Sperm integrity pre- and post-chemotherapy in men with testicular germ cell cancer

J.R. Spermon$^{1,5}$, L. Ramos$^2$, A.M.M. Wetzels$^2$, C.G.J. Sweep$^3$, D.D.M. Braat$^2$, L.A.M. Kiemeney$^{1,4}$ and J.A. Witjes$^1$

$^1$Department of Urology, $^2$Department of Obstetrics and Gynecology, $^3$Department of Chemical Endocrinology and $^4$Department of Epidemiology, Radboud University Nijmegen Medical Centre, HB Nijmegen, The Netherlands

$^5$To whom correspondence should be addressed at: Department of Urology, Rijnstate Hospital Arnhem, 6800 TA Arnhem, The Netherlands. E-mail: spermon@hotmail.com

BACKGROUND: While (partial) recovery of spermatogenesis, observed by means of standard semen analysis, has been seen in testicular cancer patients after chemotherapy with cisplatin, sperm genomic integrity and its implication for the patient’s fertility are poorly understood. METHODS: Semen and serum from 22 patients treated for testicular cancer were analysed pre- and post-chemotherapy. Besides routine semen analysis, sperm samples were evaluated by computerized karyometric image analysis (CKIA), chromomycin-A3 assay (CMA3, chromatin condensation) and TdT-mediated dUTP nick-end labelling assay (TUNEL, DNA damage). Serum FSH, LH and testosterone concentrations were measured. RESULTS: Ejaculate volume decreased post-chemotherapy ($P < 0.05$). External sperm characteristics (CKIA morphometry) and sperm counts did not deteriorate after chemotherapy. An improvement in DNA condensation was assessed after chemotherapy (37 versus 50% and 47.5 versus 63.7% for CMA3 and CKIA respectively; both $P<0.005$); yet a high percentage of TUNEL-positive sperm was found in the samples (21 versus 25% for pre- and post-chemotherapy samples respectively). These values were significantly higher than those of a convenience sample of normozoospermic males attending pre-IVF screening. Serum FSH and LH (IU/l) increased after chemotherapy compared with pretreatment levels (8.1 versus 16.7 and 4.5 vs 6.8; both $P < 0.05$, respectively). CONCLUSIONS: Despite the improvement in sperm chromatin packaging after chemotherapy, an abnormally high percentage of DNA-damaged sperm was found in these samples. As sperm quality does not reach normal levels after treatment, it remains difficult to outline the best strategy and guidance concerning fertility potential of testicular cancer patients.

Key words: chemotherapy/chromatin condensation/DNA breaks/sperm

Introduction

The introduction of cisplatin-based chemotherapy has greatly improved the cure rate for testicular germ cell tumours (Einhorn and Donohue, 1998). Because the majority of men treated are in the prime of their reproductive lives, the long-term side effects become increasingly important (Costabile, 1993).

Gonadal dysfunction is one of the most common side effects of chemotherapy (DeSantis et al., 1999). Both the endocrine and exocrine compartments of the testis are affected by chemotherapy. The serum FSH levels seem to rise immediately after initiation of chemotherapy, indicating a dysfunction of the germinal epithelium (Drasga et al., 1983; Hendry et al., 1983; Meistrich, 1986; Hansen et al., 1990; Howell et al., 1999). Although the Leydig cells of the testes are more resistant to cytotoxic damage than germinal epithelium, the increased LH levels in men suggest an endocrine dysfunction (Hansen et al., 1990; Howell et al., 1999). This probably represents a compensatory mechanism resulting from reduced negative feedback by testosterone at the hypothalamic-pituitary level, thereby reflecting a degree of impairment of testosterone production by the Leydig cells. The increased levels of LH and FSH are suggested to maintain normal testosterone levels and also support sperm production (Nijman et al., 1987; Hansen et al., 1990; Howell et al., 1999).

