Increased aneuploidy in spermatozoa from testicular tumour patients after chemotherapy with cisplatin, etoposide and bleomycin

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Testicular cancer is the most common neoplasia occurring in the young male population. The PEB (cisplatin, etoposide and bleomycin) adjuvant chemotherapy usually proposed after orchidectomy in non seminomatous tumours, and in metastatic seminomas, has improved the long-term survival of these patients. Following an azoospermic period, sperm cell recovery is generally observed after treatment delivery, but little is known about the genetic consequences on these new spermatozoa. To estimate the chromosomal consequences of this chemotherapy on sperm cells during the period of recovery of spermatogenesis, sperm cell aneuploidy was studied in testicular cancer patients, at 6–18 months after PEB adjuvant chemotherapy delivery, using fluorescence in-situ hybridization (FISH) of chromosomes 7, 16, 18, X and Y with specific DNA probes. A significant increase in the frequency of diploidy and disomy for chromosomes 16, 18 and XY was observed in treated patients compared with a healthy control group. Spermatozoa aneuploidy occurring during the spermatogenesis recovery period might be a possible side effect of the PEB regimen. Thus, practitioners should be advised to provide counselling about the need for an appropriate duration of contraception. Moreover, genetic counselling should be offered in cases of pregnancy occurring soon after the end of chemotherapy.

Key words: chemotherapy/FISH /PEB side effects/sperm aneuploidy/testicular cancer

Introduction

Testicular cancer is the most common neoplasia occurring in the young male population, and its incidence appears to be increasing (Adami *et al.*, 1994; Forman and Møller, 1994). In non seminomatous tumours (Culine *et al.*, 1997; Sternberg, 1998), and in metastatic seminomas (Warde *et al.*, 1996), adjuvant chemotherapy proposed after orchidectomy has improved the long-term survival of these patients. Chemotherapy usually consists of two to four cycles of the PEB (cisplatin, etoposide and bleomycin) regimen, according to the stage of the neoplasia. Few studies have reported the effects of PEB chemotherapy on spermatogenesis in patients treated for testicular cancer. Patients become azoospermic during weeks following treatment initiation, but spermatogenesis does not seem to be affected in the long term (Cullen *et al.*, 1996; Pont *et al.*, 1996). While spermatogenesis recovery is generally observed, little is known about the genetic consequences on these new spermatozoa.

The incidence of congenital malformation does not appear to be increased in offspring of male cancer patients following chemotherapy. However, published reports are either case studies or have small sample sizes which are not able to detect less than a 3- to 5-fold increase in relative risks (Meistrich, 1993). Moreover, few studies have assessed the genetic consequences of chemotherapy on spermatozoa. Rather, they addressed different chemotherapy regimens and their results are conflicting, showing the presence (Jenderny and Röhrborn, 1987; Genesca *et al.*, 1990a,b; Brandriff *et al.*, 1994; Monteil *et al.*, 1997; Robbins *et al.*, 1997; Martin *et al.*, 1999) or absence (Jenderny *et al.*, 1992; Martin *et al.*, 1995, 1997a,b) of chromosomal alterations. In the case of testicular cancer chemotherapy, only one group has studied the effects of the PEB regimen on sperm chromosomes. In four patients who provided sperm samples between 2 and 13 years after chemotherapy, neither structural (Martin *et al.*, 1997b) nor numerical (Martin *et al.*, 1997a) abnormalities were found. On the other hand, one patient investigated soon after the beginning of 4 cycles of PEB regimen (at 59 and 366 days), showed a significant increase in sperm chromosomal abnormalities (Martin *et al.*, 1999).

Using fluorescence in-situ hybridization (FISH) of chromosome-specific DNA probes, the aim of this study was to

compare spermatozoa aneuploidy in patients recently submitted to adjuvant PEB chemotherapy, with a control group.

Materials and methods

Patients and semen samples

Five patients with testicular tumours at the CECOS Midi-Pyrénées, Male Sterility Center in Toulouse who provided sperm samples for cryopreservation before PEB treatment, were recruited for the study. Sperm samples were obtained at 6–17 months after chemotherapy, and frozen until analysis. All patients gave their informed consent for the sperm chromosomes analysis. Semen specimens from five healthy fertile sperm volunteer donors were used as controls (mean age 37.2 years; range: 32–45 years).

