Sperm DNA integrity in cancer patients before and after cytotoxic treatment

M. Smit1,*, N.J. van Casteren1, M.F. Wildhagen2, J.C. Romijn1, and G.R. Dohle1

1Andrology Unit of the Department of Urology, Erasmus MC, PO Box 2040, 3000 CA Rotterdam, The Netherlands
2Unit Trials & Research Coordination of the Departments of Urology and Obstetrics and Gynaecology, Erasmus MC, Rotterdam, The Netherlands

*Correspondence address. E-mail: m.smit.3@erasmusmc.nl

Submitted on October 1, 2009; resubmitted on March 20, 2010; accepted on March 23, 2010

BACKGROUND: We assessed sperm DNA fragmentation index (DFI) in cancer patients before and after treatment to evaluate if sperm DNA integrity is compromised by cancer itself or its treatment.

METHODS: In a prospective study, DFI was assessed in 127 patients diagnosed with testicular germ cell tumours (TGCT), Hodgkin’s lymphoma (HL), non-Hodgkin’s lymphoma (NHL) and various malignancies. The severity of cancer and tumour markers at diagnosis was recorded. Follow-up DFI after treatment was available in 52 patients who were mostly less severely affected.

RESULTS: In patients diagnosed with TGCT, HL and various malignancies, pretreatment DFI levels were not significantly different from that of proven fertile controls, but in patients with NHL an increased DFI was found. An overall significant decrease in post-treatment DFI (13.2% range 5.0–70.5) compared with pretreatment values (17.1% range 5.1–66.6) was found ($P = 0.040$). In TGCT patients, post-treatment DFI was significantly higher in patients who were treated with radiotherapy (16.9% range 11.5–39.9) compared with that in patients treated with chemotherapy (CT) alone (10.9% range 5.5–39.9) ($P = 0.037$). In HL patients, the type of treatment or number of CT cycles was not associated with DFI. Overall, post-treatment DFI in cancer patients was not significantly different from that of proven fertile controls.

CONCLUSIONS: In this study, the presence of cancer does not seem to negatively affect the sperm DNA integrity in TGCT and HL patients; only NHL patients showed increased DFI at the time of diagnosis compared with healthy controls. Our results confirm previous reports that DFI decreases significantly following various anti-cancer treatments. In contrast, radiotherapy in TGCT patients is associated with an increase in DFI compared with CT treatment alone.

Key words: sperm DNA integrity / cancer / therapy / testicular cancer / Hodgkin’s lymphoma

Introduction

Fertility preservation has become an important issue in the counselling and therapy of cancer patients of reproductive age, particularly for cancer types with a high cure rate, such as testicular germ cell tumour (TGCT) and Hodgkin’s (HL) or non-Hodgkin’s lymphomas (NHL). More than 90% of all TGCT patients are considered cured in a 5-year period following treatment (Kopp et al., 2006). In HL and NHL patients, more than 80% of all patients are successfully treated (Diehl et al., 2003; Bernard et al., 2005).

Impaired spermatogenesis is commonly observed at the time of cancer diagnosis, both for TGCT and for lymphomas (Gandini et al., 2003; Howell and Shalet, 2005; van Casteren et al., 2009). TGCT is associated with testicular malfunction and it is postulated that the disease is part of the testicular dysgenesis syndrome also including defective spermatogenesis and cryptorchidism (Skakkebaek et al., 2001). The gonadal dysfunction observed in lymphoma is believed to be caused by a systemic effect of the disease, possibly because of cytokine activity. Increased levels of interleukins have been associated with more severe dysfunction of spermatogenesis (Rueffer et al., 2001). Cancer treatment might further affect gonadal function, though these effects may be (partly) reversible, depending on the agents and cumulative dosage used. Current chemotherapy (CT) agents and radiotherapy treatment protocols aim at cure in combination with minimal long-term morbidities such as treatment-related infertility. TGCT patients treated with at least 3 cycles of bleomycin, etoposide and cisplatin (BEP) initially all develop azoospermia with recovery of spermatogenesis in 48% after 2 years and in 80% after 5 years (Lampe et al., 1997). Alkylating agents such as mustine, vincristine,
and disease stage, pretreatment tumour markers and type of treatment has been associated with an increased number of pathological pregnancies (Trasler et al., 1985; Dobrzenska et al., 2005). However, the safety of the use of sperm cryopreservation at the time of cancer diagnosis and the use of recovered sperm from males exposed to cancer treatment has recently been questioned (Spermon et al., 2006). Assessment of sperm DNA integrity has been proposed as a means to investigate the impact of cancer and its treatment on the functional quality of spermatozoa. In animal studies, sperm DNA damage induced by gonadotoxic treatment has been associated with an increased number of pathological pregnancies (Trasler et al., 1985; Dobrzenska et al., 2005). In humans, however, there is no epidemiological proof of increased malformations in children born to parents who were formerly treated for cancer (Morris, 2002).

