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Sperm DNA integrity in testicular cancer patients

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BACKGROUND: We evaluated the impact of testicular germ cell cancer (TGCC), its treatment and length of follow-up on sperm DNA integrity. METHODS: In 96 TGCC patients, semen was collected at specific intervals until 5 years after treatment. Sperm DNA integrity was assessed by the sperm chromatin structure assay (SCSA, *n* = 193) and by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL, *n* = 159) assay. Results were expressed as DNA fragmentation index (DFI). Controls comprised of 278 military conscripts. RESULTS: Post-surgery testicular cancer (TC) patients did not differ from controls. Compared with pretreatment values, radiotherapy induced a transient increase in $SCSA_{DFI}$ (medians: 12 versus 19%; *P* = 0.03), normalizing after 3–5 years. One year or more after therapy, 5/13 (38%) of normozoospermic, irradiated patients had $SCSA_{DFI}$ >27% compared with 7% of normozoospermic controls (*P* = 0.002). More than two cycles of chemotherapy decreased DFI 3–5 years post-therapy (median $SCSA_{DFI}$: 12 versus 9.1%, *P* = 0.02; median $TUNEL_{DFI}$: 11 versus 7.5%, *P* = 0.03). CONCLUSION: Irradiation increases sperm DNA damage 1–2 years after treatment, and 38% of irradiated patients with normozoospermia had high (>27%) DNA damage, which may affect the sperm-fertilizing ability. TC *per se* is not associated with an increase of DFI, and DFI is reduced by three or more cycles of chemotherapy.

Key words: chemotherapy/radiotherapy/SCSA/sperm DNA/testicular cancer/TUNEL

Introduction

Testicular germ cell cancer (TGCC) is the most frequent malignant disease in young men. With adequate treatment, 90–95% of the patients are cured (Schmoll *et al*., 2004). The excellent cure rate puts focus on long-term effects of the treatment, such as fertility preservation.

Sperm concentration is negatively affected by cancer treatment, the extent depending on both type of therapy and dosage (Eberhard *et al*., 2004). However, with standard TGCC treatment, the impairment is transient and pretreatment values are regained within 5 years.

Cancer treatment is potentially mutagenic, and animal studies have shown sperm DNA injury following exposure to chemotherapy or radiotherapy (Witt and Bishop, 1996; Brinkworth, 2000). Less is known regarding the effect of cancer treatment on human spermatozoa. An increased proportion of aneuploid spermatozoa following chemotherapy were reported, but the effect seems to be transient (Martin *et al*., 1997; Frias *et al*., 2003; Thomas *et al*., 2004). However, it cannot be excluded that more discrete changes in the sperm genome can be more persistent. Such phenomenon could have serious implications. Despite the recovery of spermatogenesis, the fertilizing capacity of the spermatozoa might be seriously impaired. Furthermore, new powerful assisted reproduction techniques (ART) such as intracytoplasmic sperm injection (ICSI) reduce the demands of sperm quality in terms of concentration, motility, morphology and DNA integrity (Morris, 2002). A potential worry with ICSI is that by surpassing normal biological control mechanisms in fertilization, there is a risk of transmitting defect paternal DNA to the offspring. Fertility in TGCC patients is reduced even before treatment (Petersen *et al*., 1998). Hence, these patients can be expected to benefit from ICSI, thereby being at risk of transmitting therapy-induced DNA damage to the offspring.

Studies have shown that the fertilizing capacity of the spermatozoa is also dependent on the integrity of their DNA (Larson *et al*., 2001; Carrell *et al*., 2003; Bungum *et al*., 2004). Several methods for assessment of sperm DNA breaks exist, and the sperm chromatin structure assay (SCSA) is the method mostly used for clinical purposes. Studies indicated a serious impairment of fertilization *in vivo* when the SCSA DNA fragmentation index (DFI) exceeds the level of 27–30% (Larson *et al*., 2001; Bungum *et al*., 2004). Nevertheless, SCSA has been regarded as an indirect method for the assesment of sperm DNA fragmentation as it relies on the assumption that DNA denaturability mirrors the presence of DNA strand breaks. There are other more direct tests available to measure the level

of sperm DNA breaks, such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL).

