Testicular and sperm DNA damage after treatment with fludarabine for chronic lymphocytic leukaemia

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This study investigated whether chemotherapy using fludarabine (FLU) caused testicular damage and if cytotoxicity could be detected as sperm DNA damage in the single cell Comet assay. A patient with chronic lymphocytic leukaemia requesting preservation of fertility was treated with seven monthly cycles of fludarabine (45.8 mg total dose per cycle). Testicular assessments, serum follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone measurements, semen analysis and sperm Comet assays were carried out at presentation (pre-FLU therapy), after 1 and 7 months of FLU treatment, and finally at 11 months after completion of chemotherapy. We found that testicular damage occurred within a month, as indicated by reduced testicular volume, oligozoospermia, elevated FSH and LH, and lower testosterone concentrations. Spermatozoa with a large range of DNA damage were detected in the samples from both the control and treated men. DNA damage in the spermatozoa was marked by 7 months of FLU treatment. The high levels of sperm DNA damage seen during and possibly persisting after treatment suggests that caution should be exercised if the ejaculates from these men are used for in-vitro fertility treatment. Further experiments are needed to assess the biological significance of these DNA changes; it may, however, be prudent at present to be cautious when counselling these patients.

Key words: chemotherapy/Comet assay/DNA damage/fertility/ spermatозoa

Introduction

The adverse effects of conventional and high-dose chemotherapy on testicular function is well established (Chatterjee and Goldstone, 1996; Meistrich, 1998; Howell et al., 1999), but little is known about fludarabine (FLU; Schering Health Care Ltd, Burgess Hill, West Sussex, UK), a purine analogue which is commonly used in haematological malignancies (Adkins et al., 1997; Byrd et al., 1998). Cytotoxic substances used for malignancies lack tissue specificity, and spermatogenesis is often impaired, so preservation of fertility is an important issue, especially for young adults. Each year several thousand children and young persons of the reproductive age group are exposed to cancer chemotherapy and as more of these treatments are effective, there is a growing concern about the long-term consequences of testicular damage on the survivors.

The recent availability of micromanipulation techniques using a single spermatozoon [intracytoplasmic sperm injection (ICSI)] has improved the prospects of child-bearing in patients with severe oligo-asthenozoospermia (Pisarska et al., 1999). Sperm selection techniques do not allow assessment of the genetic integrity of the spermatozoon and it is clear that mutations in the paternal genome can be passed on to the children (Martin, 1996; Robbins et al., 1997). Current practice to preserve fertility for patients undergoing genotoxic chemotherapy is to cryopreserve spermatozoa with a view to using them later for assisted reproduction treatment (Tournaye et al., 1993; Lass et al., 1999; Pfeifer and Coutifaris, 1999).

Although it is well known that chemotherapy can adversely affect sperm production, the genetic consequences arising after fertilization with sperm from a treated man are less clear. Aneuploidy of the X and Y chromosomes has been reported in the children of men treated for cancer (Robbins et al., 1997; Monteil et al., 1997). The integrity of the DNA in the male genome carried by the spermatozoon is crucial for genetic health, and changes can lead to alterations in the germ line which result in the inheritance of lethal and non-lethal conditions (Perreault, 1998). A major limitation of the current assessment of male fertility is that the genetic integrity of the spermatozoon cannot be easily determined. This is particularly important in view of the known effects of chemotherapy in animal models and the view that normal human ejaculates carry a significant proportion of damaged spermatozoa (Sakkas et al., 1999).

We have taken the opportunity to see if DNA damage in spermatozoa can be detected in the semen of a patient treated with a chemotherapeutic regime effective for chronic lymphocytic leukaemia (CLL). The patient received FLU, which has not been previously reported to be gonadotoxic and would allow semen collection uncomplicated by reductions in sperm numbers. This case study allowed us to test the hypothesis that FLU causes testicular and sperm DNA damage.