In the present study, we determined sperm DNA damage in cancer patients before and following treatment to evaluate whether the cancer itself or its treatment induced changes in the genomic integrity of sperm. We evaluated the association between sperm DNA damage and disease stage, pretreatment tumour markers and type of treatment, to establish the impact of tumour characteristics and intensity of treatment on sperm DNA integrity.

Materials and Methods

Study population

Between September 2003 and September 2005, study participants were recruited among all men who visited our Andrology outpatient clinic for an attempt to cryopreserve their semen before oncological treatment. One hundred and twenty-seven non-azoospermic men were included in the study and provided written informed consent to have the sperm chromatin structure assay (SCSA) performed in addition to classic semen analysis, prior to cryopreservation and in future semen samples. All patients were categorized according to oncological diagnosis comprising TGCT, HL, NHL, leukaemia, sarcoma and various malignancies requiring CT like astrocytoma, rectal cancer and so on (other). Information regarding cancer stage, prognosis group, tumour markers at the time of diagnosis, histological tumour type and oncological treatment was obtained from patient records. TGCT patients were categorized as good prognosis if, at the time of diagnosis, the primary tumour was found in the testis or retroperitoneum, beta-human chorionic gonadotrophin (βHCG) was <1.9 IU/l (normal value 0–1.9 IU/l) and lactate dehydrogenase (LDH) was <1.5 x 449 U/l (normal value 0–449 U/l). Intermediate prognosis comprised extrapulmonary visceral metastasis in seminomas or alpha-fetoprotein (AFP) 1–10 µg/l (normal value 0–9 µg/l) or LDH <1.5–10 x 449 U/l or βHCG 5–50 IU/l in non-seminomas. Tumour markers above these thresholds or the presence of extrapulmonary visceral metastases in non-seminomas resulted in classification as poor prognosis (Mead and Stenning, 1997). For lymphomas, the Ann Arbor staging system was used, defined as cancer in a single lymph node region (stage I), two separate lymph node regions either above or below the diaphragm (stage II), cancer on both sides of the diaphragm (stage III) or disseminated involvement (stage IV) (Lister et al., 1989).

Patients were advised to repeat semen analysis at least 6 months after the last oncological treatment to assess fertility and decide whether cryopreservation of the pretreatment semen sample should be prolonged. When multiple follow-up samples were available, the most recent sample was used for the analysis. A total of 30 patients were lost to follow-up. In 43 patients, follow-up semen analysis and SCSA results could not be obtained because of progression and death, persistent azoospermia due to treatment, anorchia or retrograde ejaculation. Two patients were managed in a wait and see protocol and did not receive treatment. In 52 patients, follow-up data were available for analysis. A study flowchart is depicted in Fig. 1. For fertile controls, 22 proven fertile healthy men donated a semen sample prior to vasectomy.

Semen analysis

Semen samples were collected by masturbation prior to oncological treatment. Following liquefaction, a droplet of semen was used for gross examination of the sample. If motile, viable sperm was found, the sample was considered adequate for cryopreservation. After cryopreservation, semen analysis in the droplet of semen was carried out according to WHO guidelines (WHO, 1999). Remnant material was stored at −80°C for SCSA analysis at a later stage. Whenever patients were able to have multiple samples cryopreserved prior to therapy, results from the first semen sample were used in this study. During follow-up, semen samples were collected by masturbation after a period of abstinence of 3–5 days. Semen analysis was performed according to WHO guidelines (WHO, 1999), and material was stored at −80°C for sperm DNA integrity assessment at a later stage.