We have previously reported preliminary results on the effect of different TGCC treatment modalities on sperm DNA integrity, as assessed by SCSA (Stahl *et al*., 2004). Radiotherapy induced a transient increase of $SCSA_{DFI}$, the values normalizing after 3–5 years. Chemotherapy induced a decrease in $SCSA_{DFI}$ which, with more than two cycles of chemotherapy treatment, was observed throughout the 5 years of follow-up. In the present study, we performed both SCSA and TUNEL analysis on an extended number of ejaculates to detect the level of sperm DNA fragmentation with two independent sperm DNA integrity approaches.

Our aim was to investigate cancer therapy-induced changes in sperm DNA integrity to improve the prediction of fertility in TGCC patients. In addition, we aimed at assessing the potential risk of using sperm from men treated for cancer for ART.

Patients and methods

Patients

This study is a part of a longitudinal survey of reproductive function in males treated for TGCC, initiated in 2001. All men with TGCC, below the age of 50, diagnosed <5 years before inclusion, were eligible for the study. Fixed time intervals for delivery of semen samples were defined: T_0—after orchidectomy but before further therapy; T_6, T_{12}, T_{24}, T_{36} and T_{60}—6 to 60 months, respectively, after completed treatment. Patients entered the study at any time between T_0 and T_{60} and delivered samples at the remaining intervals.

In November 2004, 178 eligible patients had passed through the Department of Oncology, Lund University Hospital, Lund, Sweden. Twenty-five patients, not differing from the included patients in the distribution of age, histological subtype or tumor stage, refused to participate, whereas 25 patients were excluded for various reasons (bilateral disease, psychological, psychiatric reasons, hepatitis C or linguistic problems). Of the 128 patients included in the survey, one died, and four were lost during follow-up. Of the remaining 123 men, 27 were excluded from sperm analysis because of azoospermia (n = 14), retrograde ejaculation (n = 11) and development of contralateral disease after inclusion (n = 2). In the remaining 96 men, at least one semen sample was analysed.

All men participated with written informed consent according to protocols approved by the ethical review board of Lund University.

For SCSA analysis, 278 military conscripts, with a mean age of 18 years, served as controls. Data on the conscripts have been published previously (Richthoff *et al*., 2002). Twenty-four of these men were randomly selected as controls for TUNEL analysis.

Cancer treatment

The patients were treated according to the SWENOTECA protocol (Klepp *et al*., 1997; Laguna *et al*., 2001), the Swedish-Norwegian Testicular Cancer collaborative cancer care program (www.ocsyd.lu.se). Patients with nonseminomatous germ cell cancer (NSGCT) were treated with the BEP regimen (bleomycin 90 000 IU; days 1, 8, 15, to a maximum dose of 3×10^5 IU; etoposide 500 mg/m^2 and cisplatin 100 mg/m^2 per cycle, both given days 1–5, with a 3-week interval) or a similar regimen. Patients with seminomatous germ cell cancer (SGCT) were treated with EP (BEP minus bleomycin) or BEP.

The adjuvant radiotherapy (RT) was administered to a total absorbed dose of 25.2 Gy in 14 fractions to the clinical target volume of infradiaphragmal para-aortic and ipsilateral iliac lymph nodes. The dose to the remaining, lead-shielded testicle was measured at the start of the treatment. On the basis of a retrospective calculation of seven randomly selected patients in the study, the total dose to the remaining testicle was estimated not to exceed 0.5 Gy (range 0.04–0.43).

The patients were allocated into groups according to treatment given (for patient characteristics, see Table I):

(i) Surgery only (SO); nine patients with stage 1 disease, receiving no adjuvant therapy and 16 men from groups ii–iv assessed after orchidectomy, before further treatment.

(ii) Thirty-three patients with NSGCT, clinical stage (CS) I [according to the Royal Marsden Hospital staging system (Dearnaley *et al*., 2001)], receiving 1–2 cycles of adjuvant chemotherapy (ACT): [one BEP, n = 27; one CVB (etoposide replaced by vinblastin 0.3 mg/kg, maximum 22 mg/cycle), n = 2; two CVB, n = 3; two JEB, cisplatin replaced with carboplatin, n = 1].

(iii) Twenty-three patients with disseminated disease receiving more than two cycles of chemotherapy (CT) (HCT): (three BEP, n = 8; four BEP, n = 10; four EP, n = 4; more intensive CT, n = 1).

(iv) Twenty-nine patients with SGCT, CS I, receiving RT.

(v) Two patients with disseminated disease receiving both RT and CT.