Materials and methods

Subjects

A 47-year-old patient with CLL requesting preservation of fertility was studied longitudinally for 18 months that included a course of seven monthly treatment cycles of FLU chemotherapy (45.8 mg per
cycle and at 11 months after cessation of treatment). The patient had received one course of chlorambucil (2 mg daily for 2 weeks), a known gonadotoxin (Clark et al., 1995), 9 months prior to the treatment with FLU, although there was no indication that this had impaired his testis (Table I). The patient also received gonadotrophin-releasing hormone (GnRH) therapy in the form of leuproline acetate (Prostap-SR; Wyeth, South Taplow, Berks, UK) 3.75 mg i.m. monthly for 6 months starting 1 month after initiation of chemotherapy during FLU therapy in an attempt to suppress testicular function. Testicular size was assessed and blood and semen samples collected throughout the study period. The four time-points of study were (i) at presentation after the start of therapy, as evident from the reductions in testicular volume, oligozoospermia, elevated FSH and LH, and diminished testosterone concentrations compared to pretreatment values and the laboratory control. No effect of the chemotherapy, however, was seen on sperm motility or the proportion of abnormal spermatozoa. During chemotherapy, recovery of testicular function took place, so that at the end of the seventh treatment cycle semen parameters, FSH, and LH approached normal values although testosterone remained low. All semen and hormonal values appeared normal 11 months after the end of treatment.

The hormonal and semen analysis results for the patient receiving the FLU treatment are given in Table I. The results suggest that FLU induces testicular damage within a month after the start of therapy, as evident from the reductions in testicular volume, oligozoospermia, elevated FSH and LH, and diminished testosterone concentrations compared to pretreatment values and the laboratory control. No effect of the chemotherapy, however, was seen on sperm motility or the proportion of abnormal spermatozoa. During chemotherapy, recovery of testicular function took place, so that at the end of the seventh treatment cycle semen parameters, FSH, and LH approached normal values although testosterone remained low. All semen and hormonal values appeared normal 11 months after the end of treatment.

**Table I. Sperm and endocrine parameters of patient before and after fludarabine treatment**

<table>
<thead>
<tr>
<th>Timea</th>
<th>Sperm parameters</th>
<th>Endocrine parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count ($\times 10^9$/ ejaculate)</td>
<td>% motility</td>
</tr>
<tr>
<td>1</td>
<td>250</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>160</td>
<td>60</td>
</tr>
<tr>
<td>Normal value</td>
<td>50</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>

T = testosterone.

*Time-points were: (i) at presentation; (ii) at end of first monthly treatment; (iii) at end of seventh monthly treatment; and (iv) at 11 months after completion of chemotherapy.

Normal value was derived from a single sample from one control subject.

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**Results**

The hormonal and semen analysis results for the patient receiving the FLU treatment are given in Table I. The results suggest that FLU induces testicular damage within a month after the start of therapy, as evident from the reductions in testicular volume, oligozoospermia, elevated FSH and LH, and diminished testosterone concentrations compared to pretreatment values and the laboratory control. No effect of the chemotherapy, however, was seen on sperm motility or the proportion of abnormal spermatozoa. During chemotherapy, recovery of testicular function took place, so that at the end of the seventh treatment cycle semen parameters, FSH, and LH approached normal values although testosterone remained low. All semen and hormonal values appeared normal 11 months after the end of treatment.

The Comet assay was used as a measure of DNA damage. Spermatozoa assayed from the treated man at the time-points (i)–(iv) gave Comet tail lengths (µm, mean ± SEM) of 54.0 ± 1.5, 50.1 ± 0.6, 67.6 ± 0.5, and 54.0 ± 1.4 respectively. The comet tail moments were 10.0 ± 1.67, 6.7 ± 0.2, 14.8 ± 0.2, and 7.5 ± 0.1 units ($n = 100–200$ spermatozoa, mean ± SEM). The values at time-point (iii) were clearly elevated. This was in contrast to values obtained for the control subject which ranged randomly with time from 54.2–65.7 µm tail length and 4.9–6.3 units tail moment. The mean ± SD Comet data from the eight 3-monthly samples was 5.64 ± 0.56 units tail moment and 58.70 ± 4.46 µm tail length. Further information on the effect of cytotoxic treatment was obtained by inspection of the distribution of DNA damage in single spermatozoa from the ejaculate (Figure 1). The Comet tail length distribution from the eight samples from the control subject was very similar to the pre-treatment pattern from the patient. The distributions from the first and last sample taken from the control man are given in Figure 2. In the control sample and the pre-treatment sample from the patient it can be seen that DNA damage was detected in all spermatozoa (a product of the migration and the percentage of DNA in this tail) were measured by image analysis in >100 sperm cells per time-point, using a fluorescence microscope and Comet Assay II software (Perceptive Instruments, Steeple Bumpstead, Haverhill, Suffolk, UK).
and that there was a wide distribution in the level of DNA damage. In control samples, there was a clear population with low amounts of DNA damage (i.e. shorter tail lengths). The frequency distribution pattern of sperm Comet lengths was monophasic and skewed to the left with a frequency peak about 45–50 µm. The number of spermatozoa with higher amounts of DNA damage was much greater after 7 months of FLU chemotherapy (Figure 1, panel iii) and the distribution was skewed to the right with a peak at 75 µm. The population of spermatozoa containing lower amounts of DNA damage had almost disappeared. Eleven months after the withdrawal of FLU and GnRH therapy, there was a marked reversal towards the control pattern.

