the solvent control ($P > 0.05$). In NCEs the MN frequencies were between 0.02 and 0.06/100 NCE in all groups (data not shown). Thus, no discrimination between MN induced in PCE and NCE was required for the fluorescent analysis of MN with *in situ* hybridization since the NCE only contributed minimally to the total number of MN.

3.4. Bone marrow FISH analysis of MN

The results of the FISH analysis of MN are shown in Table 5. In the control group, a total of 51 MN were analysed and 23 (45.0%) of them were centromeric-positive. One signal per MN was observed in 47.9%, two signals were seen in 34.8% and ≥3 signals were seen in 17.3% of the 23 MN. After treatment of mice with the positive control aneugen colchicine, 71 (75.5%) out of the 94 MN scored were centromeric-positive confirming the predominantly aneugenic effects of colchicine. Of these, 25 MN (35.3%) had one signal, 34 MN (47.8%) contained two signals, and 12 MN (16.9%) had ≥3 signals. After treatment with the positive control clastogen mitomycin C, only 26 MN (29.2%) of 89 MN analysed were signal positive confirming the predominantly clastogenic effects of mitomycin C. Of the signal-positive MN, 14 MN (53.8%) had one signal, 9 MN (34.6%) contained two signals, and 3 MN (11.6%) had ≥3 signals.

After treatment with 12 mg/kg idarubicin, a total of 149 MN were analysed by FISH assay and 87 MN (58.3%) were signal-positive. Out of these 87 MN, 45 (51.7%) had one signal, 29 (33.3%) contained two signals, and 13 (15.0%) had ≥3 signals. In the doxorubicin 12 mg/kg group, 83 MN (62.8%) of 132 MN analysed were signal positive confirming the aneugenic effects of doxorubicin. The distribution of signals per MN was nearly similar to idarubicin treated animals. Similarly, idarubicin and doxorubicin induced 41.7 and 37.2% signal-negative MN, respectively, indicating that they were formed by DNA strand breaks and represented clastogenic activity. To correlate the FISH data with the conventional MN data, the expected percent of PCE with signals-positive and -negative MN was calculated (Fig. 8). For example, after treatment with colchicine, 1.0% MNPCE were found in the conventional MN test and 75.5% MN were signal-positive in the FISH analysis, thus 0.755% of the 1.0% MNPCE were calculated to be signal-positive and, correspondingly, 0.245% MN were calculated to be signal-negative.

4. Discussion

Studies in humans have shown that certain chemotherapy regimens increase the frequencies of aneuploidy in germinal and somatic cells [23,24], suggesting that such patients may be at higher risk for the development of secondary tumors and abnormal reproductive outcomes. Therefore, it is of general concern to decrease the risk of aneuploidy production, detection of aneugens and understanding of the causal mechanisms. In the current study aneuploidy was determined in germinal and somatic cells by the sperm-FISH assay and MN test complemented by FISH with centromeric DNA probe. In order to determine the reliability of the methods, two-model mutagens, colchicine and mitomycin C, known to be predominantly aneugenic and clastogenic, respectively, were used as positive control substances. The results of the positive and negative controls were in the same range as those of the earlier studies [16,17,25,26]. These data confirmed the sensitivity of the experimental protocol followed in the detection of aneuploidogenic effects.

It has been often discussed that chemicals with aneugenic properties can alter the progression of cell division in both meiotic and mitotic cells [27,28]. In the present study the time of development from meiotic divisions in spermatocytes to epididymal sperm was assessed by the BrdU-incorporation assay. The results clearly indicate that idarubicin and doxorubicin prolonged the duration of the meiotic divisions in mouse spermatocytes for at least 24 h. These observations therefore confirm previous results that inhibition of topoisomerase II function during different phases of the cell cycle by anthracyclines slows down cell cycle progression and causes cells to arrest at the G_2/M phase [29,30]. Such G_2/M arrest has been proposed to be due to induction of G_2 checkpoint machinery that allows damaged DNA to be repaired before cells move to the next cell cycle stage [31].

In the present experiments, idarubicin caused dose-dependent significant increases in the frequencies of disomic and diploid sperm and the increases were statistically significant at all three doses tested. Moreover, significant increases above the concurrent control in the frequencies of disomic and diploid sperm were found with the two highest doses of doxorubicin. The induction of aneuploidy showed linear dose–responses between 0 and 12 mg/kg of both compounds. This *in vivo* observation is in line with an earlier *in vitro* report on the human lymphocytes cultured from healthy individuals and cancer patients that doxorubicin led to an increase in the trisomies of chromosomes 7 and 17 [32]. Furthermore, Ganapathi et al. [33] reported that human leukemia HL-60 cells that carry monosomy 8 as the only karyotypic change

