ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Loredana Gandini, Laboratory of Seminology-Sperm Bank, Department of Experimental Medicine, University of Rome "La Sapienza", Viale del Policlinico 155, 00161 Roma, Italy.
E-mail: loredana.gandini@uniroma1.it

Keywords:
chemotherapy, chromatin, DNA damage, radiotherapy, SCSA

Received: 21-Oct-2013
Revised: 9-Jun-2014
Accepted: 20-Jun-2014

doi: 10.1111/j.2047-2927.2014.00250.x

Testicular cancer and sperm DNA damage: short- and long-term effects of antineoplastic treatment

[1]D. Paoli, [1]M. Gallo, [1]F. Rizzo, [2]M. Spanò, [2]G. Leter, [1]F. Lombardo, [1]A. Lenzi and [1]L. Gandini

[1]Laboratory of Seminology-Sperm Bank, Department of Experimental Medicine, University of Rome "La Sapienza", and [2]Laboratory of Toxicology, Unit of Radiation Biology and Human Health, ENEA Casaccia Research Centre, Rome, Italy

SUMMARY

The aim of this study was to investigate sperm DNA damage induced by chemo- and radiotherapy in patients with testicular cancer to provide data on the extent and persistence of nuclear damage that might affect individual reproductive potential. We evaluated pre- and post-antineoplastic treatment sperm DNA integrity, expressed as DNA Fragmentation Index (DFI), in a large caseload of testicular cancer patients by sperm chromatin structure assay. The mean total DFI for all patients at T0 was 18.0 ± 12.5%. Sperm chromatin profile was markedly impaired at T3 (27.7 ± 17.4%) and T6 (23.2 ± 15.3%), improving considerably at T12 and T24 (14.0 ± 8.9% and 14.4 ± 10.3%). After chemotherapy, we found a marked increase in DFI at T3 and T6 and a significant reduction at T12 and T24 in comparison with the baseline. In contrast, DFI increased at T3 and T6 after radiotherapy but the subsequent reduction was far less marked, reaching baseline values at T12 and T24. Finally, post-treatment DNA damage was not age or histotype dependent, but was more marked in the advanced stage of cancer. In this study, we showed that the chromatin profile may be affected in the months immediately following the end of the treatment, improving after 12–24 months. Our results thus indicate that post-treatment DNA damage is influenced both by the type and intensity of the therapy and by the pathological and clinical stage of the disease.

INTRODUCTION

Testicular cancer is the most common cancer in men of reproductive age. Although the international incidence varies considerably, with large variations in different countries and among different ethnic groups, there has been a general rise over the last 30–40 years (Huyghe *et al.*, 2003; Purdue *et al.*, 2005; Chia *et al.*, 2010). It is particularly prevalent in European populations and men of European ancestry (Purdue *et al.*, 2005), with the highest incidence in Northern Europe (8.0–9.0 per 100 000) and lowest in Asia and Africa (<1 per 100 000) (Chia *et al.*, 2010). As in many other Western countries, the incidence in Italy has risen, from a mean of 3.7 cases/year per 100 000 inhabitants in 1993–1995 to 5.2 in 2003–2005, an increase of 40.5% (Crocetti *et al.*, 2009). Fortunately, progress in multimodal treatments such as chemotherapy and radiotherapy in combination with surgery over the last 20–25 years have made testicular cancer one of the most treatable of all cancers, especially when diagnosed early. For example, the estimated 5-year mortality rate is 4% in the United States (Rosen *et al.*, 2011), whereas in Italy, it accounted for just 0.1% of all deaths from cancer between 1998 and 2002 (AIRTUM – Associazione Italiana Registri Tumori, www.registri-tumori.it). Today, the 5-year survival rate is above 90% (Kopp *et al.*, 2006). Post-treatment quality of life is thus an important aspect in the management of testicular cancer in young men, especially with respect to fertility and the possibility of future fatherhood.

Various studies of the effect of testicular cancer treatments on spermatogenesis have found a post-treatment impairment of semen parameters (Dohle, 2010; Trost & Brannigan, 2012). In fact, while these treatments effectively kill cancer cells, they can also affect cells with a fast replication rate, such as germ cells. Gandini *et al.* (2006) showed that the most detrimental effects of chemotherapy and radiotherapy protocols on spermatogenesis last up to 3–6 months after treatment, with 94% of chemotherapy patients showing good recovery after 12 months and 97% after 24 months.

Not only semen parameters may be affected. Some studies have also found increased sperm DNA damage and aneuploidy

in men (Ståhl *et al.*, 2004; Tempest *et al.*, 2008; O'Flaherty *et al.*, 2010) and impaired germ cell gene expression in animal models (Delbès *et al.*, 2009). To date, however, few studies have been conducted on the effects on the offspring of men who have undergone cancer treatments (Brinkworth, 2000; Ståhl *et al.*, 2011; Signorello *et al.*, 2012). Animal studies have shown that chromatin damage in spermatozoa from animals exposed to antineoplastic agents can impair the embryonic development of offspring (Trasler *et al.*, 1986; Bieber *et al.*, 2006). For this reason, and because of the youth of many testicular cancer patients, there has been great concern in recent years about the effects of antineoplastic treatment on sperm chromatin quality. Several reports have suggested that the recovery of spermatogenesis depends on the drugs used and on the cumulative dose given (Dohle, 2010) and is a function of the time since the end of the therapy (Gandini *et al.*, 2006). However, there is still a lack of data on chromatin and DNA damage after exposure to antineoplastic treatments, with the extent and duration of such damage (and thus the time necessary for its reversal) still unknown. This information is highly important, especially for oncologists having to manage young patients who may still wish to father children in the future. The aim of this study was thus to investigate sperm DNA damage induced by antineoplastic therapies in patients with testicular cancer to provide data on the extent and persistence of nuclear damage that might affect individual reproductive potential.

