Investigation of suppression of the hypothalamic–pituitary–gonadal axis to restore spermatogenesis in azoospermic men treated for childhood cancer

A.B. Thomson1, R.A. Anderson2, D.S. Irvine2, C.J.H. Kelnar1, R.M. Sharpe2 and W.H.B. Wallace1,3

1 Section of Child Life and Health, Department of Reproductive and Developmental Sciences, University of Edinburgh, Edinburgh EH9 1LW and 2 MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, Edinburgh EH3 9ET, UK
3 To whom correspondence should be addressed at: Department of Haematology/Oncology, Royal Hospital for Sick Children, 17 Millerfield Place, Edinburgh EH9 1LW, UK. E-mail: Hamish.Wallace@luht.scot.nhs.uk

BACKGROUND: Does suppression of the hypothalamic–pituitary–gonadal (HPG) axis restore spermatogenesis in men rendered azoospermic following treatment of childhood cancer? METHODS: Seven men with azoospermia secondary to treatment for childhood cancer, median age (range), 22.2 (18–25.3) years, aged 10.4 (4.4–13.3) years at original diagnosis, participated. Each subject underwent semen analysis and testicular biopsy, followed by treatment with medroxyprogesterone acetate (MPA), 300 mg i.m. repeated after 12 weeks, with 800 mg testosterone pellets s.c. on day 1 to suppress the HPG axis. Hormone and semen analysis was performed every 6 weeks for 48 weeks. A second testicular biopsy was performed at week 48. RESULTS: Before HPG axis suppression, mean plasma LH was 9.0 ± 1.8 U/l, testosterone 17.9 ± 1.5 nmol/l and FSH 22.4 ± 4.4 U/l. Median (range) venous plasma and seminal plasma inhibin B levels were 10.0 (7.8–35) and 11.2 (7.8–770) ng/l respectively. During HPG suppression, FSH and LH levels were undetectable for ≥12 weeks followed by a gradual return to pretreatment concentrations by 48 weeks. All men remained azoospermic at study completion and complete absence of germ cells on biopsies was demonstrated by immunocytochemistry for all specimens pre- and post-HPG axis suppression. CONCLUSIONS: HPG axis suppression with MPA–testosterone for ≥12 weeks did not restore spermatogenesis in azoospermic men treated with gonadotoxic radiotherapy and chemotherapy for childhood cancer.

Key words: cancer/childhood/hormone suppression/spermatogenesis/testis

Introduction

Advances in the treatment of childhood cancer mean that most children can realistically hope for long-term survival. With a 70% overall survival rate, the prevalence of long-term survivors in the young adult population is now estimated to be 1 in 1000 (Wallace, 1997). Of increasing concern among survivors is the deleterious impact that chemotherapy and radiotherapy has on future fertility irrespective of pubertal status at the time of treatment (Heikens et al., 1996; Mackie et al., 1996; Howell and Shalet, 1998; Waring and Wallace, 2001; Thomson et al., 2002). For prepubertal boys, fertility preservation through semen cryopreservation is not an option and consequently, attention is focusing on the development of techniques that might preserve or restore fertility potential in boys being subjected to gonadotoxic cancer therapy.

A number of approaches to this problem have been investigated, based on the idea that suppression of spermatogenesis might protect the normally rapidly dividing germ cell population from damage. Suppression of the rat hypothalamic–pituitary–gonadal (HPG) axis by administration of the GnRH analogue, goserelin, before and during chemotherapy with procarbazine, enhanced recovery of spermatogenesis (Ward et al., 1990). Similarly, protection of spermatogenesis in rats subjected to treatment with procarbazine, cyclophosphamide and radiotherapy has been demonstrated using a number of hormones including testosterone alone (Delic et al., 1986) or in combination with estrogen (Kurdoglu et al., 1994); GnRH analogues in combination with testosterone (Pogach et al., 1988) or the anti-androgen flutamide (Kangasniemi et al., 1995; Meistrich et al., 1995).

Furthermore, recovery from spermatogenic damage in rats induced by radiotherapy or procarbazine treatment has been shown to be enhanced by treatment with GnRH analogues or testosterone even when administered after the gonadotoxic agent (Pogach et al., 1988; Meistrich and Kangasniemi 1997; Meistrich et al., 1999). The mechanisms by which such hormonal manipulation offers protection or enhancement of recovery of spermatogenesis are unclear. Hormonal analysis following irradiation in rats has shown a marked increase in intratesticular testosterone levels and it has been postulated
A.B.Thomson et al.