The PEB chemotherapeutic regimen (based on body surface area) was 120 mg/m² etoposide and 20 mg/m² cisplatin from day 1 to day 5, and 30 mg bleomycin on days 2 and 9. This regimen was repeated two, three or four times at monthly intervals according to the tumoral grade.

Sample preparation

After thawing, semen samples were washed twice in a buffer solution (0.1 mol/l Tris–HCl, pH 8.0) and, following the final centrifugation at 400 g, were resuspended in 0.5–2.0 ml buffer. One drop of the suspension was smeared onto a clean glass microscope slide and allowed to air-dry. Slides were either stored in a slide box and kept at –20°C or used immediately in preparation for FISH.

The slides were dehydrated through 70, 85 and 100% ethanol for 2 min each. After air-drying, the sperm decondensation was performed using a published method (Wyrobek et al., 1990), with minor modifications. Briefly, slides were incubated at room temperature for ~8 min (range: 5–30 min) in 10 mmol/l dithiothreitol (DTT; Sigma-Aldrich, Saint Quentin Fallavier, France) in 0.1 mol/l Tris–HCl, pH 8.0, and for 12 min (range: 5–45 min) in 10 mmol/l lithium 3,5-diiodosalicylic acid (LIS; Sigma-Aldrich) and 1 mmol/l DTT in 0.1 mol/l Tris–HCl, pH 8.0. The slides were then briefly rinsed in 0.1 mol/l Tris–HCl, pH 8.0, allowed to air-dry, and then fixed with methanol:acetic acid (3:1) for a minimum of 20 min and air-dried.

FISH

Directly-labelled alpha satellite centromere-specific probes (Vysis, Downers Grove, IL, USA) were used for this FISH assay: (i) Spectrum Green CEP 7 alpha, Spectrum Orange CEP 16 alpha in a two-colour hybridization; and (ii) Spectrum Green CEP X alpha, Spectrum Orange CEP Y alpha, Spectrum Green CEP 18 alpha and Spectrum Orange CEP 18 alpha in a three-colour hybridization. The DNA was denatured in 70% formamide for 5 min at 74°C, dehydrated through an increasing concentration (70, 85 and 100%) of cold ethanol, air-dried and placed for 2 min on a hot plate with a surface temperature of 42°C. The labelled probes were added to hybridization buffer according to the manufacturer's instructions. The hybridization solution was dropped onto the sperm preparation, and a coverslip (22×22 mm) was added and sealed with rubber cement. Hybridizations were conducted overnight in a dark moist box, at 42°C. The slides were then washed in 0.4× standard saline citrate (SSC), pH 7.0, at 74°C for 2 min, then for 1 min in 0.1% Nonidet P-40 (NP-40; Sigma-Aldrich)/2×SSC at room temperature and air-dried. 4,6-Diamidino-2-phenylindole (DAPI) counterstain (12 µl) was applied to each slide, and a coverslip added and sealed with rubber cement.

Scoring and analysis

The slides were examined using a Zeiss Axioplan 2 epifluoresence microscope equipped with a triple band pass filter for DAPI, fluorescein isothiocyanate (FITC) and tetramethyl-rhodamine isothiocyanate (Rhodamine). The Spectrum Green CEP X alpha, CEP 7 alpha appeared green, the Spectrum Orange CEP Y alpha, CEP 16 alpha appeared red, and the Spectrum Green CEP 18 alpha/Spectrum Orange CEP 18 alpha appeared yellow. The chromosome 18 probe was used as an internal control to distinguish between sex chromosome disomy and diploidy.

To avoid scoring bias, the same stringent scoring criteria were used by one examiner (P.D.M.) on both groups. Sperm nuclei were scored as having two signals when they displayed two signals of equal size and intensity, separated by at least the diameter of one signal (Martin and Rademaker, 1995) and well positioned within the sperm head. Overlapping spermatozoa or sperm heads without a well-defined boundary were not evaluated. Nullisomies of one of the tested chromosomes were not directly scored and were considered as equivalent to the incidence of disomies (Downie et al., 1997; Egozcue et al., 1997). A total of 10 000 consecutive sperm nuclei were scored from each combination of probes.