MATERIALS AND METHODS

Patients

The study was approved by our University Hospital's institutional review board. We studied chromatin integrity and semen quality in 254 patients with testicular cancer who cryobanked spermatozoa at the Seminology Laboratory-Sperm Bank, Department of Experimental Medicine, University of Rome "La Sapienza" after orchiectomy and before beginning chemotherapy or radiotherapy.

This longitudinal study evaluated patients pre- and post treatment at the following times: T0 (about 1 month after orchiectomy and before beginning cancer treatment) and 3 (T3), 6 (T6), 9 (T9), 12 (T12) and 24 (T24) months after the end of treatment. Pre-treatment DNA fragmentation index (DFI) could not be assessed for some patients, as the volume of their sperm sample was sufficient for cryobanking only. Specifically, of these 254 patients, 139 patients were evaluated pre-treatment, with 82 of these also providing samples at various times post treatment, and 115 patients were assessed post treatment only. Post-treatment recruitment of patients was based on the recovery of spermatogenesis, as an adequate number of spermatozoa is essential for analysis of chromatin integrity.

Cancer treatment

A total of 141 patients (38 seminoma and 103 non-seminoma) underwent chemotherapy. Of these, 110 were treated with BEP (bleomycin, etoposide and cisplatin). The chemotherapy dose and administration regimen were as follows: days 1, 2, 3, 4 and 5: cisplatin 20 mg/m^2 i.v. and etoposide 100 mg/m^2 i.v.; days 2, 9 and 16: bleomycin 18 mg/m^2 i.v. every 3 weeks. From one to four BEP cycles were administered: five patients underwent one cycle, 46 two cycles, 44 three cycles and 15 four cycles. Twenty-three of the remaining patients underwent one/two carboplatin cycles and eight patients underwent one to five cycles of other chemotherapy regimens: cisplatin or bleomycin and polychemotherapy (cisplatin, vepesid, bleomycin).

One hundred and nine seminoma patients and four non-seminoma patients were treated with radiotherapy of the lumbar–aortic lymph nodes (with shielding of the remaining testicle). The protocol involved a mean dose of 2600 cGy (range 1800–4320 cGy).

A longitudinal study was carried out the short- and long-term effects of chemo- and radiotherapy on chromatin integrity and semen parameters of all testicular cancer patients. We studied 139 patients at T0, 59 patients at T3, 54 at T6, 60 at T9, 75 at T12 and 75 at T24. We then separately evaluated the effects of chemotherapy or radiotherapy on chromatin integrity.

Semen analysis

Semen samples were collected by masturbation directly into a sterile plastic container after 2–7 days of sexual abstinence. They were examined by light microscope according to World Health Organization criteria (World Health Organization, 2010). The following variables were taken into consideration: total sperm count ($n\times10^6$), total motility (%) and morphology (% abnormal forms). One aliquot of each of the raw semen samples was treated for evaluation of sperm chromatin structure. The aliquots were washed twice with NaCl 0.9% solution and the pellets resuspended in TNE buffer containing 10% glycerol to a final sperm concentration of 2×10^6/mL and transferred to Eppendorf snap-cap tubes. The tubes were stored at −80°C until flow cytometry (FCM) analysis.

Assessment of DNA integrity

Sperm DNA integrity was evaluated by sperm chromatin structure assay (SCSA), strictly following the procedure described in the literature (Spanò *et al.*, 2000; Evenson *et al.*, 2002). On the day of analysis, samples were quickly thawed in a 37 °C water bath and used immediately. A total of 1–2 $\times$ 10^6 cells were treated with a pH 1.2 detergent solution containing 0.1% Triton X-100, 0.15 mol/L NaCl and 0.08 mol/L HCl for 30 sec and then stained with 6 mg/L of purified acridine orange (AO; Molecular Probes, Eugene, OR, USA) in a pH 6.0 phosphate-citrate buffer. Cells were analysed by a FACS Vantage flow cytometer (Becton Dickinson, San José, CA, USA), equipped with an air-cooled argon ion laser. A total of 10 000 events were accumulated for each measurement. Under these experimental conditions, when excited with a 488-nm light source, AO intercalated with double-stranded DNA emits green fluorescence and AO associated with single-stranded DNA emits red fluorescence. Sperm chromatin damage can thus be quantified by FCM measurement of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red (fragmented DNA) versus green (DNA stainability) fluorescence intensity cytogram patterns.