Table I. Patient diagnoses and their exposure to gonadotoxic treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Chlorambucil TD, g (g/m²)</th>
<th>Procarbazine TD, g (g/m²)</th>
<th>Vinblastine TD, g (g/m²)</th>
<th>Cytarabine TD, g (g/m²)</th>
<th>Radiotherapy TD (Gy)</th>
<th>Sperm concentration (×10⁹/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ALL</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.76 (2)</td>
<td>Cr/TBI 24/14.4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>ALL/estis relapse</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.93 (2)</td>
<td>Cr/testis 18/24</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>HD</td>
<td>0.9 (0.67)</td>
<td>16.8 (11.2)</td>
<td>134 (96)</td>
<td>–</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>HD</td>
<td>0.5 (0.504)</td>
<td>8.4 (8.4)</td>
<td>79.2 (72)</td>
<td>–</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>HD</td>
<td>0.5 (0.504)</td>
<td>10 (8.4)</td>
<td>86.4 (72)</td>
<td>–</td>
<td>Upper mantle 30</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>HD</td>
<td>0.34 (0.504)</td>
<td>6.3 (8.4)</td>
<td>54 (72)</td>
<td>–</td>
<td>Medias/neck 35</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>B-cell NHL</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.2 (2)</td>
<td>Cr/TBI 6/14.4</td>
<td>0</td>
</tr>
</tbody>
</table>

ALL = acute lymphoblastic leukaemia; HD = Hodgkin’s disease; NHL = Non-Hodgkin’s lymphoma; TD = total dose; Cr = cranium; TBI = total body irradiation.

that suppression of the HPG axis promotes multiplication and differentiation of spermatogonia by lowering testosterone concentrations within the testis (Meistrich and Kangasniemi, 1997).

While there is significant evidence for the success of protection/recovery strategies in rats, clinical studies in man have to date been inconclusive (Johnson et al., 1985; Waxman et al., 1987; Masala et al., 1997) and there have been no trials investigating the effects of post-gonadotoxic hormonal suppression. The present study has investigated whether suppression of the HPG axis in men rendered azoospermic by treatment for childhood cancer might restore spermatogenesis, using both semen analysis and testicular biopsy as endpoints.

Materials and methods

The study was approved by the Lothian Paediatric and Reproductive Medicine Research Ethics Sub-Committee and all patients gave written informed consent.

Patients

A review of the oncology database at the Royal Hospital for Sick Children, Edinburgh for men rendered azoospermic secondary to treatment for childhood cancer, identified seven men aged 22.2 (18–25.3) [median (range)] years. All men were invited to participate in the study regardless of the underlying malignancy or cytotoxic therapy and all seven accepted. The median age at original diagnosis was 10.4 (4.4–13.3) years with a disease-free survival of 8.4 (3.3–14.77) years. The underlying malignancies were acute lymphoblastic leukaemia (n = 2), Hodgkin’s disease (n = 4) and non-Hodgkin’s lymphoma (n = 1). A summary of the patients’ diagnoses with details of the gonadotoxic chemotherapy and radiotherapy received is given in Table I.

Clinical assessment and routine haematological and biochemical assessment was performed on each patient to ensure that there was no evidence of disease relapse or second primary malignancy, or other reason likely to impair spermatogenesis. None of the patients had a family history of impaired spermatogenesis.

Assessment of testicular function

Pubertal maturation was assessed according to the Tanner criteria and testicular volume (ml) was measured using a Prader orchidometer (Tanner and Whitehouse, 1976). The mean value of the two testes was taken to represent the subject’s testicular volume. Venous blood samples were collected (20 ml), and LH, FSH and testosterone levels determined using an automated immunoassay analyser (Bayer Immuno 1, Bayer plc., Newbury, Berks, UK). Inhibin B was measured as previously described (Groome et al., 1996), with the limit of assay sensitivity being 7.8 pg/ml. Semen samples were collected in a room adjacent to the laboratory, by masturbation into sterile wide-mouthed non-toxic containers, following an abstinence period of ≥48 h. Samples were centrifuged at 3000 g for 30 min and the pellet examined to confirm azoospermia (World Health Organization, 1999). Seminal plasma was stored at −70°C until assayed for inhibin B (Anderson et al., 1998). Testicular biopsy under general anaesthetic was undertaken on all patients at the start of the study to exclude obstructive azoospermia. The specimens were fixed in Bouin’s fixative and after routine processing and paraffin embedding, sections were cut at 5 μm and examined. A second biopsy of the same testis was performed at the end of the study.