Sperm DNA integrity assessment

The SCSA was used to assess sperm DNA integrity. O’Flaherty et al. (2008) recently concluded that SCSA and deoxynucleotidyl transferase-mediated dUTP nick end-labelling are significantly correlated and suggested that any of these assays can be used to determine DNA damage in cancer patients. SCSA was performed essentially as described by Evenson and Jost (2000), using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). In brief, frozen samples were quickly thawed, diluted to a concentration of 1–2 x 10⁶ sperm cells per ml, exposed to acidic detergent solution and stained with acridine orange. A reference sample, treated similarly, was run prior to the actual sample measurements and used to adjust the voltage gains of the flow cytometer FL3 and FL1 photomultipliers that analyse red and green fluorescence, respectively. The voltage gains were adjusted to obtain stable mean red (X) and mean green (Y) values of the reference sample at 110 and 370 channels, respectively, with a maximum deviation of five channels. An aliquot of reference sample was run after every 5–10 samples. The voltage gains were re-adjusted whenever the fluorescent signal of the reference sample drifted. Data collection of the fluorescent pattern in 5000 cells was performed at 3 min after acid treatment. Debris, bacteria and leucocytes were gated out during acquisition as recommended by Evenson and Jost (2000). The extent of DNA damage is expressed as the DNA fragmentation index (DFI), reflecting the ratio of red
fluorescence to total fluorescence. Cell Quest Pro and WinList software were used to calculate the DFI of each sample. Each sperm sample was measured in duplicate and the mean values of the results were used in the analysis.

Statistics

All variables were tested for normal distribution using the one sample Kolmogorov–Smirnov test. Because the majority of semen analysis and SCSA variables were not normally distributed, non-parametric tests were used for all variables. We aimed to assess whether pre- and post-treatment semen analysis and DFI values were statistically different using the Wilcoxon-paired test. Possible associations between cancer characteristics, timing of semen sampling after therapy and sperm quality parameters were evaluated using Spearman's signed rank coefficient of correlation. Differences in classic semen analysis and SCSA variables between patients groups and in comparison with proven fertile controls were assessed with the Mann–Whitney U-test. All results are expressed as median with range. A P-value of <0.05 was considered statistically significant.

Results

Pretreatment

Pretreatment semen analysis results and sperm DFI for patients with various types of cancer are depicted in Table I.

In TGCT patients, 41 non-seminomas and 11 seminomas were diagnosed. No significant differences were found in semen variables or DFI between the two histological TGCT types. In the TGCT patients with a poor or intermediate prognosis ($n = 7$), total sperm count and sperm concentration were significantly lower compared with TGCT patients with a good prognosis, $n = 45$, [8.2 x 10^6 (2.2–105.6) versus 50 x 10^6 (1.9–898.0) $P = 0.040$ and 4.9 x 10^6/ml (0.5–33.0) versus 16.0 x 10^6/ml (1.0–749.0) $P = 0.049$, respectively].
Higher DFI values were found in the intermediate/poor prognosis patients [25.4% (12.1–42.0)] compared with patients with good prognosis [13.6% (4.0–65.3)], but the difference was not statistically significant (P = 0.086). Serum AFP, βHCG and LDH at diagnosis were 9.0 μg/l (1.0–12 264.0), 4.7 IU/l (0.2–88 915.0) and 342.0 U/l (146.0–3698.0), respectively. Both total sperm count and sperm concentration were significantly negatively correlated with serum βHCG at diagnosis (r = −0.286, P = 0.049 and r = −0.306, P = 0.034, respectively). Serum LDH level at diagnosis was significantly correlated with DFI (r = 0.354, P = 0.017).

In HL patients, stage I/II disease was diagnosed in 24 patients, whereas seven men were diagnosed with stage III/IV disease. In the NHL patient group, stage I/II was found in 10 patients and 5 patients were diagnosed with stage III/IV disease. For both HL and NHL patients, no association between disease stage and pretreatment semen parameters or DFI was observed.

Post-treatment

In 73 patients, no follow-up data were available because no spermatozoa were available due to death, azoospermia, anorchoia or retrograde ejaculation (n = 43) or semen analysis could not be obtained (n = 30). When compared with the 52 patients in whom follow-up data were available, NHL, leukaemia and high-dose treatment were overrepresented in the former group. Median follow-up time was 1.1 years (0.5–3.3), since semen cryopreservation and 0.8 years (0.2–3.1) since the last oncological treatment. DFI levels were not significantly correlated with the time since the last treatment to semen sampling. Overall, total sperm count and sperm concentration decreased significantly at follow-up [from 80.1 × 106 (2.2–915.6) to 22.9 × 106 (0.1–612.0) P < 0.001 and from 30.0 × 106/ml (0.5–327.0) to 9.9 × 106/ml (0.1–102.0) P < 0.001, respectively]. Progressive motility and normal morphology were not significantly different after treatment. A significant decrease in DFI following treatment in the population with available follow-up was found [from 17.1% (5.1–66.7) to 13.2% (5.0–70.5) P = 0.040].