Adopting the guidelines described by Evenson *et al.* (2002) and using the dedicated SCSASoft software (SCSA Diagnostics, Volga, SD, USA) for offline data analysis, we expressed the extent of DNA denaturation in terms of DFI, the ratio of red to total (red plus green) fluorescence intensity, that is the level of denatured DNA over total DNA. The DFI value was calculated for each sperm cell in a sample, and the percentage of spermatozoa

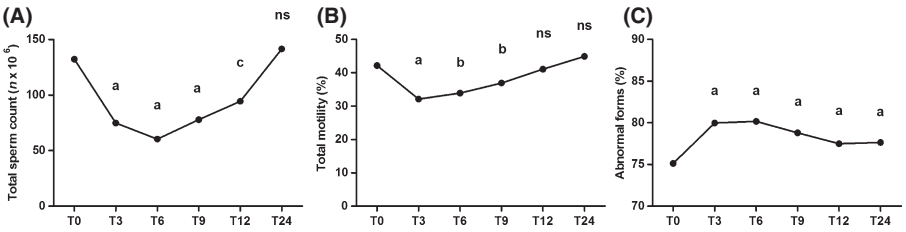

Figure 1 Variation in mean semen parameters of all testicular cancer patients after chemotherapy and radiotherapy over time (in months). a: $p < 0.001$ in comparison to T0; b: $p < 0.01$ in comparison to T0; c: $p < 0.05$ in comparison to T0; ns: not significant.

with detectable DFI values was evaluated from the resulting DFI frequency distribution histogram. The statistical DFI threshold for infertility is established in the Georgetown Male Factor Infertility Study (Evenson *et al.*, 1999; Zinaman *et al.*, 2000).

We also considered the fraction of highly DNA-stainable (HDS) cells, representing the percentage of immature spermatozoa. These events exhibit a green fluorescence intensity higher than the upper border of the main cluster of the sperm population with non-detectable DFI. The percentage of these parameters was calculated by setting an appropriate gate on the scattergram (abscissa: red fluorescence, fragmented DNA; ordinate: green fluorescence, native DNA stainability). Samples were measured twice and the results reported refer to the mean value of the two FCM measurements.

Statistical analysis

All quantitative results are expressed as means and SD. The Kolmogorov–Smirnov test was used to evaluate the normal distribution of all variables. We aimed to assess whether pre- and post-treatment semen analysis and DFI values were statistically different. As some semen parameters and SCSA variables were not normally distributed, we used also non-parametric tests for paired o unpaired data to evaluate the differences between two mean values. The different DFI values at the baseline (T0) and post therapy were categorized by age, histotype and stage.

Patients were grouped by age into ≤30 and >30 years, by histotype into seminoma and non-seminoma and by stage into pathological stage (pT1 and pT2) and clinical stage (Stage I and Stage II). The results at T3 and T6 were combined (T3 + T6) and compared against T0. To compare the results for DFI parameters in the groups pT1 and pT2 and stage I and stage II, we subsequently calculated the relative efficacy for DFI parameter X as $(X_t - X_0)/X_0$, where X_0 is the pre-therapy value and X_t is the value of the DFI parameter at time T3 + T6. Spearman correlations were calculated between the DFI and sperm parameters (total sperm number, total motility, absolute value of motile spermatozoa/ejaculate and abnormal forms). A two-tailed p-value below 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Multivariate analyses (ANOVA, logistic regressions) were performed between DFI at T3 + T6 and the independent variables included in the model (treatment, histotype, pathological and clinical stage and DFI at T0).

RESULTS

Patients

We evaluated 254 testicular cancer patients aged 14–49 years (mean ± SD = 29.9 ± 6.2), of whom 147 (57.9%) had seminoma and 107 (42.1%) had non-seminoma. Patients were also classified on the basis of pathological and clinical stage (TNM classification). Testicular descent into the scrotum at the time of birth was found to be normal in 236 patients (92.9%), while 18 (7.0%) had a history of cryptorchidism [17 unilateral (10 on the left and 7 on the right) and one bilateral]. Of these, 12 had seminoma and six had non-seminoma. The tumour was in the left testis in 121 (47.6%) patients and in the right testis in 133 (52.4%).

Semen analysis

There was a significant decrease in total sperm number in the population as a whole from T0 ($132.3 \pm 116.8 \times 10^6$/ejaculate) to T12 ($94.5 \pm 80.7 \times 10^6$/ejaculate) ($p = 0.027$). Differences between sperm numbers at T0 ($132.3 \pm 116.8 \times 10^6$/ejaculate) and T24 ($141.5 \pm 98.5 \times 10^6$/ejaculate) were not statistically significant, indicating that sperm quality had returned to pre-treatment values. Total motility at T0 was 42.2 ± 13.4%; this showed a significant decrease at T3 (32.1 ± 17.8%, $p = 0.0003$), T6 (33.9 ± 18.0%, $p = 0.007$) and T9 (36.9 ± 13.0%, $p = 0.004$) but had returned to near pre-treatment values by T12 (41.1 ± 13.1%) ($p = 0.502$) and showed a non-significant increase by T24 (44.9 ± 10.8) ($p = 0.201$). In contrast, abnormal forms increased significantly from T0 (75.1 ± 6.7%) to T24 (77.6 ± 5.5%) ($p < 0.0001$), with the most marked increase at T3 (80.0 ± 8.7%) and T6 (80.2 ± 8.1%) (Fig. 1).

DNA integrity

Sperm chromatin structure assay analysis was used to detect sperm chromatin damage, that is increased susceptibility to partial DNA denaturation induced by a weak acid treatment. DNA chromatin integrity is expressed by the parameters DFI and HDS. The mean DFI for all patients at T0 was 18.0 ± 12.5%. This increased significantly at T3 (27.7 ± 17.4%) ($p < 0.0001$) and T6 (23.2 ± 15.3%) ($p = 0.011$), returning to the baseline value by T9 (17.3% ± 8.2) ($p = 0.570$). There was a further significant decrease at T12 and T24 (14.0 ± 8.9% and 14.4 ± 10.3% respectively) ($p = 0.009$; $p = 0.016$) (Fig. 2A). The mean percentage of HDS cells was 17.4 ± 9.5% for all patients at T0, dropping significantly at T9 (14.5 ± 9.0%) ($p = 0.007$) and continuing to drop significantly at T12 and T24 (11.9 ± 5.8%; 8.0 ± 3.0%) ($p < 0.0001$), indicating an increased fraction of spermatozoa with higher chromatin condensation as a function of time after therapy.