HPG axis suppression

All men underwent a period of suppression of the HPG axis, designed to induce hypogonadotrophic hypogonadism with reduced intratesticular testosterone levels for a period of ~24 weeks, followed by a recovery period of 24 weeks. Following testicular biopsy, subjects were administered depot medroxyprogesterone acetate (DMPA, 300 mg i.m.; Pharmacia and Upjohn, Milton Keynes, UK) and testosterone pellets (4×200 mg s.c.; NV Organon, Oss, The Netherlands). Administration of DMPA was repeated 12 weeks later. Subjects were reviewed at 6 weekly intervals throughout the 48 weeks of the study for clinical assessment, blood sampling and semen analysis.

Immunohistochemistry of testicular tissue

The objective of the immunohistochemical analysis was to investigate whether or not any germ cells, in any developmental stage, were present in the testes of patients before or after HPG suppressive treatment. This was achieved using immunoeexpression of the MAGE-57B antigen and androgen receptor (AR). The MAGE-57B antigen is expressed in early germ cells, strongly in the spermatogonial and weakly in early spermatocytes (Aubry et al., 2001). AR is expressed in the nuclei of all Sertoli cells but not in the nuclei of any germ cells that might be present (Saunders et al., 1996).

Unless otherwise stated, all incubations were undertaken at room temperature. Sections were deparaffinized in xylene, rehydrated in graded ethanol and washed in water. A temperature-induced antigen retrieval step was required for AR only. The sections were pressure-cooked in 0.01 mol/l citrate buffer, pH 6.0 for 5 min at full pressure, allowed to stand for 20 min, cooled in running tap water and washed twice in (5 min each wash) in Tris-buffered saline [TBS: 0.05 mol Tris–HCl, pH 7.4, 0.85% (w/v) NaCl]. Endogenous peroxidase activity was then blocked by immersing sections in 3% (v/v) H₂O₂ in methanol.
Hormone restoration of spermatogenesis

Table II. Patient characteristics before hypothalamic–pituitary–gonadal suppression

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tanner stage</th>
<th>Testicular volume (ml)</th>
<th>FSH (U/l)</th>
<th>LH (U/l)</th>
<th>Testosterone (nmol/l)</th>
<th>Inhibin B (venous) (ng/l)</th>
<th>Inhibin B (seminal) (ng/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>12</td>
<td>18.2</td>
<td>6.6</td>
<td>21.9</td>
<td>35.0</td>
<td>770</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
<td>45.1</td>
<td>19</td>
<td>17.7</td>
<td>15.5</td>
<td>27.7</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>10</td>
<td>23.9</td>
<td>9.3</td>
<td>21.5</td>
<td>&lt;7.8</td>
<td>14.2</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>10</td>
<td>23</td>
<td>8</td>
<td>20.5</td>
<td>&lt;7.8</td>
<td>8.2</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5.5</td>
<td>25.2</td>
<td>8.8</td>
<td>10.2</td>
<td>&lt;7.8</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>8</td>
<td>9.8</td>
<td>6.3</td>
<td>16.6</td>
<td>12.2</td>
<td>N/O</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>12</td>
<td>11.6</td>
<td>4.9</td>
<td>17.2</td>
<td>N/O</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td>Mean (SEM)</td>
<td>5</td>
<td>11</td>
<td>22.4 (4.4)</td>
<td>9.0 (1.8)</td>
<td>17.9 (1.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N/O = values not obtained.