Testicular exocrine function is even more affected by chemotherapy. The differentiating spermatogonia appear to be most vulnerable to the cytotoxic effects of chemotherapy (Meistrich, 1986). Previous studies have shown that the majority of patients with testicular germ cell tumours have reduced sperm counts at diagnosis (before chemotherapy) (Baker et al., 2005), and this will further deteriorate during treatment (Drasga et al., 1983; Hendry et al., 1983; Fossa et al., 1985; Kreuser et al., 1986; Nijman et al., 1987; Hansen et al., 1990; Botchan et al., 1997; Lampe et al., 1997; Howell et al., 1999). The duration and severity of the spermatogenic depression depends upon the dose and duration of chemotherapy and baseline testis function prior to therapy. Despite an early depression in spermatogenesis, a reasonable number of patients show recovery within 1–2 years.
after treatment with variable sperm counts in their ejaculates (Nijman et al., 1987; Costabile, 1993; Howell et al., 1999). On this point, knowledge is lacking in two ways. First, it is doubtful whether classical semen analysis gives the right information about the status of semen from men with testis carcinoma. Second, it is not clear whether the cancer itself is also capable of inducing changes in the genomic integrity of the spermatozoon. Sperm DNA strand breaks induced during spermiogenesis or incompletely matured sperm with abnormally condensed chromatin may contribute to high rates of damaged sperm in the ejaculate.

The present study was set up to obtain more information about these problems. We used both conventional and new methods to determine the changes in the quality of ejaculated spermatozoa and applied them in samples obtained before and after exposure to cisplatin-based chemotherapy. Sperm genetic integrity, defined as normal condensed, undamaged DNA, was measured using computerized karyometric image analysis (CKIA), proven to be an objective method to study the morphology, DNA density and chromatin texture of sperm samples and individual spermatozoa (see Ramos et al., 2002a). Chromomycin A3 (CMA3) and the TdT-mediated dUTP nick-end labelling assay (TUNEL) were used to assess the DNA condensation (packaging) and damage rate (DNA breaks) respectively (Sun et al., 1997; Sakkas et al., 1998; Esterhuizen et al., 2000; Ramos et al., 2002b; Ramos et al., 2004).

Materials and methods

Patients and controls

The subjects of this study were patients who had hemi-orchiectomy and chemotherapy in the past as a result of testicular cancer. Of these patients, frozen sperm samples and serum were available, both obtained between hemi-orchiectomy and subsequent chemotherapy (Figure 1). All the included subjects received bleomycin, etoposide and cisplatin on a 5-day regimen for advanced stages of disease. Treatment consisted of etoposide at a total dose of 500 mg/m² per cycle (100 mg/m² days 1 through 5) and cisplatin at a total dose of 100 mg/m² per cycle (20 mg/m² days 1 through 5) for four cycles. Bleomycin was administered at a dose of 30 mg weekly for 9 weeks (total dose of bleomycin, 270 mg in three cycles). All patients gave written informed consent before inclusion in the study. This research was reviewed and approved by the institutional ethics committee.

Control semen samples were obtained from males attending our fertility centre for semen analysis before starting fertility treatment (with a female factor involved). After anonymization, the remainder of normospermic (WHO, 1999) semen samples (n = 13) were cryopreserved for research purposes and used as controls for CKIA, CMA3 and TUNEL assays.

Collection and workup of samples

Semen and blood samples were stored before the start of chemotherapy. For this study, new semen samples from patients post-chemotherapy were collected during a 6-month period. In the same interval of time, semen samples of the controls were collected and stored under exactly the same conditions to avoid cryopreservation bias in the assessments. In short, cryopreservation of sperm was performed as follows: samples were diluted 1 : 1 with TEST Yolk Buffer medium (TYB, Irvine Scientific, Santa Ana, CA, USA) and cooled in liquid nitrogen (LN2) vapour phase for 15 min before plunging the straws into LN2. Concomitantly with the second semen sample (see Figure 1 for the timing of sampling), a new serum sample (20 ml in EDTA) was taken and stored at −40°C until assayed. The paired (before and after treatment) semen and blood samples were analysed at the same time, with the exception of the routine semen analysis parameters that were determined in the fresh samples.