The data were analysed by the χ^2 test. A P-value < 0.05 was considered significant.

Results

Clinical data, sperm counts and treatments are reported in Table I. Except for one patient (B) the differences in sperm count before and after chemotherapy were quite moderate in the studied patients. Analysis of 20 000 sperm cells was carried out for each patient and control. Diploidy and disomy for chromosomes 7, 16, 18, X, Y and XY, for each PEB-treated patient and control are shown in Table II.

Frequency of disomy 16, 18 and XY was significantly increased in patients compared with the control group (Table II). As for diploidy detected by two-colour or three-colour FISH, a significant increase was also noticed (Table II). The same values of diploidy obtained in two-colour and in three-colour FISH preparations for each patient and control is a good reflection of the validity of these results (Table II).

Discussion

Adverse effects of chemotherapeutic regimens on spermatogenesis have been extensively studied. With regard to PEB adjuvant chemotherapy proposed in testicular cancer, published results have demonstrated that two or three cycles have no long-term effect on fertility (Cullen et al., 1996; Pont et al., 1996). To our knowledge, few studies have dealt with genetic damages caused by PEB regimens in recovered spermatozoa. This study was designed to compare sperm cell aneuploidy in patients recently submitted to adjuvant testicular cancer chemotherapy with that in control volunteers. A healthy control group was chosen for practical and ethical reasons, as spermatozoa delivered before chemotherapy were used only for gamete cryopreservation. Moreover, it has been demonstrated previously that testicular cancer patients have no increase in chromosomal sperm abnormalities prior to any treatment (Martin et al., 1997a,b; Alvarez et al., 1999). In order to detect a putative reproductive risk to testicular chemotherapy patients,

Table I. Clinical data and sperm count in cisplatin, etoposide and bleomycin (PEB)-treated patients

Patient	Age (years)	Sperm count before CT ($\times 10^6$/ml)	No. of PEB regimens	Tumour type	Sperm count after CT ($\times 10^6$/ml)	Time between CT and FISH study (months)
T	27	32.8	2	S/CE	81.0	11
P	30	84.0	3	CE	30.0	6
S	37	22.0	3	S	17.6	8
G	36	14.0	4	CE	24.0	17
B	21	44.0	3	CE	6.0	9

CE = carcinoembryonic; CT = chemotherapy; FISH = fluorescence in-situ hybridization; S = seminoma.

Table II. Disomy and diploidy frequencies in post cisplatin, etoposide and bleomycin chemotherapy (PEB-CT) patients and in controls

%	Post PEB-CT patients						Controls					
	T	P	S	G	B	Mean (%)	Mean (%)	1	2	3	4	5
disomy 7	0.10	0.05	0.04	0.11	0.05	0.07	0.044	0.09	0.03	0.02	0.05	0.03
disomy 16	0.10	0.10	0.08	0.09	0.08	0.09[*]	0.046	0.04	0.04	0.06	0.03	0.06
disomy 18	0.06	0.04	0.03	0.06	0.03	0.044[**]	0.014	0.01	0.00	0.01	0.02	0.03
disomy X	0.02	0.02	0.03	0.03	0.04	0.028	0.030	0.01	0.05	0.04	0.03	0.02
disomy Y	0.01	0.04	0.02	0.07	0.02	0.032	0.008	0.00	0.00	0.01	0.01	0.02
disomy XY	0.14	0.13	0.15	0.27	0.24	0.186[***]	0.072	0.07	0.08	0.07	0.05	0.09
Diploidy[a]	0.29	0.29	0.16	0.11	0.42	0.254[***]	0.094	0.10	0.06	0.10	0.09	0.12
Diploidy[b]	0.49	0.24	0.16	0.20	0.28	0.274[***]	0.080	0.07	0.04	0.06	0.13	0.10

Values are expressed as percentages.
[a]Chromosomes 7 and 16 two-colour FISH.
[b]Chromosomes X, Y and 18 three-colour FISH.
[*]$P < 0.05$; [**]$P < 0.01$; [***]$P < 0.001$.

a decision was made to study the earliest period after chemotherapy in which recovery of spermatogenesis is detected. As detection of chromosomal abnormalities has not been reported after the second year following PEB treatment (Martin *et al.*, 1997a,b), the current study was focused on the critical period from the recovery of spermatogenesis to the second-year threshold.