In a number of semen samples, the biological material was too sparse to allow both TUNEL and SCSA analyses. A total of 193 samples from 95 of the 96 patients were analysed with SCSA, and 159 samples from 90 of the 96 patients were analysed by TUNEL (Figure 1).

Semen analysis

Fresh semen samples were collected, and within an hour post-ejaculation, sperm concentration was assessed according to WHO guidelines 1999 (World Health Organization, 1999), and the results in both TGCC patients and controls were in accordance with previous reports.

An aliquot was stored at –80°C for the subsequent SCSA and TUNEL analysis.

SCSA

The SCSA analysis is based on the phenomenon that chromatin with abundant DNA strand breaks has a tendency to denaturate when exposed to acid detergent, whereas normal chromatin remains stable. Acridine Orange stains the native double-stranded DNA and the single-stranded nucleic acids, and in excitation of blue light, the intact DNA emits green fluorescence, whereas the denaturated DNA emits red fluorescence. The extent of DNA denaturability is expressed as the DFI, being the ratio of red to total (red plus green) fluorescence intensity. DFI hereby expresses the proportion of cells containing denaturated DNA (Evenson *et al*., 2002). Five thousand cells were analysed by FACSort (Becton Dickinson, San Jose, CA, USA). The analysis was performed as previously described (Stahl *et al*., 2004). An intra-laboratory coefficient of variation (CV) of 4.5% was found.

TUNEL

The TUNEL assay quantifies the incorporation of fluorescently labelled dUTP at breaks in double-stranded DNA, utilizing a reaction catalysed by terminal deoxynucleotidyl transferase. TUNEL positivity in somatic cells reflects apoptosis, but the origin of sperm DNA strand breaks detected by TUNEL remains unclear (Sakkas *et al*., 2002; Perreault *et al*., 2003). The TUNEL analysis was performed according to the manufacturer's (Roche Diagnostics GmbH, Manheim, Germany)

Table I. Clinical data regarding the 96 testicular germ cell cancer patients included in the present study

Treatment	No adjuvant therapy (n = 9)	ACT (n = 33)	More than 2 cycles of chemotherapy (n = 23)	Radiotherapy (n = 29)	Chemotherapy and radiotherapy (n = 2)	Total (n = 96)
Median age (range)	29 (20–41)	29 (16–42)	28 (20–48)	36 (21–47)	48 (46–49)	30 (16–49)
NSGCT	8	33	18			59
SGCT	1		5	29	2	37
Stage I	9	33	6[a]	29	2[a]	79
Stage II			10			10
Stage III			3			3
Stage IV			4			4

ACT, 1–2 cycles of cisplatin-based chemotherapy; NSGCT, non-seminomatous germ cell cancer; SGCT, seminomatous germ cell cancer.
Stage refers to the clinical stage according to the Royal Marsden Hospital Staging System (14).
[a]Presenting with more advanced disease after initial staging.

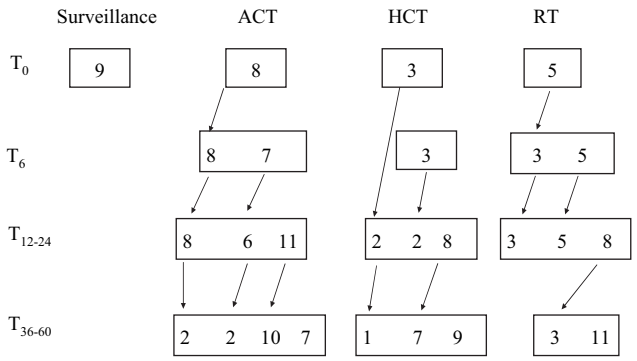

Figure 1. Flow chart illustrating distribution of the 193 semen samples, delivered by 96 TGCC patients at different time-points; T_0: before post-operative cancer treatment; T_6, $T_{12–24}$, $T_{36–60}$: 6, 12–24 and 36–60 months, respectively, post-treatment. The patients were divided according to treatment; surveillance; ACT, 1–2 cycles of chemotherapy; HCT, more than two cycles of chemotherapy; RT, radiotherapy. The arrows connect samples delivered by the same patient. Semen samples were assessed by either SCSA or TUNEL or both. If two samples were delivered by a patient in one time category, $T_{12–24}$ or $T_{36–60}$, it is presented as one sample in the figure.

protocol with slight modifications as previously described (Erenpreiss *et al*., 2004). A total of 10 000 events were accumulated for each measurement and analysed by FACSort (Becton Dickinson) flow cytometer; the same was used for SCSA. The intra-laboratory CV of 8.6% was found.