The three largest patient categories, TGCT, HL and NHL, were analysed separately.

Of the 25 TGCT patients with available follow-up data, 17 patients were treated with 3 × 4 × BEP alone, whereas eight patients were treated with either a combination of 3 × 4 × BEP and 13–25 fractions of 2.0 Gy radiotherapy (RT) (n = 3) or RT alone (n = 5) on the retroperitoneum. DFI at follow-up was significantly higher in patients who were treated with RT or a combination of CT and RT [16.9% (11.5–39.9)] compared with patients treated with BEP CT alone [10.9% (5.5–37.1) P = 0.037]. In the whole TGCT group, DFI decreased from 15.1% (6.7–52.0) to 12.0% (5.5–39.9) upon treatment but the difference was not statistically significant (P = 0.061).

In the 15 patients with HL in whom follow-up semen analysis was available, two patients were treated with ABVD only, and 13 patients were treated with 15–20 fractions of 2.0 Gy RT in addition to ABVD. RT was administered to the groin in one patient, whereas all other RT was localized above the diaphragm. Only sperm concentration was shown to decrease significantly following therapy from 77.0 × 106/ml (11.0–327) to 40.0 × 106/ml (5.9–102.0) (P = 0.041). The other sperm characteristics and the DFI did not change significantly following treatment. Post-treatment DFI was not significantly different in patients who were treated with CT alone or a combination of CT and RT. The number of CT cycles was not associated with the DFI at follow-up.

In the NHL patient category, three patients were treated with a combination of 6 × CHOP CT and 20 fractions 2 Gy RT and 2 patients received only high-dose CT (either 8 × CHOP or 6 × CHOP followed by third line CT). Because of the small number of patients, no statistical analysis was performed.

In TGCT, HL and NHL groups, the number of patients with pre- and post-treatment DFI levels above 30% were calculated and depicted in Table II.

Table II Pre- and post-treatment sperm DFI in patients in whom follow-up data were available for analysis and fertile controls.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>DFI pretreatment &lt;30%</th>
<th>DFI pretreatment &gt;30%</th>
<th>n</th>
<th>DFI post-treatment &lt;30%</th>
<th>DFI post-treatment &gt;30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGCT</td>
<td>52</td>
<td>41 (79%)</td>
<td>11 (21%)</td>
<td>25</td>
<td>22 (88%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>HD</td>
<td>31</td>
<td>22 (71%)</td>
<td>9 (29%)</td>
<td>15</td>
<td>14 (93%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>NHL</td>
<td>15</td>
<td>10 (67%)</td>
<td>5 (33%)</td>
<td>5</td>
<td>5 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

TGCT, testicular germ cell tumours; HL, Hodgkin’s lymphoma and NHL, non-Hodgkin’s lymphoma.

DFI in cancer patients versus controls

Overall, both pre- [17.1% (5.1–66.7)] and post-treatment [13.2% (5.0–70.5)] DFI levels in cancer patients were not significantly different from that of proven fertile controls [15.3% (6.4–25.7)] (Fig. 2). No statistical differences in DFI between proven fertile controls and TGCT patients, before or after treatment, were found. The same holds true for HL patients. In NHL patients, however, pretreatment DFI was significantly higher compared with proven fertile controls (P = 0.008) (Fig. 2).

Discussion

We determined pretreatment DFI levels in 127 cancer patients and compared this with the results obtained for proven fertile controls. Sperm DNA integrity was found to be compromised in NHL patients but was not significantly changed in patients diagnosed with TGCT or HL. This finding is in agreement with a recent study by Ribeiro et al. who found that DNA fragmentation was not significantly increased in 48 TGCT patients compared with proven fertile controls (Ribeiro et al., 2008). In contrast, several other studies have reported increased
sperm DNA damage in pretreatment sperm samples of patients with TGCT and HL compared with controls (O’Donovan, 2005; Spermon et al., 2006; Meseguer et al., 2008; O’Flaherty et al., 2008; Stahl et al., 2008). This discrepancy may have been caused by low numbers of patients studied, selection bias in patients who were offered semen cryopreservation or selection bias of controls. To our knowledge, our study cohort contains the largest number of patients in whom pretreatment DFI was assessed. All patients who were offered semen cryopreservation were eligible for study inclusion. Consequently, our cohort consisted of patients with normal prevalence and variation in severity of cancer stage and prognosis. It should be emphasized that at clinical presentation, patients with poor prognosis and high-stage disease are the minority. We used proven fertile controls but acknowledge that population-based controls are the most appropriate controls because proven fertile controls may have lower DFI levels than men on average.