To confirm these results, we selected 95 patients from the original caseload of 254 for whom both baseline and one or more post-treatment (T3, 6, 9, 12, 24) measurements were available. Statistical analysis against T0 (18.2%) revealed that DFI rose at T3 (29.6%, $p < 0.0001$) and T6 (25.1%, $p = 0.013$), returned to the baseline at T9 (17.9%, $p = 0.311$) and dropped significantly at T12 (14.7%, $p = 0.034$) and non-significantly at T24 (15.8%, $p = 0.588$). These results were similar to those for the caseload

Figure 2 Variation in DFI values pre- and post therapy over time (in months): (A) all testicular cancer patients (254), (B) follow-up of 95 testicular cancer patients. a: $p < 0.001$ in comparison to T0; b: $p < 0.01$ in comparison to T0; c: $p < 0.05$ in comparison to T0; ns: not significant.

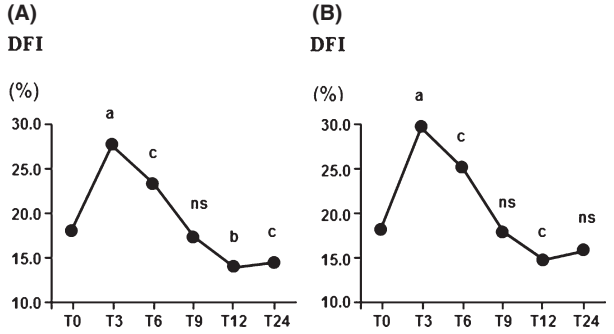

Table 1 Effect of treatment on sperm chromatin integrity by age group. Comparison of two age groups (≤30 and >30 years) pre- (T0) and post therapy (T3 + T6)

	DFI (%)		*p*
	≤30 years	>30 years	
T0[a]	18.5 (12.7) (*n* = 87) [b]	17.1 (12.3) (*n* = 52) [b]	0.44[c]
T3 + T6[a]	26.4 (17.4) (*n* = 69) [b]	24.1 (15.0) (*n* = 44) [b]	0.68[c]
p value	0.001[c]	0.003[c]	

[a]Mean (standard deviation). [b]Number of patients. [c]Mann–Whitney test.

as a whole, and in fact there was no statistically significant difference in DFI between the 95- and 254-patient caseload at any time point ($p > 0.05$) (Fig. 2B).

As we found greater treatment-induced chromatin damage at T3 and T6, to evaluate the effect of age, histotype and pathological and clinical stage on chromatin integrity we compared the DFI of the 139 patients at T0 against the 113 T3 + T6 patients, to carry out a robust, numerically significant analysis.

Age

Patients were divided into subgroups of ≤30 and >30 years. No statistically significant differences in DFI were seen between the two subgroups pre- and post therapy (Table 1).

Histotype

At T0 there were 81 seminoma and 58 non-seminoma patients; no significant differences in DFI were seen between histotypes ($p = 0.236$). Comparison of post-treatment values revealed high DFI in both histotypes (seminoma 25.7% vs. non-seminoma 25.3%); the difference was not significant ($p = 0.973$).

Pathological stage

At T0 there were 85 pT1 and 46 pT2 patients, with no significant differences in DFI between the two stages ($p = 0.222$). Comparison of the baseline and post-treatment (T3 + T6) values revealed a statistically significant post-treatment increase in DFI for both stages (pT1 18.3% vs. 23.7%, $p = 0.003$, pT2 17.2% vs. 28.4%, $p = 0.001$). The magnitude of the increase in DFI between the two stages was assessed by calculating the relative difference (RD), revealing a larger increase in pT2 than in pT1 (65% vs. 30%).

Clinical stage

At T0 there were 100 stage I and 31 stage II patients, with no significant differences in DFI between the two stages ($p = 0.301$). Comparison of the baseline and post-treatment (T3 + T6) values revealed a statistically significant post-treatment increase in DFI for stage I (17.2% vs. 25.1%, $p < 0.0001$) and a non-significant increase for stage II (19.0% vs 26.8%, $p = 0.084$). The RD revealed a similar increase in both stages (45.9% vs. 41.1%).

We thus found that post-treatment DNA damage is not affected by age or histotype, but is dependent on the stage and is more marked in a more advanced pathological stage. These results suggest that even if pre-treatment DFI is similar for both stages, a more advanced pathological stage makes patients more susceptible to treatment-induced damage. We also carried out ANOVA and logistic regressions, which showed no statistical correlation between DFI at T3 + T6 and the independent variables included in the model (treatment, pT stage, clinical stage and DFI at T0). This could be because of the low numbers of subjects in some groups.

Finally, we assessed the correlation between DNA damage and semen parameters using Spearman's correlation on the 139 T0 patients and 113 (T3 + T6) post-treatment patients. We found a negative correlation between DFI and total sperm count ($r = -0.21$, $p = 0.012$) and DFI and total motility ($r = -0.35$, $p < 0.0001$) and a positive correlation between DFI and abnormal forms ($r = 0.18$, $p = 0.029$) at T0, maintained post treatment. In addition to raw data on percentage motility, we also considered absolute values in terms of millions of motile spermatozoa per ejaculate (obtained by multiplying the total spermatozoa per ejaculate by the percentage of sperm motility). This revealed a negative correlation between the percentage of mobile spermatozoa and DFI ($r = -0.27$, $p = 0.001$).