Statistical analysis

Statistical analysis was performed using the SPSS 11.0 software (SPSS, Chicago, IL, USA). To obtain sufficient numbers of individuals, the results of samples collected at T_{12} and T_{24} were combined into one time category $T_{12–24,}$ and samples collected at T_{36} and T_{60} were combined into $T_{36–60}$. If two samples were delivered by a patient in one time category, the mean value was used in the analysis. The group descriptive values were expressed as medians and ranges. The data were treated in a cross-sectional manner (Mann–Whitney *U*-test). SO (post-surgery) values were compared with those of controls in regard to both DFI and TUNEL. For each therapy, group comparisons between SO and the three different time categories were made, a total of nine comparisons for DFI and TUNEL, respectively.

The proportion of men at $T_{12} – T_{60}$ with $SCSA_{DFI}$ ≥27% was calculated, and for normozoospermic (sperm concentration ≥20 × 10^6/ml) men, the odds ratio for such high $SCSA_{DFI}$, as compared with controls, was calculated for each therapy group by means of binary logistic regression analysis. If one patient had delivered more than one ejaculate, the first sample was used for analysis.

Spearman's rho was calculated to correlate the results of SCSA and TUNEL.

$P < 0.05$ was considered statistically significant.

Results

Surgery only

Semen from TGCC patients before post-surgery treatment, SO patients, did not differ from that of controls in regard to sperm DNA integrity (Tables II and III and Figures 2 and 3).

Chemotherapy

ACT

Patients receiving ACT did not differ from SO at any time-point, neither in $SCSA_{DFI}$ nor in $TUNEL_{DFI}$ (Tables II and III and Figures 2 and 3).

HCT

Between 1 and 2 years after therapy, $TUNEL_{DFI}$ was significantly lower than in the SO group. $SCSA_{DFI}$ at $T_{12–24}$ was also lower compared to SO, but without reaching statistical significance.

Between 3 and 5 years after therapy, both $TUNEL_{DFI}$ and $SCSA_{DFI}$ were significantly lower than in the SO group (Tables II and III and Figures 2 and 3).

RT

At $T_{12–24}$, $SCSA_{DFI}$ was significantly higher in patients receiving RT than in SO. $TUNEL_{DFI}$ at $T_{12–24}$ was also higher compared with SO, without reaching statistical significance. At $T_{36–60}$, neither $SCSA_{DFI}$ nor $TUNEL_{DFI}$ differed between RT and SO (Tables II and III and Figures 2 and 3).

The data for patients receiving combined therapy were too few to analyse.

Proportion of patients with $SCSA_{DFI}$ >27%

Among the normozoospermic controls, 6.9% had $SCSA_{DFI}$ ≥27%, and in normozoospermic TGCC patients treated by

Table II. $TUNEL_{DFI}$ and sperm concentration in testicular germ cell cancer patients according to treatment and follow-up time

	Control group	SO	ACT			HCT			RT		
		$T_{0–60}$	T_6	$T_{12–24}$	$T_{36–60}$	T_6	$T_{12–24}$	$T_{36–60}$	T_6	$T_{12–24}$	$T_{36–60}$
Patients (*n*)	24	19	12	22	19	3	10	17	4	13	13
Median sperm concentration (10^6/ml) (range)	57 (13–320)	21[a] (2.6–86)	21 (5.3–38)	31 (1.0–66)	32 (2.5–104)	0.6 (0.5–1.4)	7.1 (3.5–58)	23 (5.4–92)	4.2 (3.1–13)	25 (5.5–112)	39 (7.5–174)
$TUNEL_{DFI}$ median (range)	11 (2.5–31)	11 (2.7–28)	9.1 (1.7–23)	9.6 (4.1–30)	11 (6.3–22)	3.1 (2.9–17)	7.5[b] (1.2–19)	7.5[c] (4.1–27)	18 (4.5–52)	18 (2.4–44)	11 (4.5–33)

ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SO, surgery only; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.
[a]$P = 0.001$ in comparison to controls.
[b]$P = 0.03$ in comparison to SO.
[c]$P = 0.02$ in comparison to SO.