The current study, using 7, 16, 18, X and Y -specific chromosome probes demonstrated a significant increase in sperm aneuploidy (disomy 16, 18, XY and diploidy) in treated patients compared with control men.

To date, only one other group has reported a study on sperm damage after PEB chemotherapy in patients treated for testicular cancer. This group (Martin *et al.*, 1997a) studied disomy for chromosomes 1, 12, X, Y, XY and diploidy by FISH in four testicular cancer patients both before and 2 to 13 years after treatment with PEB. These authors did not find any increased risk of numerical chromosomal abnormalities. Sperm karyotyping was used to confirm this result, and the possibility of an increased risk of structural abnormalities in the same patients was excluded (Martin *et al.*, 1997b). However, in a patient submitted to four PEB cycles, the study of sperm cell chromosomes by FISH before, during (59 days) and after (366 days) chemotherapy showed an increased aneuploidy, which is in agreement with the current results. In fact, a significant increase of the XY disomy and of diploid sperm cells was noticed (Martin *et al.*, 1999). The current results led to a similar observation showing a significant increase in the

frequency of aneuploidy during the first 18 months following initiation of the PEB chemotherapy.

Among the drugs used, etoposide appears to be a good candidate for aneuploid sperm induction as it interacts with topoisomerase II during anaphase I and II of meiosis. Topoisomerase II activity is required for removing regions of DNA catenation between (sister and non-sister) chromatids. This decatenation enables segregation of homologous chromosomes and sister chromatids (Murray and Szostak 1985; Holm *et al.*, 1989). Etoposide inhibits topoisomerase II activity by forming a DNA–topoisomerase II–drug ternary complex, and induces a non-disjunction of recombinant chromosomes (Rose *et al.*, 1990) and of sister chromatids (Downes *et al.*, 1991). Experiments *in vivo* showed that etoposide treatment results in an increased risk of aneuploid germ cells, in mouse oocytes (Mailhes *et al.*, 1994) and also in spermatocytes (Kallio and Lahdetie, 1996). Abnormal chromosome segregation has been shown with bleomycin in yeast (Sora *et al.*, 1983; Käfer, 1990) and in *Drosophila melanogaster* (Traut, 1980), but the main effect of the drug is the induction of DNA double-strand breaks resulting in structural chromosome abnormalities, as shown in hamster primary embryonic cells (Xiao and Natarajan, 1999) and in human lymphocytes (Ellard *et al.*, 1995). No increased risk of aneuploidy was shown with cisplatin (cis-diamminodichloroplatinum II) in either male or female germ cells of *D. melanogaster* (Brodberg *et al.*, 1983) or in murine metaphase II oocytes (Higdon *et al.*, 1992).

The impact of the drugs used is further demonstrated by

Table III. Spermatozoan chromosomes after chemotherapy in cancer patients

Study	Pathology	No. of cases	Chemotherapy	Time after treatment	Type of study	Increased frequency of anomalies	
						Structural	Numerical
Jenderny and Röhrborn (1987)	Cancer (type ?)	1	PEB + D-Act + CY-CH-V-MT	26 months	Hamster test	+	+
Genesca *et al.* (1990a)	Non seminoma	2	PVB PVB + PEB	2 to 5 years	Hamster test	+	+
Genesca *et al.* (1990b)	Rhabdomyosarcoma	1	CYVADIC ×13	5 years			
	Ewing sarcoma	1	VAC ×12	5 years	Hamster test	+	−
	Wilms' tumour	2	RT ± D-Act	11 and 18 years			
Jenderny *et al.* (1992)	Seminoma	1	PVB	9 months	Hamster test	−	−
Brandriff *et al.* (1994)	Hodgkin's disease	3 3	MOPP ×2–6 MOPP/RT ×2–3	3 to 20 years	Hamster test	+	+
Martin *et al.* (1995)	Lymphoma	1	MACOP-B	3 years	Hamster test FISH	−	− −
Martin *et al.* (1997a)	Embryonal carcinoma	4	PEB	2 to 13 years	FISH		−
Martin *et al.* (1997b)	Embryonal carcinoma	4	PEB	2 to 13 years	Hamster test	−	−
Robbins *et al.* (1997)	Hodgkin's disease	6	NOVP ×3	DT 100 days	FISH		+−
Monteil *et al.* (1997)	Hodgkin's disease	1	Vinblastine + RT	DT 38 days	FISH		++
Martin *et al.* (1999)	Non seminoma	1	PEB ×4	DT 1 year	FISH		++
Current study	Non seminoma	5	PEB	6–17 months	FISH		+