We also examined the impact of the different treatments by dividing the caseload into two treatment subgroups: chemotherapy (CH) group, consisting of 141 patients who had undergone chemotherapy, and radiotherapy (RT) group, consisting of 113 patients who had undergone radiotherapy of the lumbar–aortic lymph nodes. More specifically, the CH group consisted of 74 patients at T0, 22 at T3, 25 at T6, 30 at T9, 39 at T12 and 43 at T24, whereas the RT group consisted of 65 patients at T0, 37 at T3, 29 at T6, 30 at T9, 36 at T12 and 32 at T24.

Chemotherapy group

The mean DFI value for this group at T0 was 18.9 ± 13.5%. This underwent a significant increase at T3 (25.5 ± 16.7%, $p = 0.028$), while at T6 (23.9 ± 17.3%) the increase against the baseline was not statistically significant. There was a return to the baseline value at T9 (18.9 ± 9.2%, $p = 0.374$) and a significant decrease at T12 and T24 (11.7 ± 7.3%, $p = 0.002$ and 13.4 ± 10.4%, $p = 0.007$ respectively) (Fig. 3A). The HDS cell value for this group at T0 was 16.4 ± 8.9%. This underwent a significant decrease at T12 (12.6 ± 5.5%, $p = 0.038$) and T24 (7.7 ± 2.9%, $p < 0.0001$), indicating improved chromatin condensation with increasing time since the end of treatment. The increase in DNA damage from T0 to T3 + T6 as a function of treatment intensity is shown in Table 2. Comparison of patients undergoing 1–2 and 3–4 PEB cycles revealed an increase in post-treatment DFI at higher treatment doses, although this was not

Figure 3 Variation in total DFI values over time (in months): (A) after chemotherapy, (B) after radiotherapy. a: $p < 0.001$ in comparison to T0; b: $p < 0.01$ in comparison to T0; c: $p < 0.05$ in comparison to T0; ns: not significant.

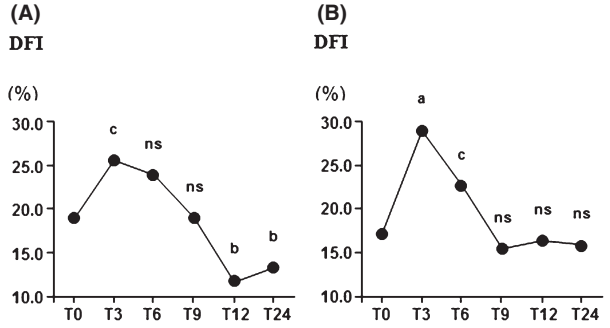

Table 2 Sperm DNA damage increase from T0 to T3 + T6 and treatment intensity

Chemotherapy	DFI (%)		RD	*p* value
	T0 (pts)	T3 + T6 (pts)		
1–2 BEP cycles	19.9 (29)	28.4 (19)	42.7	0.0748
3–4 BEP cycles	16.6 (29)	32.5 (14)	95.8	0.0011
p value	0.7855	0.3920		

DFI, DNA fragmentation index; RD, relative difference.

statistically significant. The RD revealed a larger increase for 3–4 PEB cycles than for 1–2 PEB cycles (95.8% vs. 42.7%).

Radiotherapy group

The mean DFI value for this group at T0 was 17.1 ± 11.3%. There was a significant increase at T3 (28.9 ± 18.0%, $p = 0.0003$) and T6 (22.7 ± 13.8%, $p = 0.036$). There was a non-significant decrease from the baseline at T9 (15.5 ± 6.6%, $p = 0.876$), T12 (16.4 ± 9.9%, $p = 0.658$) and T24 (16.0 ± 10.1%, $p = 0.602$) (Fig. 3B). The HDS cell value for this group at T0 was 18.5 ± 10.1%. This dropped significantly at T9 (12.4 ± 8.5%, $p = 0.0006$) and continued to drop significantly at T12 and T24 (11.1 ± 6.0%, $p < 0.0001$; 8.6 ± 3.0%, $p < 0.0001$). Again, this indicated improved chromatin condensation with increasing time since the end of treatment.

As most patients undergoing radiotherapy received a dose of around 2550 cGy, the effect of radiotherapy intensity could not be evaluated.

DISCUSSION

Fertilization and embryonic development are biological events which depend on numerous factors, including sperm quality. An excessive number of sperm DNA strand breakages has particularly negative consequences for the reproductive process. Such chromatin abnormalities may be the outcome of apoptotic processes, oxidative stress or faulty protaminization. Antineoplastic therapies are an important cause of sperm DNA damage. Interest in the toxic effects of chemotherapeutic agents on embryonic development has generally focussed on the mother, while the paternal aspect has often been underrated. Few studies have investigated male-mediated teratogenicity (Brinkworth, 2000), but above all the little available data provide conflicting information on sperm chromatin damage induced by these treatments. Radiotherapy and chemotherapy can in fact impair reproductive function through both cytological and molecular effects including impaired spermatogenesis, resulting in oligozoospermia and azoospermia (Trost & Brannigan, 2012), and increased aneuploidy for up to 18–24 months after treatment (Martin *et al.*, 1999; De Mas *et al.*, 2001; Tempest *et al.*, 2008). While the main aim of cancer treatment must of course be to cure the cancer itself, the future quality of life of such patients must not be neglected, given the increased survival rates permitted by technological advances.