Assessment of semen parameters

Semen analysis was performed in fresh samples as described previously (Menkveld et al., 2001). In brief, concentration and motility were measured using a Makler counting chamber. For morphology assessment, semen was mixed on a slide with Methylene Blue/eosin, smeared and flame-fixed. Only vital cells (eosin negative) were evaluated for morphology using WHO criteria (1992 for pre-chemotherapy samples and 1999 for post) (WHO, 1992, 1999). Furthermore, we used the CKIA for the quantification of sperm nuclear characteristics in the frozen samples. For a detailed description, see Ramos et al. (2002a). The karyometric parameters were grouped into three categories: (1) morphometric parameters, which describe size, form and shape of the nuclei; (2) densitometric parameters, related to staining intensity of DNA content; and (3) chromatin texture parameters, related to the stain distribution pattern.

Assessment of DNA damage and chromatin condensation

Sperm DNA damage was evaluated by TUNEL to evaluate the percentage of cells with DNA fragmentation in the total sample. The TUNEL reaction detects single- and double-DNA strand breaks. Defects in chromatin condensation that increase the accessibility of the DNA to fluorochromes were detected by CMA3. The more positive cells for CMA3, the more poorly condensed sperm DNA was present (i.e. abnormal or immature cells). The results in this study are given as the percentage (and range) of normal condensed sperm (CMA3-negative cells) in the samples. At least 200 cells were evaluated per sample for the TUNEL and CMA3-staining assays. Our methods have been described in detail before (Ramos and Wetzels, 2001; Ramos et al., 2004).

Assessment of testicular function

Testicular function was evaluated by measuring LH (IU/l), FSH (IU/l) and testosterone (nmol/l) in serum. FSH and LH were determined with the fluorescence Immuno Enzymatic Assay (Abbott, IL, USA) using the Random Access Analyser type AxSYM. Testosterone was measured after extraction using the direct Radioimmuno assay (Swinkels et al., 1988).

Figure 1. Patient inclusion and timing of sampling (T) following testicular carcinoma (TC) diagnosis.
### Patient characteristics

Routine semen analyses pre- and post-chemotherapy treatment

Because of the different criteria used in the evaluation of morphology, before and after chemotherapy (i.e. a shift to a more strict evaluation criteria for the post-chemotherapy samples), no statistical evaluation for conventional sperm morphology was performed. The decrease in the percentage cells with normal morphology after chemotherapy is probably not only due to a diminished spermatogenesis but also to the above-mentioned technical changes. Using the threshold values for teratospermia considered in our centre at the time of evaluation, 22 and 47% of the samples presented teratospermia before and after completion of chemotherapy, respectively. Two patients became azoospermic after chemotherapy treatment (9%).

### Results

**Patients**

From 51 patients with stored sperm and serum obtained before the start of chemotherapy, 22 agreed to participate in the study. The relevant patient characteristics are summarized in Table I.

### Routine semen analyses

The effect of chemotherapy on routine sperm parameters is summarized in Table II. The only statistically significant difference found was a lower ejaculate volume after chemotherapy. Additionally, a significant lower percentage of spermatozoa with normal morphology after chemotherapy is probably not only due to a diminished spermatogenesis but also to the above-mentioned technical changes. Using the threshold values for teratospermia considered in our centre at the time of evaluation, 22 and 47% of the samples presented teratospermia before and after completion of chemotherapy, respectively. Two patients became azoospermic after chemotherapy treatment (9%).

### Sperm DNA analyses

The results obtained by CKIA, CMA3 and TUNEL are summarized in Table III. The percentage of cells with normal morphology by CKIA was equally distributed before and after treatment. Sperm DNA condensation improved after chemotherapy according to CKIA and CMA3 assays. The number of DNA-damaged cells (TUNEL positive) did not change statistically after chemotherapy treatment. Nevertheless, both percentages (chromatin condensation and DNA-damaged cells) were significantly different (abnormal) for patients compared with controls.