[a]Hamster test = fusion of human spermatozoa with hamster oocytes.
CY-CH-V-MT = cyclophosphamide, chlorambucil, vinblastine, methotrexate; CYVADIC = cyclophosphamide, adriamycin, vincristine, dicarbazine; D-Act = D-actinomycin; DT = during treatment; FISH = fluorescent in-situ hybridization; MACOP-B = methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone; MOPP = nitrogen mustard, vincristine, procarbazine, prednisone; NOVP = novanthrone, vincristine, vinblastine, prednisone; PEB = cisplatin, etoposide, bleomycin; PVB = cisplatin, vinblastine, bleomycin; RT = radiotherapy; VAC = vincristine, adriamycin, cyclophosphamide.

discrepancies between various chemotherapeutic regimens used in different cancers (Table III). In two studies (Jenderny and Röhrborn, 1987; Genesca *et al.*, 1990a), interspecific fertilization of hamster eggs was used to show a significant increase in the incidence of stable structural chromosome abnormalities (deletions, translocations, inversions). This was seen in the spermatozoa of two patients treated for non seminoma testicular cancer by PVB (cis-platinum diamine-dichloride, vinblastine, bleomycin) or PVB + PEB, 2 and 5 years after treatment, and also four additional patients, treated 5 to 18 years earlier by chemotherapy and/or radiotherapy for sarcoma or Wilms' tumour (Genesca *et al.*, 1990b). Conversely, in the study of a PVB-treated testicular cancer patient (Jenderny *et al.*, 1992), no increase in chromosome structural abnormalities was found. Indeed, the earliest studies are controversial with regard to numerical chromosome aberrations. Some reports claimed an increase in numeric chromosome anomalies (Jenderny and Röhrborn, 1987; Genesca *et al.*, 1990a; Brandriff *et al.*, 1994), while others did not (Genesca *et al.*, 1990b; Jenderny *et al.*, 1992). All of these studies used the interspecific hamster test, and it is possible that the low number of sperm cells analysed might have biased these results.

Using FISH studies, an increased frequency of hyper-haploid spermatozoa has been found during chemotherapy treatment by NOVP (novanthrone, oncovin, vinblastine, prednisone) in eight Hodgkin's disease patients studied using the three-chromosomes hybridization in-situ method (Robbins *et al.*, 1997) and in one patient treated for the same disease by vinblastine and radiotherapy (Monteil *et al.*, 1997). It is noteworthy that FISH studies carried out during or up to 2 years following chemotherapy, demonstrate an increase in sperm aneuploidy. This could indicate an effect in some stem cells that disappeared with time.

Whatever the cause and duration of sperm aneuploidy, the consequences could be spontaneous abortion, stillbirth or birth defects in chromosomally abnormal children (Hassold *et al.*, 1996). In fact, under experimental conditions, no selection of chromosomally abnormal spermatozoa occurs during fertilization, and numerous studies (Genesca *et al.*, 1990a,b; Brandriff *et al.*, 1994; Marchetti *et al.*, 1999) have shown (by using several methods) that aneuploid spermatozoa are as competent as normal spermatozoa for fertilizing mouse oocytes.

It is believed that patients must be informed of these facts, so that genetic counselling can be offered. With regard to PEB chemotherapy, while the hypothesis of a transient chromosomal side effect appears possible, more studies on sperm chromosomes are required before and during the follow-up of the treatment. Definitive conclusions will help practitioners in counselling about the necessity and duration of contraception after chemotherapy treatment, while a chromosomal examination could be proposed in cases of pregnancy of these patients' partners, soon after the end of chemotherapy.

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