(both from BDH Laboratory Supplies, Poole, UK) for 30 min, followed by two 5 min washes in TBS. Sections were incubated for 30 min with the appropriate normal serum diluted 1:5 in TBS containing 5% bovine serum albumin (BSA; Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) to block non-specific binding sites. Normal swine serum (NSS) and normal rabbit serum (NRS) (both from Diagnostics Scotland, Carluke, UK) were used for AR and MAGE-57B respectively. Primary antibodies were added to the sections at the appropriate dilution in either NSS–TBS–BSA (for AR 1:2000: AR N-20, Santa Cruz Biotechnology SC0816, Santa Cruz, CA, USA) or NRS–TBS–BSA (for MAGE-57B: 1:50, source of antibody) and incubated overnight at 4°C in a humidified chamber. The sections were washed twice in TBS and then incubated for 30 min with anti-rabbit or anti-mouse horse-radish peroxidase-labelled polymer (EnVision: Dako, Ely, UK) for AR and MAGE-57B respectively. Sections were washed twice (5 min each) in TBS and immunostaining was developed using liquid diaminobenzidine (Dako) until staining was optimal, when the sections were counterstained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and cover-slipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). As negative controls, slides were processed as above except that the appropriate normal serum was substituted for the primary antibody.

Immunostained sections were examined and a mean of 132 (range 7.8–284 ng/l) in the subject with the highest inhibin B pretreatment, and rose from <15 ng/l to low but detectable concentrations (range 16–35 ng/l) in the five other subjects (venous serum reference range: mean 257, 95% CI 231–284 ng/l). Serum inhibin B concentrations fell towards

### Results

#### Clinical features

All seven men were Tanner stage 5 with reduced testicular volumes of 11 (5–12) ml [mean (range), Table II]. There were no changes in clinical features during the study and no changes in testicular volume.

### HPG axis suppression

MPA–testosterone treatment was well tolerated and there were no adverse effects. One man reported increased libido during the first 12 weeks of the study. Pretreatment serum FSH levels were elevated, 22.4 ± 4.4 U/l (mean ± SEM, reference range: 1.5–9), in keeping with damage to the seminiferous epithelium (Table II). Serum LH concentrations pretreatment were 9.0 ± 1.8 U/l (reference range: 1.5–9) and testosterone 17.9 ± 1.5 nmol/l (reference range: 10–30), indicating compensated Leydig cell dysfunction (Table II). Serum and seminal plasma inhibin B concentrations were barely detectable in all but one subject (subject no. 1: 35 and 770 ng/l respectively; Table II).

FSH was suppressed to undetectable concentrations during MPA–testosterone treatment for 12 weeks, and remained partially suppressed during the subsequent 12 weeks (Figure 1). Thereafter, there was a gradual rise by weeks 42–48 to 19.5 ± 3.6 U/l, which was not significantly different from pretreatment concentrations. LH showed a similar pattern to FSH, with suppression to undetectable concentrations for 12 weeks, followed by gradual recovery to 8.9 ± 1.6 U/l at 48 weeks (Figure 1). There was no statistically significant difference between LH concentrations pretreatment and at 48 weeks.

Conversely, testosterone concentrations rose initially following MPA–testosterone administration to a peak of 29.7 ± 1.9 nmol/l at 6 weeks, close to the upper limit of the normal range. This was followed by a gradual decline to a nadir of 10.2 ± 2.0 nmol/l at 30 weeks, with a subsequent slight rise to 13.8 ± 1.9 nmol/l at weeks 42–48. The latter was not significantly different from the pretreatment concentration.

Serum inhibin B concentrations increased from 35 to 60.9 ng/l at 12 weeks in the subject with the highest inhibin B pretreatment, and rose from <15 ng/l to low but detectable concentrations (range 16–35 ng/l) in the five other subjects (venous serum reference range: mean 257, 95% CI 231–284 ng/l). Serum inhibin B concentrations fell towards
Figure 1. Serum concentrations of FSH, LH and testosterone in the seven patients before (time 0) and during combined treatment with medroxyprogesterone acetate and testosterone. Values are means ± SEM. Dashed lines show the limit of assay sensitivity (FSH, LH) or the upper and lower limits of the normal range (testosterone). Hormone levels at 42–48 weeks were not significantly different from pretreatment (week 0) values in each instance.

The end of the study, becoming undetectable in all subjects other than the individual with the highest pretreatment concentration at weeks 42–48. This same individual was the only subject with readily detectable seminal plasma inhibin B concentration pretreatment (770 versus <20 ng/l in the others, seminal plasma reference range: mean 2279, 95% CI 698–3864 ng/l) (Anderson and Sharpe, 2000). Seminal plasma inhibin B was not determined during MPA–testosterone treatment as the volume of the ejaculate was insufficient. In all subjects, seminal plasma inhibin B concentrations were undetectable at the end of the study.