With testicular cancer, which often affects young men, reproductive problems that might arise after treatment are an important issue. Sperm chromatin impairment is of particular interest as it may be an infertility factor and could even be associated with genomic instability, with negative consequences for any offspring. Information on such factors is thus of considerable translational value in enabling the adequate counselling of patients as to their future reproductive potential.

Literature evidence in this area is contradictory, owing to the different methods used to study sperm DNA damage, the different diseases and treatments under investigation and, above all, the relatively small caseloads. A prospective longitudinal study carried out by Ståhl *et al.* (2004) used SCSA to evaluate the persistence of any sperm DNA impairment following antineoplastic treatment in 74 testicular cancer patients and 278 controls. Semen examinations were carried out after orchiectomy and before chemo- or radiotherapy and 6, 12, 24, 36 and 60 months after the end of treatment. Patients were divided into subgroups on the basis of their treatment: surgery and monitoring, chemotherapy, chemotherapy plus radiotherapy and radiotherapy alone. The authors found a significant but transient increase in DFI in the first 2 years after radiotherapy, which normalized after 3–5 years, and a reduction in DFI up to 5 years post chemotherapy. This curious result was explained by the considerable vulnerability of germ cells to chemotherapy, which might cause the elimination of sperm cells with DNA damage.

Another prospective study by O'Donovan (2005) evaluated chromatin integrity pre- and post therapy in various cancers including testicular cancer, lymphoma (Hodgkin and non-Hodgkin) and leukaemia. This study involved a semen examination, COMET assay and a chromatin condensation assay by cytofluorometry using propidium iodide. Semen samples were examined before therapy and 3, 6 and 12 months after the end of treatment with various antineoplastic agents. There was a significant reduction in DNA integrity and a non-significant reduction in chromatin condensation after treatment; however, the study involved a very small caseload of 14 fertile men and 33 cancer patients, of whom just 13 had testicular cancer.

Another study by Ståhl *et al.* (2009) evaluated patients with various neoplastic diseases to establish if DNA integrity was affected by the type of treatment, carrying out a semen examination and SCSA on the semen samples of 58 testicular cancer patients and 137 controls. Patients were divided into subgroups on the basis of the treatment (mean time 3 years, low- and high-dose chemotherapy, combined chemotherapy/radiotherapy and radiotherapy alone). The authors found no significant differences in pre- and post-treatment DFI, demonstrating that DNA integrity was not affected by the treatment.

However, O'Flaherty *et al.* (2010) in a prospective study obtained different results. These authors studied 16 patients

with testicular cancer after orchiectomy who underwent BEP and 16 patients with Hodgkin's disease who underwent ABVD. The controls consisted of 11 healthy male volunteers. Sperm DNA integrity was evaluated using COMET assay at various times after the end of chemotherapy: 0, 6, 12, 18 and 24 months. This longitudinal study found that chemotherapy had a negative impact on both Hodgkin's disease and testicular cancer patients, with increased sperm DNA fragmentation 6 months after the end of treatment in comparison with the baseline; this value remained elevated up to 18–24 months.

Smit *et al.* (2010) evaluated DNA integrity in patients with various cancers, including 52 testicular cancer patients before treatment and 25 after a follow-up of 0.5–3.3 years after the end of treatment. Patients were divided into subgroups on the basis of their treatment: BEP, BEP combined with radiotherapy, or radiotherapy alone. This study found a drop in post-therapy DFI when considering all testicular cancer patients, but a significant increase after radiotherapy in comparison with chemotherapy alone.

A different study (Romerius *et al.*, 2010) evaluated sperm DNA integrity in 99 childhood cancer survivors with different oncological diagnosis who received different types of treatment. The median age (range) at diagnosis was 10 years (0.1–17 years) and at the time of examination 30 years (20–46 years). These authors found that childhood cancer per se is associated with increased sperm DNA damage. In fact, the increased DFI in the group that received neither radiotherapy nor chemotherapy might imply that childhood cancer patients had some kind of genomic instability. The study also found that DFI might be additionally increased by radiotherapy and reduced by cytotoxic treatment.

A more recent study by O'Flaherty *et al.* (2012) evaluated chromatin packaging defects in cancer survivors after chemotherapy. Various methods were used to evaluate chromatin integrity in addition to SCSA. All tests were carried out at T0 and 6, 12, 18 and 24 months after the end of treatment. The study involved just 16 patients with testicular cancer after orchiectomy, 15 patients with Hodgkin's disease, 11 infertile controls and 11 fertile controls. In the testicular cancer patients, high levels of DNA damage were found up to 24 months post therapy, with low pretherapy chromatin compaction in comparison with fertile controls. These data suggest that the chromatin structure is less resistant because of reduced DNA compaction.

A recent multicentre prospective study by Bujan *et al.* (2013) evaluated semen parameters of 129 testicular cancer patients before and after antineoplastic treatment, of whom 53 patients underwent sperm DNA integrity testing by SCSA and TUNEL at the baseline and 3, 6, 12 and 24 months after the end of chemo- or radiotherapy. The authors found only HDS to be increased at T6 in radiotherapy patients, indicating impaired chromatin condensation shortly after the end of the treatment.