### Hormone analyses

Elevated serum FSH and LH levels were measured after chemotherapy compared with pretreatment levels (Table IV) without a significant change in the testosterone level. There was a positive correlation between LH and FSH level both before ($r = 0.99; P < 0.01$) and after treatment ($r = 0.87; P < 0.01$). No correlation between testosterone and LH or FSH was found.

### Discussion

In this study, the effect of cisplatin-based chemotherapy (<400 mg/m²) was evaluated using conventional semen parameters and DNA-integrity-related tests in sperm samples of testicular cancer patients. It was found that treatment with cisplatin has no effect on sperm count, or morphometric characteristics of spermatozoa. An improvement in sperm DNA condensation was found, but surprisingly, elevated rates of DNA damage were also observed after treatment. These values were abnormally elevated compared to fertile semen samples.

Recent literature is in agreement with our findings. After completion of chemotherapy, (partial) recovery of spermatogenesis occurs within 2 years and may continue thereafter (Draisma et al., 1983; Fossa et al., 1985; Nijman et al., 1987; Lampe et al., 1997; Spermon et al., 2003). In our series, sperm concentration and total sperm count were not significantly affected after chemotherapy, and approximately 50% of the patients still had at least $20 \times 10^9$ sperm cells/ml, with only two (9%) patients presenting azoospermia. These observations are all made after using cisplatin <400 mg/m². In patients receiving more than 400 mg/m² total dose cisplatin, a significant decrease in spermatogenesis has been described (DeSantis et al., 1999).

It is not just the chemotherapy that causes impairment of the spermatogenesis. The literature describes that 50–70% of the testicular cancer patients were subfertile or had impaired spermatogenesis before the start of chemotherapy (Hendry et al., 1983; Baker et al., 2005). This impaired spermatogenesis was neither related to stage of disease nor to the duration or severity of symptoms attributed to testicular cancer (Hendry et al., 1983; Fossa et al., 1985; Hansen et al., 1990; Lampe et al., 1997). This study confirms that a minority of patients who have been hemi-orchiectomized for testicular cancer are normospermic at the time of diagnosis. Berthelsen and Skakkebaek showed that in 24% of their cases, there were irreversible changes such as spermatogenic arrest (azoospermia), and in...
Sperm integrity pre- and post-chemotherapy treatment

Table III. Sperm integrity pre- and post-chemotherapy treatment

<table>
<thead>
<tr>
<th></th>
<th>Median pretreatment (range)</th>
<th>Median post-treatment (range)</th>
<th>P-value*</th>
<th>Control (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKIA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% normal morphometry</td>
<td>30.9 (17.0-53.0)</td>
<td>28.6 (18.0-56.0)</td>
<td>0.78</td>
<td>53.9 (41.1-66.0)</td>
</tr>
<tr>
<td>% normal DNA condensation</td>
<td>47.5 (28.0-85.0)</td>
<td>63.7 (24.0-94.0)</td>
<td>0.005</td>
<td>81.3 (73.5-89.9)</td>
</tr>
<tr>
<td>% normal chromatin texture</td>
<td>39.0 (19.0-88.0)</td>
<td>50.6 (15.0-89.0)</td>
<td>0.23</td>
<td>69.4 (53.1-88.6)</td>
</tr>
<tr>
<td>% total normal cells</td>
<td>7.8 (0.8-81.2)</td>
<td>10.4 (0.0-60.0)</td>
<td>0.40</td>
<td>40.5 (29.6-53.1)</td>
</tr>
<tr>
<td>CMA3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% normal chromatin condensation</td>
<td>37.0 (12.0-60.0)</td>
<td>50.0 (10.0-74.0)</td>
<td>0.001</td>
<td>69.8 (45.0-91.0)</td>
</tr>
<tr>
<td>% damaged cells</td>
<td>21.0 (8.0-66.0)</td>
<td>25.0 (10.0-47.0)</td>
<td>0.48</td>
<td>9.7 (4.0-14.0)</td>
</tr>
</tbody>
</table>

CKIA, computerized karyometric image analysis; CMA3, chromomycin A3; TUNEL, TdT-mediated dUTP nick-end labeling assay.