Our results are in agreement with previous findings that pretreatment sperm concentration in TGCT patients is significantly lower compared with that of HL and NHL patients and that there is no interrelationship between the TGCT histological type and pretreatment semen quality (Gandini et al., 2003; van Casteren et al., 2006). It has been suggested that spermatogonia with normal chromatin structure following CT, although some authors report abnormal post-treatment sperm DNA damage levels compared with healthy controls (O’Donovan, 2005; Spermon et al., 2006; Stahl et al., 2006). It has been suggested that spermatogonia with abnormal chromatin might be more susceptible to CT and are thus eliminated by the treatment. Remaining normal spermatogonia are responsible for the restoration of spermatogenesis after a recovery time (Spermon et al., 2006). Our observation that the time since the last oncological treatment was not associated with post-treatment DFI levels in our study, may further indicate that normal spermatogonia with normal chromatin structure are responsible for the recovery of spermatogenesis. Studies among childhood cancer survivors indicate that the sperm DNA integrity in men with recovered spermatogenesis is not different compared with healthy, fertile controls (Thomson et al., 2002; van Beek et al., 2007). Fertility status follow-up after cancer treatment can be troublesome due to variable recovery rates of spermatogenesis and anorchia or retrograde ejaculation due to oncological treatment. Our study results were further influenced by selection bias because a significant number of patients were lost to follow-up and the majority of patients included were categorized as good prognosis or low grade. Despite these shortcomings, we were able to analyse the impact of cancer and its treatment on the sperm DNA integrity in as many as 52 patients. In the present study, we observed a significant decrease of DFI levels following oncological therapy. As shown before, irradiation therapy of TGCT patients resulted in more sperm DNA damage than did CT of TGCT patients (Stahl et al., 2006). It is presumed that sperm DNA damage can be induced by direct gonadal irradiation or by radiation scatter during treatment despite shielding of the gonads. In HL patients, however, irradiation therapy was not associated with increased post-treatment DFI in our study.

It is common practice to recommend patients to postpone conception for 6–12 months after therapy to bypass possible negative effects of radiation and CT such as single gene mutations and chromosomal translocations in spermatogonia (Meistrich, 1993). A longer period up to 24 months has been suggested by others because of the persistence of increased aneuploidy after CT (De Mas et al., 2001). Our results support previous reports that sperm DNA damage is significantly reduced in post-treatment semen samples compared with pretreatment samples after a median follow-up time of 1.1 year. Moreover, we found that the presence of cancer does not seem to negatively affect the sperm DNA integrity in TGCT and HL patients, whereas NHL patients showed increased DFI at the time of diagnosis and compared with proven fertile controls. On the basis of our results and a review of the literature, RT in TGCT patients may have a more profound negative impact on the sperm DNA integrity resulting in increased DFI levels, persisting 1–2 years following radiation treatment, than CT alone (Stahl et al., 2006). Finally, we have shown that about one-third of TGCT, HL and NHL patients have increased DFI levels above 30% at the time of diagnosis.
Following treatment only 0–12% of these patients have increased DFI levels. DFI levels above 30% are associated with significantly lower pregnancy chances in vivo (Evenson et al., 1999), which can be overcome by the use of IVF/ICSI (Bungum et al., 2007). Moreover, apart from diminished in vivo fertilizing potential in patients with high DFI levels, it is unclear if high DFI in patients who achieve pregnancies have a negative impact on the offspring. One could argue that SCISA may be unable to assess the subtle changes associated with the cancer or therapy-induced damage of the paternal genome and that it is therefore unjustified to conclude that the use of spermatozoa of cancer survivors with low DFI in IVF/ICSI is safe. Further, larger studies that evaluate aneuploidy rate, genomic stability and IVF/ICSI offspring follow-up will be needed to further address the safety and timing issues of the use of spermatozoa in IVF/ICSI in cancer survivors formerly exposed to cancer treatment.

Acknowledgement

The authors wish to thank Joke Veldhoven for her assistance with the collection of semen samples and SCISA determinations.

References


Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 2001; 16:972–978.