In our longitudinal study, we evaluated sperm DNA integrity and spermatogenesis in a large caseload of testicular cancer patients pre- and post treatment. We found a post-treatment reduction in total sperm number from the baseline up to T12 and in motility up to T9; these parameters then improved progressively, returning to near-baseline values by T24. The percentage of abnormal forms increased more strongly at T3, T6 and T9, dropping at T12 and T24, even if there was still a significant difference with respect to T0. These data are in agreement with a previous study we conducted on the effects of chemo- and radiotherapy on spermatogenesis (Gandini *et al.*, 2006), in which we demonstrated that spermatogenesis recovery is a function of the time since the end of the therapy, with 94% of chemotherapy patients and 93% of radiotherapy patients showing good recovery after 12 months and 97 and 94%, respectively, after 24 months. We also showed that the recovery of spermatogenesis after chemo- or radiotherapy was not a function of pre-therapy sperm parameters. Given the recovery of spermatogenesis 12–24 months after the treatment end, we considered the possibility of a time-dependent difference in sperm chromatin integrity between pre- and post-chemotherapy/radiotherapy. No significant difference in pre-treatment (T0) sperm DFI in cancer patients was found between the two histotypes (seminoma and non- seminoma), between the two pathological stages (pT1 and pT2) or between the two clinical stages (I and II). Analysis at T3, T6, T9, T12, T24 demonstrated that the sperm chromatin profile is markedly impaired 3 and 6 months after the treatment end, returning to the baseline at T9 and improving considerably at T12 and T24. This is supported by the results of the HDS analysis used to assess sperm DNA compaction indirectly. HDS levels at T12 and T24 are significantly lower than at T0, indicating increased chromatin condensation with increasing time since the end of the treatment.

As our data demonstrate that sperm DNA damage is greatest at T3 + T6, we also investigated if the treatment effects on sperm chromatin integrity were influenced by age, histotype or stage, finding that post-treatment DNA damage is not age or histotype dependent but is a function of pathological and clinical stage and is more marked in a more advanced pathological stage. Although this needs to be confirmed in larger caseloads, it is a particularly interesting finding, as it suggests that spermatozoa from patients with greater tumour-induced impairment of the testicular structure are more vulnerable to damage caused by antineoplastic treatments. We also examined the impact of the different treatments on sperm chromatin integrity by dividing the caseload into two treatment subgroups: chemotherapy and radiotherapy. After chemotherapy, we found a marked increase in DFI at T3 and T6 and a significant reduction at T12 and T24 in comparison with the baseline, thus revealing a clear improvement in the chromatin profile at the later times. In contrast, after radiotherapy DFI increased at T3 and T6 but the reduction was far less marked, reaching the baseline values at T12 and T24. These results are in line with previous literature studies, suggesting that radiotherapy induces greater damage because of dispersion of radiation during the treatment. Finally, we investigated the correlation between DNA damage and pre- and post-treatment semen parameters, finding a negative correlation between DFI and total sperm number, total motility and number of motile spermatozoa and a positive correlation between DFI and abnormal forms. These data demonstrate for the first time that sperm chromatin damage is more extensive when there is greater quantitative and qualitative pre- and post-treatment impairment of spermatogenesis. We also demonstrated that the chromatin profile may be affected in the months immediately following the end of the treatment, improving after 12–24 months.

It should be stressed that our results refer only to testicular cancer and only to the antineoplastic treatments specific for this disease. Different treatments can in fact cause different types of nuclear damage and the methods currently in use for the study

of chromatin profile provide only an incomplete picture of such damage, especially as these treatments can cause long-term changes in gene expression profile and methylation pattern, with a possible impact on embryonic development.

ACKNOWLEDGEMENTS

The authors wish to thank Marie-Hélène Hayles for her assistance in the English translation of the manuscript, Prof. Franco Culasso for his assistance with the statistical analysis and Dr. Patrizia Eleuteri (ENEA) for her technical support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FINANCIAL SUPPORT

This work was supported by a grant from the Italian Ministry of Education and Research (MIUR-PRIN) and the University of Rome "La Sapienza" Faculty of Medicine.

AUTHOR CONTRIBUTIONS

D.P. and L.G. conceived and designed the experiments; D.P. and L.G. drafted the article; L.G. approved the submitted and final versions; M.G., F.R. and G.L. acquired and analysed the data; D.P., L.G., F.L., A.L. and M.S. revised the manuscript critically.

REFERENCES

Bieber AM, Marcon L, Hales BF & Robaire B. (2006) Effects of chemotherapeutic agents for testicular cancer on the male rat reproductive system, spermatozoa, and fertility. *J Androl* 27, 189–200.

Brinkworth MH. (2000) Paternal transmission of genetic damage: findings in animals and humans. *Int J Androl* 23, 123–135.

Bujan L, Walschaerts M, Moinard N, Hennebicq S, Saias J, Brugnon F, Auger J, Berthaut I, Szerman E, Daudin M & Rives N. (2013) Impact of chemotherapy and radiotherapy for testicular germ cell tumors on spermatogenesis and sperm DNA: a multicenter prospective study from the CECOS network. *Fertil Steril* 100, 673–680.

Chia VM, Quraishi SM, Devesa SS, Purdue MP, Cook MB & McGlynn KA. (2010) International trends in the incidence of testicular cancer, 1973-2002. *Cancer Epidemiol Biomarkers Prev* 19, 1151–1159.

Crocetti E; AIRTUM Working Group. (2009) Numbers. Testis cancer is the most frequent neoplasia among young men. *Epidemiol Prev* 33, 145.