Figure 2. Haemotoxylin and eosin staining: (a) and (b) show testis morphology pre- and post-hypothalamic–pituitary–gonadal axis suppression respectively, and demonstrate complete absence of all germ cell types, in contrast to the abundant germ cells in the normal seminiferous epithelium of a healthy adult man (c). Scale bars = 50 μm.

Semen analysis

All men remained azoospermic throughout the study.
Testicular tissue
Light microscopy
Examination of testicular tissue pre- (Figure 2a) and post- (Figure 2b) HPG axis suppression indicated complete absence of all germ cells, in contrast to the abundant different germ cell types in the normal seminiferous epithelium of a healthy adult man (Figure 2c). This was representative of all seven cancer survivors.

Immunocytochemistry
AR
The nuclei of all cells within the seminiferous epithelium of all patients immunoexpressed AR (Figure 3a, brown staining), and this was comparable before MPA–testosterone treatment and at the end of the study (Figure 3b), in contrast to AR negative cells evident in the testis of a healthy adult man (Figure 3c). This finding suggested that only Sertoli cells were present within the tubules of the cancer patients. Using the methodology applied in the current study, all Sertoli cells in the human testis immuno stain for AR with similar intensity (Saunders et al., 1996), although application of modified methods can reveal differences in staining intensity between different Sertoli cells in the normal adult testis (Suarez-Quian et al., 1999).

MAGE-57B antigen
Immunoeexpression of MAGE-57B was negative in all specimens, pre- and post-HPG axis suppression, in contrast to the abundant immunopositive germ cells seen in the healthy adult control (Figure 3d–f). This confirms the absence of spermatogonia in the cancer patients.

Discussion
This study demonstrates that suppression of the HPG axis for ≥3 months, in men rendered azoospermic by gonadotoxic chemotherapy or radiotherapy for childhood cancer, did not result in restoration of spermatogenesis, assessed by both semen analysis and testicular biopsy. In rats, it has been shown that some germ cells survive cytotoxic therapy and that the resulting azoospermia is a consequence of the inability of those spermatogonia that are present to proliferate and differentiate (Kangasniemi et al., 1995). Suppression of the HPG axis facilitates recovery of spermatogenesis following such treatment, and it has been hypothesized that this is the result of a reduction in intratesticular testosterone concentrations (Meistrich and Kangasniemi, 1997). This can be achieved by administration of steroid hormones, or GnRH agonists or antagonists with or without testosterone before, during or after chemotherapy or radiotherapy and such regimens have been demonstrated to enhance recovery of spermatogenesis in rats (Ward et al., 1990; Delic et al., 1986; Pogach et al., 1988; Kangasniemi et al., 1995; Meistrich et al., 1995; Meistrich, 1998). Application of this approach to humans makes the important assumption that the mechanism of cytotoxic chemotherapy or radiotherapy-induced testicular damage is similar in both species, and it has been assumed that like the rat, men might retain a population of spermatogonial stem cells from which spermatogenesis could be regenerated. The mechanism and permanency of impaired spermatogenesis induced by some forms of chemotherapy/radiotherapy in the human may differ more substantially from the rat than was previously appreciated.

The success of hormonal treatment to aid recovery of spermatogenesis in rats subjected to chemotherapy is believed to be based on lowering intratesticular testosterone levels. While the prepubertal testis is relatively quiescent, there is a steady turnover of early germ cells, which undergo spontaneous degeneration before maturation is reached (Muller and Skakkebaek, 1983). This relatively low activity, compared with the adult, does not protect the prepubertal testis from the deleterious impact of cytotoxic therapy, as the present data confirm. The slow turnover of germ cells and their subsequent degeneration in the prepubertal testis may be partly due to low levels of intratesticular testosterone, which is required to complete the end-stages of spermatogenesis (Chemes, 2001). The lack of protection afforded to the prepubertal testis, at a time when testosterone levels are low, would suggest that additional environmental factors play a role in the successful recovery of spermatogenesis in rats and the vulnerability of the prepubertal human testis to cytotoxic therapy. In this regard, our studies with the marmoset, a primate surrogate for man, have demonstrated that activation of testicular cell function occurs well before puberty and is largely gonadotrophin-dependent, but that spermatogonial replication appears to be independent of gonadotrophin stimulation (Kelner et al., 2002).