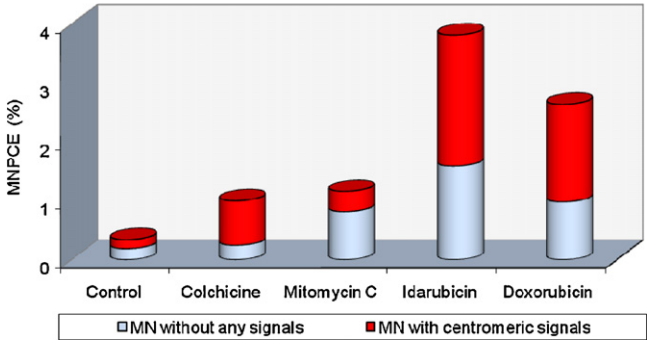

Fig. 8. Illustration of the contribution of clastogenicity (blue) and aneugenicity (red) to the induced MN frequencies in animals treated with colchicine (2 mg/kg), mitomycin C (2 mg/kg), idarubicin (12 mg/kg) and doxorubicin (12 mg/kg). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

acquired 7q21 markers upon exposure to doxorubicin. Additionally, cytogenetic findings in the bone marrow showed trisomy 8 in patients received courses of systemic chemotherapy with idarubicin containing regimen [34]. Structural chromosomal aberrations of chromosomes 1, 9 and 16 have been related to chemotherapeutic drugs containing anthracycline [35]. Moreover, monosomy 7, 7q−, and unbalanced translocation including chromosome 7 were observed in patients who received anthracycline-containing chemotherapy [36].

To determine if subacute treatment with low doses of idarubicin or doxorubicin would have an effect because earlier prophase stages would be included in idarubicin and doxorubicin treatment. Individual doses of 0.25, 0.5 and 1 mg/kg of each compound were injected on 12 consecutive days and sperm were sampled 23 days after the last treatment. It was found that a total of 6 and 12 mg/kg idarubicin applied to the entire prophase of meiosis significantly increased disomic and diploid sperm frequencies, while a total dose of 3 mg/kg was negative. Similarly, a total of 12 mg/kg doxorubicin applied to the entire prophase of meiosis significantly increased disomic and diploid sperm frequencies, while a total dose of 3 and 6 mg/kg doxorubicin were negative (Tables 3 and 4). In contrast, a single dose of 3 mg/kg idarubicin and 6 mg/kg doxorubicin applied to spermatocytes during MMI/MMII gave a positive result (Tables 1 and 2). These data suggest that earlier prophase stages contribute relatively less to idarubicin and doxorubicin-induced aneuploidy in male germ cells.

The sperm-FISH assay for disomy or diploidy is capable of detecting effects induced during both meiotic divisions and to compare the sensitivity of both meiotic divisions [37,38]. In the sperm FISH assay, it was found that idarubicin caused noticeable increases above the control of autodiploid sperm (XX88 and YY88). After treatment with idarubicin, autodiploid sperm resulting from arrest of MMII were more frequent than diploid sperm resulting from arrest during MMI (XY88). Thus, the second meiotic division was more sensitive to idarubicin treatment than the first meiotic division. A similar observation was made for doxorubicin. The conclusion that second meiotic divisions were more sensitive than first meiotic divisions is also supported by the observed frequencies of sex chromosome disomic. Sperm with signals of XX8 or YY8 were more frequent than sperm with signals of XY8 for both chemicals. This general higher sensitivity of MMII spermatocytes to induction of diploidy and disomy can be explained in two ways. Firstly, it is a real effect and can be attributed to the short time interval that elapses between MMI and MMII without interkinesis, which requires rapid disassembling and reassembling of microtubules to form a new spindle. This phase may be especially vulnerable to adverse chemical influences. Secondly, it is a result of the timing of sperm sampling, i.e. inherent in the present test protocol. Sperm are capacitated in the epididymis for about one week. With the present protocol, the first wave of sperm entering the epididymis is sampled. If samplings would be obtained at later time intervals (24–27 days after treatment) the contribution of cells exposed to the aneugen during MMI could be greater. However, MMI and MMII occur within less than 24 h so that this second explanation is rather unlikely.

The results of MN test have shown that idarubicin and doxorubicin produce dose dependent increases in MN formation in mouse bone marrow *in vivo* and an increase in centromeric-negative and centromeric-positive stained MN, indicating the induction of both clastogenicity and aneugenicity. The results of clastogenicity confirm the findings of previous *in vivo* studies, where increases in MN formation and structural chromosomal aberrations in mouse somatic and germinal cells, over a similar dose range, was observed for doxorubicin [39] and idarubicin [40]. This study also confirms the aneugenic effect of doxorubicin *in vitro* reported by Dhawan et al. [41], where in lymphocytes from healthy individuals, an increase in the kinetochore-positive MN, using CREST serum containing anti-kinetochore antibodies, was observed.

Among other classes of topoisomerase inhibitors, etoposide, merbarone, camptothecin, topotecan and irinotecan have been studied previously by using the MN test complemented with centromeric DNA probe for FISH analysis of mouse erythrocyte MN. The results of these studies indicate that these compounds act as genotoxic agents and produce chromosomal damage that ends up as MN. Although the mechanism by which camptothecin causes this adverse genetic effect appear related to a large part to clastogenicity [42], the adverse genetic effects of etoposide, topotecan, irinotecan and merbarone were related equally to clastogenic and aneugenic events [20,42]. While the mechanism of clastogenicity of the complex-stabilizing topoisomerase inhibitors etoposide, camptothecin, topotecan and irinotecan is understood as an accumulation of enzyme-mediated DNA cleavages, the clastogenicity of the noncomplex-stabilizing topoisomerase inhibitor merbarone may be a secondary effect of the inhibition of decatenation. Both the clastogenic and the aneugenic potential of these agents can give rise to chemotherapy resistance or the development of secondary tumors and abnormal reproductive outcomes in patients treated with drug regimens that include these drugs.