All calculated by the Wilcoxon signed rank test.

Table IV. Hormone analyses pre- and post-chemotherapy treatment

<table>
<thead>
<tr>
<th></th>
<th>Median pretreatment (range)</th>
<th>Median post-treatment (range)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (IU/l)*</td>
<td>8.05 (0.2–49.3)</td>
<td>16.65 (3.5–62.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>LH (IU/l)*</td>
<td>4.46 (0.2–27.0)</td>
<td>6.81 (1.3–32.5)</td>
<td>0.04</td>
</tr>
<tr>
<td>Testosterone (nmol/l)^3</td>
<td>18.7 (1.7–44.0)</td>
<td>15.8 (6.7–26.0)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Reference range in our laboratory: *1.5–7.5 IU/l; †1.4–8.5 IU/l; ‡11–45 nmol/l.

*Calculated by paired two-tailed t-test, while the remaining data were analysed by the Wilcoxon signed rank test.

over half of the remaining patients, there was a potentially reversible depression of spermatogenesis (Berthelsen and Skakkebaek, 1983). Most likely there is a pre-existing impairment of spermatogenesis in the contralateral testis in men with testicular cancer. These findings support the hypothesis that testicular germ cell cancer may be aetologically linked to other male reproductive abnormalities as part of the so-called ‘testicular dysgenesis syndrome’ (Hoei Hansen et al., 2003). Other study confirms that a minority of patients are normospermic prior to diagnosis (Baker et al., 2005).

Recovery of spermatogenesis after chemotherapy continued for several years (Lampe et al., 1997). The probability of spermatogenesis recovery increased from 48% by 2 years to 80% by 5 years. As the storage time of cryopreserved semen has no deleterious effect on semen quality (Rofeim and Gilbert, 2005), all the observed difference between pre- and post-chemotherapy (in our study) is probably not a consequence of the storage time between first and second samples.

There are two major limitations of routine semen analysis. First, there is a lack of standardization and there is a significant observer bias (Neuwinger et al., 1990; Cooper et al., 1992; Kruger et al., 1995) and second, the standard morphology analysis probably does not describe the integrity of the sperm DNA. To bypass the observer bias, we evaluated sperm morphology with qualitative computer measurements (CKIA). CKIA also gives information about sperm DNA condensation and chromatin texture. With this technique, we found that although the external features in the morphometric parameters were equally distributed before and after chemotherapy, there were changes in internal characteristics (DNA stainability and condensation). Abnormal sperm DNA condensation is known to be adversely correlated to male fertility potential (Auger et al., 1993; Esterhuizen et al., 2000; Agarwal and Said, 2003). Despite the significant increase of cells with normal DNA condensation after chemotherapy, chromatin condensation as indicated by low CMA3 values (<60% negative) and CKIA are still poor if compared with normal donors. With respect to DNA damage, no adverse effect was measured as a result of chemotherapy. For this result also, it is worth noting that the patient group scored significantly higher than the fertile donors. These observations support the study by O’Donovan (2005) who used propidium iodide for DNA condensation measurements. In our series, none of the sperm samples had normal CMA3 values before the start of chemotherapy, in contrast to eight of 22 patients who did so after chemotherapy. Available literature does not offer an obvious explanation for this observation. Based on animal studies, we hypothesize that spermatogonia with abnormal chromatin, as a consequence of the disease, might be more susceptible to chemotherapy (Seaman et al., 2003), thus being eliminated by treatment. The remaining normal, and more viable, germ cells (spermatogonia) are responsible for the partial restoration of spermatogenesis after a recovery time. More research is necessary on this point, not in the last place to enable effective treatment: abnormalities in chromatin condensation may contribute to failures in sperm decondensation after penetration into the oocyte and subsequently result in fertilization failure (Esterhuizen et al., 2000).