In one study in which cyclophosphamide was administered as immunosuppressive therapy for nephrotic syndrome in adult men, preservation of fertility was achieved via supraphysiological testosterone therapy (Masala et al., 1997). Of 15 men treated with cyclophosphamide, five received testosterone to suppress testicular function before and during the 8 month cycle of chemotherapy. All men were azoospermic or severely oligozoospermic within 6 months of commencing cyclophosphamide. Nine of the 10 men who received cyclophosphamide alone remained azoospermic 6 months after the end of immunosuppressive therapy, whereas sperm concentrations returned to normal in all five of the men who received testosterone therapy. High dose cyclophosphamide is known to be associated with impaired spermatogenesis, which is often temporary, and it is probable that in this study the simultaneous administration of testosterone with cyclophosphamide provided some protection or hastened the recovery of spermatogenesis. It would be interesting to have long-term follow-up data on the 10 patients who received cyclophosphamide-only treatment to enable a direct comparison with the natural history of recovery of sperm production. In contrast, other studies have failed to show similar benefits in humans. For example, suppression of testicular function with a GnRH agonist, alone or in combination with testosterone during gonadotoxic chemotherapy treatment for lymphoma, did not confer any protective benefit or enhance recovery of spermatogenesis (Johnson et al., 1985; Waxman et al., 1987). A number of reasons may be considered for the lack of successful outcome in the aforementioned studies. The number of patients and controls studied was small and the cancer therapies variable, in contrast to monotherapy.
Figure 3. Androgen receptor (AR) staining: (a) and (b) show AR immunopositive (brown staining) in all cell nuclei within the seminiferous tubules pre- and post-hypothalamic–pituitary–gonadal axis suppression respectively, and demonstrate that all cell nuclei within the seminiferous tubules are AR immunopositive (brown staining), thus excluding the presence of AR negative germ cells, as evident in the testis of a healthy adult man (c). Inset shows negative control in which the primary antibody was omitted. Scale bars = 50 µm. MAGE-57B staining: (d) and (e) show no evidence of expression of the MAGE-57B antigen (all cells stained blue), both pre- and post-HPG axis suppression, in contrast to the abundant germ cells immunopositive (brown staining) for MAGE-57B demonstrated in the healthy adult control (f). Insert shows negative control in which the primary antibody was omitted. Scale bars = 50 µm.
with cyclophosphamide for a non-malignant condition. Treatment regimens may not have been sufficiently gonadotoxic to cause sterility, so no recovery effect could be seen or, conversely, the agents were so gonadotoxic that permanent ablation of all germ cells was induced. Waxman et al. studied the protective effects of a GnRH agonist during the treatment of 20 men with cytotoxic chemotherapy for advanced Hodgkin’s disease (Waxman et al., 1987). Following administration of the GnRH agonist, standard GnRH testing demonstrated adequate suppression of LH, but not of FSH, throughout the chemotherapy treatment. Follow-up assessment of the men after a 3 year interval showed that all remained severely oligozoospermic (Waxman et al., 1987). In another study, the effect of GnRH agonist administration during combination chemotherapy for advanced lymphoma was evaluated in six patients (Johnson et al., 1985). By 6 years post treatment, only one patient demonstrated any evidence of spermatogenesis. While the present study explored the delayed suppression aspect of this hypothesis, no human studies have combined pre-chemotherapy suppression with continued suppression for a significant length of time following chemotherapy.

A number of steroid hormone combinations have been used to suppress the HPG axis in rats and successfully restore spermatogenesis after chemotherapy, including MPA in combination with testosterone (Velez de la Calle et al., 1990; Jegou et al., 1991). Low dose testosterone, MPA or GnRH analogues alone have been shown to stimulate recovery of spermatogenesis in rats following sterilization with radiotherapy. However, the addition of testosterone to GnRH analogues may reduce the effectiveness of GnRH analogues (Shetty et al., 2000). The combination of testosterone with MPA may also have a reduced effect compared with either agent alone, although this combination results in a profound reduction in intratesticular testosterone concentrations in men (McLachlan et al., 2002). Although further study is warranted, the appropriate choice of hormone suppression will require careful consideration. Long-acting gonadotrophin analogues, such as goserelin, have been shown to be ineffective at suppressing FSH long term in normal men, with recovery of FSH and resumption of spermatogenesis occurring within 2–3 months (Behre et al., 1992; Bhasin et al., 1994).