Table III. $SCSA_{DFI}$ and sperm concentration in testicular germ cell cancer patients according to treatment and follow-up time

	Control group	SO	ACT			HCT			RT		
		$T_{0–60}$	T_6	$T_{12–24}$	$T_{36–60}$	T_6	$T_{12–24}$	$T_{36–60}$	T_6	$T_{12–24}$	$T_{36–60}$
Patients (*n*)	278	25	15	24	21	3	12	17	8	16	14
Median sperm concentration (10^6/ml) (range)	52 (0.5–390)	23[a] (0.6–86)	15 (3.0–38)	29 (1.0–66)	30 (2.5–104)	0.6 (0.5–1.4)	7.8 (3.5–58)	23 (5.4–92)	2.7 (0.1–13)	14 (0.7–112)	34 (7.5–174)
$SCSA_{DFI}$ median(range)	11 (1.7–62)	12 (7.1–72)	10 (5.4–28)	13 (8.0–45)	14 (4.1–29)	18 (16–31)	9.7[b] (4.7–33)	9.1[c] (4.0–34)	16 (8.0–47)	19[d] (7.8–45)	14 (9.7–64)

ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SCSA, sperm chromatin structure assay; SO, surgery only; SO, surgery only.
[a]$P < 0.001$ in comparison to controls.
[b]$P = 0.09$ in comparison to SO.
[c]$P = 0.02$ in comparison to SO.
[d]$P = 0.03$ in comparison to SO.

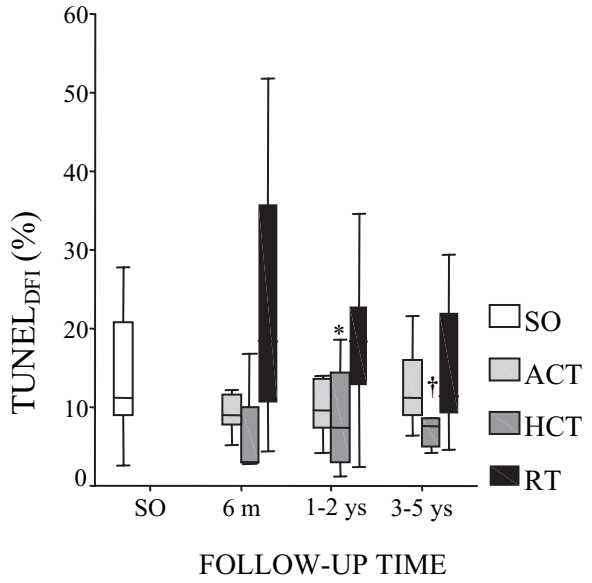

Figure 2. Box and whisker plot showing $TUNEL_{DFI}$ in TGCC patients related to treatment and follow-up time. Bars indicate median values. Boxes represent interquartile intervals and whiskers represent 95% confidence intervals. ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SO, surgery only. *HCT $T_{12–24}$ < SO, $P = 0.03$; †HCT $T_{36–60}$ < SO, $P = 0.02$.

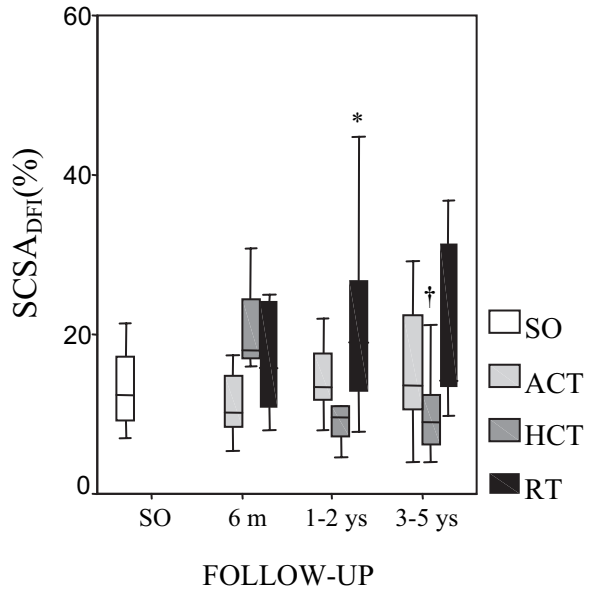

Figure 3. Box and whisker plot showing $SCSA_{DFI}$ in TGCC patients related to treatment and follow-up time. ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SO, surgery only. Bars indicate median values. Boxes represent interquartile intervals and whiskers represent 95% confidence intervals. *RT $T_{12–24}$ > SO, $P = 0.03$; †HCT $T_{36–60}$ < SO, $P = 0.02$.