**Discussion**

This is the first report to suggest that FLU, a widely used chemotherapeutic treatment, impairs spermatogenesis and potentially male fertility, a property shared with many other cytotoxic treatments (Chatterjee and Goldstone, 1996; Meistrich, 1998; Howell et al., 1999). However, these changes cannot be conclusively attributed to FLU, as the patient was also receiving GnRH, which produces atrophy of the testes by withdrawal of gonadotrophin support. As there was no evidence of a fall in gonadotrophin secretion in this patient, it strongly suggests that the testicular effects seen were due to FLU. More detailed studies are needed to confirm these changes.

In animal models, cytotoxic treatment of the male is associated with genetic damage in the spermatozoa, which is transferred to the offspring. These mutations vary in severity, being expressed diversely as embryo lethality, developmental abnormalities, or changes in coat colour (Perreault, 1998). In contrast, genetic effects of cytotoxic drugs or radiation upon the human male genome, which may be transmitted to the children, have not been conclusively demonstrated, and their occurrence is still controversial (Meistrich, 1993).

Surprisingly, DNA damage in spermatozoa obtained from normal human ejaculate has been detected by several methods (Sakkas et al., 1999). A major drawback of many of these methods is that spermatozoa and somatic cells in the human ejaculate are heterogeneous, and therefore single-cell analysis would be more informative. The terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) technique labels DNA breaks in spermatozoa, and most probably identifies apoptotic DNA strand breaks carried over from meiosis. The numbers of TUNEL-labelled spermatozoa are related to infertility (Sun et al., 1997).

Recently, Comet analysis of single cells has been introduced and is extensively used to measure single- and double-strand DNA breaks in somatic cells (Olive, 1999). This technique has been applied to spermatozoa, and the method described in the present study allows the detection of double-strand breaks in single spermatozoa (Haines et al., 1998b). We have confirmed the observations of others that DNA damage can be detected by Comet in spermatozoa; our data also show that spermatozoa in the normal ejaculate carry a range of DNA damage. In the patient sample we cannot discount the possibility that this may in part be due to the previous treatment with
chlorambucil (Clark et al., 1995). However, low numbers of spermatozoa containing high amounts of DNA damage were also present in a similar proportion of spermatozoa from the untreated control man. The origin of this damage is not clear and may arise during spermatogenesis or in the male reproductive tract during transport from the testis to the ejaculate (Aitken, 1999; Sakkas et al., 1999).

In the present study we have shown for the first time by direct measurement that DNA damage in spermatozoa is substantially increased by cytotoxic treatment. A particular concern is whether the DNA damage has been resolved 11 months after the end of treatment. We are not able to conclude this is longer than one spermatogenic cycle it may be a permanent effect (Morris et al., 1995). However, low numbers of spermatozoa containing high amounts of DNA damage were also present in a similar proportion of spermatozoa from the untreated control man. The origin of this damage is not clear and may arise during spermatogenesis or in the male reproductive tract during transport from the testis to the ejaculate (Aitken, 1999; Sakkas et al., 1999).

In conclusion, this case study indicates that FLU induces testicular damage within a month and markedly increases DNA damage carried by spermatozoa by 7 months of treatment. As the numbers of spermatozoa carrying high amounts of DNA damage may persist after discontinuation of treatment, the DNA damage may be permanent. This observation is worrying and the possibility of biological effects being transferred to the offspring must be considered and investigated further. Fertility counselling of patients undergoing cytotoxic chemotherapy should take into account these effects and it may be pertinent to reappraise the most appropriate timing of semen collection for fertility treatment.

References
Chatterjee, R. and Goldstone, A.H. (1996) Gonadal damage and effects on

Figure 2. The frequency distribution of DNA damage in spermatozoa measured by Comet tail length. The histograms represent the analysis of 100 spermatozoa from the ejaculate of a man with normal semen characteristics who was not receiving any prescribed medication. The sample collections were separated by 24 months.
fertility in adult patients with haematological malignancy undergoing stem cell transplantation. Bone Marrow Transplant., 17, 5–11.


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