Inhibin B mediates non-steroidal negative feedback from the testes, reflecting the number of sperm produced and regulating FSH secretion (Andersson et al., 1999; Peterson et al., 1999; Anderson and Sharpe, 2000; Kolb et al., 2000). Inhibin B secretion in the adult requires the presence of germ cells (Andersson et al., 1999). Inhibin B concentrations were barely detectable in the azoospermic patients, despite the preservation of Sertoli cells. This provides further evidence for an essential role of the germ cell–Sertoli cell interaction in the production of inhibin B and confirms the value of inhibin B as a non-invasive marker of spermatogenesis following cytotoxic therapy. Inhibin B was also undetectable in seminal plasma in most subjects, as previously found in men with azoospermia of other aetiologies (Anderson et al., 1998).

Although the gonadotoxic effect of chemotherapy depends upon dosage and drugs administered, and radiotherapy-induced damage upon field of irradiation and dose received, it is difficult to reliably predict the extent of testicular damage and which azoospermic patients may show recovery of spermatogenesis. Our study population comprised an unselected group of seven men rendered azoospermic secondary to treatment for childhood cancer. Testicular biopsies from all seven patients demonstrated complete absence of spermatogonia, yet survival of stem cells is a prerequisite for endocrine restoration of spermatogenesis. It was felt to be unethical to exclude men from the trial on the basis of Sertoli cell-only biopsy specimens for several reasons. Testicular volume in these men was markedly reduced and thus it was justified to take only a small piece of testicular tissue, to eliminate any impact which a reduction in testicular tissue may have on Leydig cell numbers. Small islands of spermatogenesis may be present but in our study were absent from the biopsied tissue. Survival of germ cells following apparently sterilizing chemotherapy is evident from a number of studies. Temporary azoospermia and late recovery of spermatogenesis following chemotherapy have been reported, indicating the survival of stem cells (Viviani et al., 1985; Wallace et al., 1991; Pryzant et al., 1993), although permanent azoospermia tends to follow procarbazine and alkylating agent-based regimens, typical of treatment for Hodgkin’s disease (Whitehead et al., 1982; da Cuhna et al., 1984; Bramswig et al., 1990). Similar histological findings have been reported in other studies following treatment for Hodgkin’s disease with procarbazine-based regimens (Chapman et al., 1979; Charak et al., 1990). With advances in assisted reproduction techniques, the development of testicular sperm extraction (TESE) combined with ICSI offers potential for paternity in these young men. Chan and co-workers report the use of TESE–ICSI to retrieve sperm from men with long-standing azoospermia and achieve a pregnancy (Chan et al 2001). Seventeen men, median age (range) 37.4 (28–54) years, had undergone sterilizing chemotherapy treatment 16.3 (6–34) years previously. Of the 17 men, 13 demonstrated Sertoli cell-only on biopsy and the remaining four were described as having hypospermatogenesis. Using microdissection TESE techniques, sperm retrieval was achieved in seven subjects, three (43%) of whom demonstrated Sertoli cell-only on testicular histology and four (57%) with hypospermatogenesis. The seven subjects underwent nine TESE combined with ICSI procedures resulting in a clinical pregnancy in three (33%) and a live birth in two (22%). These encouraging results suggest that microscopic visualization of the seminiferous tubules may enable identification of areas of continued spermatogenesis within the testis and sperm retrieval using microdissection techniques. This reiterates the importance of not excluding men from hormone restoration clinical trials or from assisted reproduction techniques on the basis of a Sertoli cell-only biopsy.

Although small islands of germ cell spermatogonia may exist in the sections of testes that were not biopsied, it is likely that in our patients, the severity of the cytotoxic-induced germ cell loss is such that recovery of spermatogenesis is simply impossible. This does not exclude the possibility that earlier intervention and HPG axis suppression might have been beneficial. However, it seems more probable that HPG axis suppression to restore spermatogenesis may be more successful.
in patients in whom the testicular insult is less severe and in whom there is some preservation of spermatogonial stem cells.

Acknowledgements

We gratefully acknowledge the generous support of Pharmacia Corporation and the Child Growth Foundation in funding this study.

References


Hormone restoration of spermatogenesis


Submitted on November 6, 2001; accepted on March 15, 2002