RT, this proportion was 38%. The odds ratio for $SCSA_{DFI}$ ≥27 was significantly increased only in the RT group (Table IV).

Table IV. Number of men with $SCSA_{DFI}$ >27% in relation to total number of subjects investigated

	Proportion of men with normozoospermia (%)	Odds ratio in comparison with controls (95% CI)
Controls	16/233 (6.9)	Reference
ACT	3/24 (12)	1.9 (0.5–7.2)
HCT	0/7 (0)	Not done
RT	5/13[a] (38)	8.5 (2.5–29)

ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SCSA, sperm chromatin structure assay; SO, surgery only.
For the controls and the patients, the calculations were only made for those having normozoospermia (sperm concentration ≥20×10^6/ml). Odds ratios (95% CI) between the different treatment groups and controls are given.
[a]$P = 0.002$ in comparison to controls.

SCSA–TUNEL correlation

$SCSA_{DFI}$ and $TUNEL_{DFI}$ correlated significantly (Spearman's rho = 0.41; $P = 0.01$).

Discussion

In the present prospective study, sperm DNA integrity was investigated in 96 TGCC patients and correlated with treatment and time of follow-up. TGCC patients, before post-surgical treatment, had no increase of defective sperm DNA compared with controls. Adjuvant abdominal radiotherapy induced a transient increase in the proportion of sperm with DNA strand breaks. The normozoospermic RT patients had 8.5 times increased odds ratio for $SCSA_{DFI}$ ≥27% compared with controls, indicating a therapy-induced decrease of fertility *in vivo* despite normal sperm concentration.

Three or more courses of chemotherapy induced a permanent decrease of DFI.

This is the largest study on sperm DNA integrity in cancer patients, and two methods—SCSA and TUNEL—were applied. In samples analysed with both methods, a moderate correlation between the results of the two analyses was found. However, when comparing different treatment groups, identical trends were found regardless of method.

Sperm DNA integrity has been receiving increasing attention. Discrete DNA injuries have been demonstrated in human spermatozoa and shown to affect the fertilization ability *in vivo*, and possibly even *in vitro*, regardless of standard semen parameters (Larson *et al*., 2000; Bungum *et al*., 2004). Furthermore, it is known that defective paternal genome can be transmitted to the offspring (Cram *et al*., 2000), but the significance of iatrogenic DNA damage, induced by cancer treatment, is unknown. Large follow-up studies on the offspring of cancer survivors have not shown any adverse effects of cancer treatment. However, these studies were based on children born after natural conception (Blatt, 1999; Meistrich and Byrne, 2002), and there is a fear that the ICSI procedure, surpassing the biological control system of natural conception, imposes a risk of transmitting defect DNA.

In contrast to our study results, previous studies indicated that TGCC *per se* was associated with impairment of sperm

DNA integrity. Three studies used proven fertile men as controls (Fosså *et al*., 1997; Kobayashi *et al*., 2001; O'Donovan, 2005), and because fertility is associated with a low level of sperm DNA damage, our approach of using a group of unselected males as controls might explain the divergent results. Finally, Gandini *et al*. (2000), comparing sperm DNA integrity, assessed with TUNEL, in an unselected healthy population with Mb Hodgkin and TGCC patients found a statistically significant higher $TUNEL_{DFI}$ in both cancer groups. However, when comparing the Gandini study with ours, the differing conclusions are explained with the difference in controls ($TUNEL_{DFI}$ 2.5 versus 11%) and not in cancer patients. In the present study, the median $TUNEL_{DFI}$ in controls was at the same level as their median $SCSA_{DFI}$. The finding of the same DFI in SO patients and in controls cannot be explained by age difference between the two groups. TGCC men were older than controls, which should rather lead to a higher DFI among these men because this sperm characteristic is known to increase by age (Spano *et al*., 1998).

In clinical terms, a normal level of sperm DNA damage before therapy in TGCC patients indicates that the use of cryopreserved semen constitutes no increased risk of transmitting damaged DNA compared with non-TGCC ICSI-candidates.

The genetic risks of paternal exposure to irradiation are unclear. Whereas animal models have demonstrated both the transmission of radiotherapy-induced genetic damage and a subsequent increase in both early embryonic loss and malformations (Brinkworth, 2000), the potential hazards for humans are less evident. Following oncological treatment, no such risk has been detected (Meistrich and Byrne, 2002; Tawn *et al*., 2005). Observations from the Chernobyl accident are inconsistent regarding a possible increase in the rate of malformations or genetic diseases, but data suggested an increase in germline mutations (Dubrova, 2003).