In the literature, controversial results have been published on sperm DNA damage in testicular cancer patients. Stahl et al. (2004) reported a significantly lower DNA fragmentation index (DFI) among 16 patients compared with controls (healthy males) after completion of chemotherapy (7.3 versus 11%; \( P = 0.028 \)) using the sperm chromatin structure assay. Although our controls always scored better than the patients, our data confirm that chemotherapy treatment does not produce an increase in DNA damage in spermatozoa. Also in the same line as for our findings using the TUNEL assay, Gandini et al. (2000) reported a significant increase of DNA fragmentation in 30 testicular cancer patients post-orchiectomy compared to healthy controls (11 versus 2.5%), but their reference values for the controls are very low compared to our TUNEL results (2.5 versus 9.7% respectively). Despite the fact that...
chromatin is better packed after treatment, no decrease in the percentage of sperm DNA-damaged cells was observed and remained high compared with healthy controls. This is in accordance with the results obtained by O’Donovan (2005) with use of the Comet assay. Our results led us to postulate that there might be an intratesticular alteration in the apoptotic control system as a reaction to neoplastic cell proliferation or that the chemotherapeutical treatment might affect the removal of abnormal sperm by apoptosis. Consequently, damaged cells that should have been eliminated during spermatogenesis can be found in the ejaculates (abortive apoptosis) (Sakkas et al., 1999, 2003). Based on our own results and these other publications, it seems prudent to advise the use of sperm recovered after spermatogenesis recovery (post-chemotherapy) in case these males require assisted reproduction. However, we should still be concerned about risks to the progeny, as data from follow-up studies of children born from chemotherapy-treated fathers are scarce.

Normal spermatogenesis also depends on normal endocrine balance. Testosterone, regulated by LH, is an absolute requirement for normal spermatogenesis. Our study demonstrates that standard doses of chemotherapy do not lead to a significant decrease of the testosterone level. However, the LH values were significantly elevated, indicating a compensatory reaction to the decreased Leydig cell function, resulting in a constant testosterone level. In agreement with others, we found biochemical evidence of germinal epithelial failure of the contralateral testis, indicated by increased FSH levels after treatment (Howell et al., 1999; Gerl et al., 2001). The observed correlation between LH and FSH is not surprising, given the relative susceptibility of Leydig cells and germinal epithelium to damage and suggests interaction between each other (Howell et al., 1999).

There is no doubt that testicular cancer and its treatment have serious impact on gonadal function in these young patients. Fertility is therefore a major concern, and healthcare providers are increasingly aware of the need to improve the quality of life of cancer patients by maintaining their reproductive capacity. In the past, recovery of high sperm counts were evidence of germinal epithelial failure of the contralateral testis, indicated by increased FSH levels after treatment (Howell et al., 1999; Gerl et al., 2001). The observed correlation between LH and FSH is not surprising, given the relative susceptibility of Leydig cells and germinal epithelium to damage and suggests interaction between each other (Howell et al., 1999).

In conclusion, sperm count and morphology (CKIA) was not affected in the majority of patients treated with chemotherapy for testicular cancer. Although an improvement in the chromatin condensation was found in sperm, the percentage of DNA-damaged cells did not decrease after chemotherapy. Sperm DNA integrity in general remained poor compared to healthy controls. It should be elucidated whether the observed changes in sperm integrity represents a real post-chemotherapy removal of an abnormal germ cell subpopulation or whether the post-chemotherapy matured sperm presents other types of genomic damage not detectable by the current methods. Although our results call for further investigations, it seems prudent to evaluate semen from cancer patients, not only by routine analysis but also by genomic integrity analysis, as external characteristics of spermatozoa do not necessarily correlate with their DNA integrity.

Acknowledgements

The authors thank the fertility laboratory and especially Hannie Robben en Leonie van den Hoven for their technical assistance and cryopreservation of the samples.

References