De Mas MP, Daudin M, Vincent MC, Bourrouillou G, Calvas P, Mieusset R & Bujan L. (2001) Increased aneuploidy in spermatozoa from testicular tumour patients after chemotherapy with cisplatin, etoposide and bleomycin. *Hum Reprod* 16, 1204–1208.

Delbès G, Chan D, Pakarinen P, Trasler JM, Hales BF & Robaire B. (2009) Impact of the chemotherapy cocktail used to treat testicular cancer on the gene expression profile of germ cells from male Brown-Norway rats. *Biol Reprod* 80, 320–327.

Dohle GR. (2010) Male infertility in cancer patients: review of the literature. *Int J Urol* 17, 327–331.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P & Claussen OP. (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod* 14, 1039–1049.

Evenson DP, Larson KL & Jost LK. (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparison with other techniques. *J Androl* 23, 25–43.

Gandini L, Sgrò P, Lombardo F, Paoli D, Culasso F, Toselli L, Tsamatropoulos P & Lenzi A. (2006) Effect of chemo- or radiotherapy on sperm parameters of testicular cancer patients. *Hum Reprod* 21, 2882–2889.

Huyghe E, Matsuda T & Thonneau P. (2003) Increasing incidence of testicular cancer worldwide: a review. *J Urol* 170, 5–11.

Kopp HG, Kuczyk M, Classen J, Stenzl A, Kanz L, Mayer F, Bamberg M & Hartmann JT. (2006) Advances in the treatment of testicular cancer. *Drugs* 66, 641–659.

Martin RH, Ernst S, Rademaker A, Barclay L, Ko E & Summers N. (1999) Analysis of sperm chromosome complements before, during, and after chemotherapy. *Cancer Genet Cytogenet* 108, 133–136.

O'Donovan M. (2005) An evaluation of chromatin condensation and DNA integrity in the spermatozoa of men with cancer before and after therapy. *Andrologia* 37, 83–90.

O'Flaherty C, Hales BF, Chan P & Robaire B. (2010) Impact of chemotherapeutics and advanced testicular cancer or Hodgkin lymphoma on sperm deoxyribonucleic acid integrity. *Fertil Steril* 94, 1374–1379.

O'Flaherty CM, Chan PT, Hales BF & Robaire B. (2012) Sperm chromatin structure components are differentially repaired in cancer survivors. *J Androl* 33, 629–636.

Purdue MP, Devesa SS, Sigurdson AJ & McGlynn KA. (2005) International patterns and trends in testis cancer incidence. *Int J Cancer* 115, 822–827.

Romerius P, Ståhl O, Moëll C, Relander T, Cavallin-Ståhl E, Gustafsson H, Löfvander Thapper K, Jepson K, Spanò M, Wiebe T, Lundberg Giwercman Y & Giwercman A. (2010) Sperm DNA integrity in men treated for childhood cancer. *Clin Cancer Res* 16, 3843–3850.

Rosen A, Jayram G, Drazer M & Eggener SE. (2011) Global trends in testicular cancer incidence and mortality. *Eur Urol* 60, 374–379.

Signorello LB, Mulvihill JJ, Green DM, Munro HM, Stovall M, Weathers RE, Mertens AC, Whitton JA, Robison LL & Boice JD Jr. (2012) Congenital anomalies in the children of cancer survivors: a report from the childhood cancer survivor study. *J Clin Oncol* 30, 239–245.

Smit M, van Casteren NJ, Wildhagen MF, Romijn JC & Dohle GR. (2010) Sperm DNA integrity in cancer patients before and after cytotoxic treatment. *Hum Reprod* 25, 1877–1883.

Spanò M, Bonde JP, Hjøllund HI, Kolstad HA, Cordelli E & Leter G. (2000) Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. *Fertil Steril* 73, 43–50.

Ståhl O, Eberhard J, Jepson K, Spano M, Cwikiel M, Cavallin-Ståhl E & Giwercman A. (2004) The impact of testicular carcinoma and its treatment on sperm DNA integrity. *Cancer* 100, 1137–1144.

Ståhl O, Eberhard J, Cavallin-Ståhl E, Jepson K, Friberg B, Tingsmark C, Spanò M & Giwercman A. (2009) Sperm DNA integrity in cancer patients: the effect of disease and treatment. *Int J Androl* 32, 695–703.

Ståhl O, Boyd HA, Giwercman A, Lindholm M, Jensen A, Kjær SK, Spanò M & Giwercman A. (2011) Risk of birth abnormalities in the offspring of men with a history of cancer: a cohort study using Danish and Swedish national registries. *J Natl Cancer Inst* 103, 398–406.

Tempest HG, Ko E, Chan P, Robaire B, Rademaker A & Martin RH. (2008) Sperm aneuploidy frequencies analysed before and after chemotherapy in testicular cancer and Hodgkin's lymphoma patients. *Hum Reprod* 23, 251–258.

Trasler JM, Hales BF & Robaire B. (1986) Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny. *Biol Reprod* 34, 275–283.

Trost LW & Brannigan RE. (2012) Oncofertility and the male cancer patient. *Curr Treat Options Oncol* 13, 146–160.

World Health Organization. (2010) *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. WHO Press, Geneva, Switzerland.

Zinaman MJ, Brown CC, Selevan SG & Clegg ED. (2000) Semen quality and human fertility: a prospective study with healthy couples. *J Androl* 21, 145–153.