Our study demonstrated the significant sensitivity of spermatozoa to radiotherapy. An estimated total dose of <0.5 Gy in 14 fractions was enough to induce long-standing, although not permanent, sperm DNA damage. The proportion of spermatozoa with DNA strand breaks was significantly higher in RT patients the first 2 years after therapy. One year or more after RT, patients with a normal sperm count had 8.5 times higher odds ratio of $SCSA_{DFI}$ ≥27% compared with controls, which may indicate *in vivo* infertility, which cannot be foreseen by judging the sperm concentration only. Although there are still some controversies regarding the impact of high DFI on fertility, the available data indicate that $SCSA_{DFI}$ ≥27% reduces the probability of *in vivo* fertilization, either by natural conception or by intrauterine insemination, almost to zero with ICSI being the most efficient way of achieving pregnancy (Bungum *et al*., 2004).

The median age of the RT patients was 36 years, when even a transient reduction of fertility can have consequences in terms of fatherhood. If these men present with infertility despite normal semen parameters, sperm DNA integrity should be assessed, and use of cryopreserved sperm might be indicated in cases of high level of sperm DNA damage.

The decrease in $SCSA_{DFI}$ following chemotherapy observed in our previous report (Stahl *et al*., 2004) was confirmed with the TUNEL analysis. This is inconsistent with several animal models in which chemotherapy induced sperm gene mutations and transmittable chromosome aberrations (Marchetti *et al*., 2001; Hales *et al*., 2005). Both cisplatin and etoposide were shown to induce sperm DNA injury and germ cell apoptosis (Sjoblom *et al*., 1998; Zhang *et al*., 2001; Cherry *et al*., 2004; Stumpp *et al*., 2004). However, mouse models and the human clinical setting differ in many aspects. Experimental studies are mostly monotherapeutical, using either a high single dosage or chronic low-dosage exposition (Sjoblom *et al*., 1998; Brinkworth, 2000; Hales *et al*., 2005). Most importantly, animal studies focus on the acute gonadotoxicity, which at least in humans differs completely from the long-term effects.

Few studies on humans address the issue of sperm chromatin in relation to anticancer therapy. The induction of sperm aneuploidy has been described after both radiotherapy and chemotherapy, but no permanent changes have been described (Martin *et al*., 1997; De Mas *et al*., 2001; Frias *et al*., 2003). Thomas *et al*. (2004) reported the absence of increase in sperm aneuploidy rates after anticancer therapy in 14 TGCC and 14 lymphoma patients, investigated between 7 months and 7 years after treatment with RT, CT or both.

The effect of cancer therapy on sperm DNA integrity is even less studied. A study on non-azoospermic adult childhood cancer survivors, compared with 66 proven fertile men, found no significant difference regarding sperm DNA integrity using the TUNEL assay (Thomson *et al*., 2002).

The decrease in the proportion of spermatozoa with DNA breaks seemed to occur after cessation of the spermatogenic arrest caused by CT. We, therefore, suggest that this effect may be exerted via the spermatogonial stem cells. One can hypothesize that spermatozoa with DNA strand breaks arise from stem cells with defective DNA repair mechanisms, which thereby make them more vulnerable to chemotherapy. Cisplatin was previously shown to increase apoptosis of germ cells (Cherry *et al*., 2004) and may add to the elimination of spermatogonia with DNA breaks. Whether sperm DNA is affected in the same way by other chemotherapy combinations remains to be investigated. The clinical application of our findings is restricted to patients receiving bleomycin, etoposide and cisplatin, for whom our study results indicated no increased risk in using post-therapy sperm for *in vitro* fertilization, including ICSI, and that normal fertility can be expected in those achieving full recovery of sperm count. However, semen cryopreservation should be performed before treatment, because neither the full extent of the therapy nor the degree of sperm recovery for the individual patient can be foreseen at the initiation of treatment.

In conclusion, irradiation induced an increase in the number of sperms with DNA damage, lasting for at least 1–2 years post-therapy, whereas more than two cycles of chemotherapy reduced the proportion of sperms with impaired DNA integrity. Further studies need to be performed for other patient groups, for example, those being treated for cancer in childhood and adolescence, because other treatment regimens as well as age at treatment might influence the effect on sperm DNA integrity.

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