Compounds that inhibit topoisomerase at various stages of its catalytic cycle have a clear tendency to cause stranded DNA breaks, which primarily result in the formation of centromere-negative MN [42]. Similarly the demonstration that anthracyclines are effective topoisomerase inhibitors suggests that anthracyclines elicit their clastogenic effects through this mechanism. However, the induction of centromere-positive MN by anthracyclines indicates that there may be another mechanism through which anthracyclines can induce genotoxic effects, an observation that also underscores the importance of using the FISH modification of the MN assay to determine the origin of the induced MN. The possible mechanism by which anthracyclines may exert aneugenic effects is through inhibition of topoisomerase II which could result in a missegregation of chromosomes during mitosis. Topoisomerase II has been demonstrated to be necessary for the proper separation of sister chromatids during mitosis [43] with both non-disjunction and breakage occurring in its absence [44]. Thus, anthracyclines may be able to inhibit two key roles of topoisomerase II, its ability to properly segregate newly replicated chromosomes as well as its function to religate transient double-stranded DNA breaks. However, inhibition of topoisomerase II resulting in an unsuccessful resolution of sister chromatids would be expected to result primarily in non-disjunction rather than chromosome loss [44].

The present mouse bone marrow MN studies showed that exposure to 3 mg/kg doxorubicin yielded a significant increase in MNPCE. However, in the sperm FISH assay, it was found that the lowest positive dose, which caused disomic or diploid sperm, was 6 mg/kg of doxorubicin. This observation suggests that bone marrow MN test is the more sensitive than the sperm FISH assay. It must of course be noted that the assays measure different endpoints. Chromosome loss and breakage is measured in the MN test, and non-disjunction is detected in the sperm-FISH assay. Therefore, the present data confirm the general paradigm of hazard assessment, that the positive outcome of the bone marrow MN test is an indicator of the genotoxic potential of a compound in germ cells. However, to quantify aneuploidy induced in germ cells is important for risk assessment purposes. Taking into account the fundamental differences between the meiotic process and the mitotic process, e.g. differences in spindle formation, requirement for chromosome pairing, formation of chiasmata to allow recombination, prolonged duration of meiosis in oocytes [45], it will always be necessary to confirm the aneugenic potential of a chemical detected *in vitro* by studies in somatic and in germinal cells *in vivo*. Theoretically, the differences in cell biology may give rise to qualitative differences

in response of germ cells and somatic cells to aneugens and the possibility of unique germ cell aneugens should not be neglected.

5. Conclusion

By using the BrdU-incorporation assay it could be shown that the meiotic delay caused by idarubicin and doxorubicin was about 48 and 24 h, respectively. With the sperm-FISH analysis, it could be shown that idarubicin and doxorubicin induce aneuploidies during meiosis that result in disomic sperm and both compounds cause complete meiotic arrest that results in diploid sperm. Importantly, idarubicin was more aneuploidogenic than doxorubicin at the same tested doses. The dose–response curves for disomic and diploid endpoints were linear for both compounds. The prevalence of autodiploid (XX88, YY88) sperm and disomic XX8 or YY8 sperm indicates that the second meiotic division was more sensitive to both compounds than the first meiotic division. The results suggest also that earlier prophase stages contribute relatively less to idarubicin and doxorubicin-induced aneuploidy. Similarly, somatic cell was also sensitive to the adverse genetic effects of both compounds and idarubicin was more toxic than doxorubicin at the same tested doses. The dose–response curves for MNPCE and bone marrow suppression endpoints were linear for both compounds. By using FISH analysis with the centromeric DNA-probe for erythrocyte MN it could be shown that idarubicin and doxorubicin are aneugens as well as clastogens in somatic cells *in vivo*. Both the aneugenic and the clastogenic potential of etoposide and merbarone in somatic cells can give rise to secondary malignancy in cancer patients and medical personnel exposing to these drugs.

Awareness of the genetic hazard from aneugens has always been present, however, generally accepted and validated methods to test for this genetic endpoint have not been available. The main reason is that the targets for gene mutations and chromosomal aberrations are DNA and chromatin while aneuploidy-induction has multiple targets, i.e. tubulin, spindle fibres, centrioles, centromeres, teleomeres, motor proteins, and cell-cycle checkpoint proteins. The results of the current study and our previous data base show that the aneuploidy assays such as the sperm-FISH assay and MN test complemented by FISH with centromeric DNA probes have been to some extent validated to be recommended for the assessment of adverse effects of aneugenic chemicals. However, proper validation of these methods would require more extensive testing such as a ring test in different laboratories, using unlabelled chemicals, etc., which will be considered extensively in our future studies.

Conflict of interest

The author declares that no competing interests exist.

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