Testing the Gonadal Regression-Cytoprotection Hypothesis

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ABSTRACT

Germinal damage is an almost universal accompaniment of cancer treatment as the result of bystander damage to the testis from cytotoxic drugs and/or irradiation. Cancer treatment for the most common cancers of the reproductive age group in men has improved such that most are now treated with curative intent, and many others are treated with likelihood of prolonged survival, so that the preservation of fertility is an important component of post-treatment quality of life. This has led to the consideration of developing adjuvant treatments that may reduce the gonadal toxicity of cancer therapy. One dominant hypothesis has been based on the supposition that the immature testis was resistant to cytotoxic damage. Hence, if hormonal treatment were able to cause spermatogenic regression to an immature state via an effective withdrawal of gonadotrophic secretion, the tests might be maintained temporarily in a protected state during cytotoxic exposure. However, clinical studies have been disappointing but have also been unable to test the hypothesis definitively thus far, due to the inability to completely suppress gonadotrophin secretion. Similarly, experimental models have also given conflicting results and, at best, a modest cytoprotection. To definitively test this hypothesis experimentally, we used the fact that the functionally hpg mouse has complete gonadotrophin deficiency but can undergo the induction of full spermatogenesis by exogenous FSH and hCG. Thus, if complete gonadotrophin deficiency were an advantage during cytotoxic exposure, then the hpg mouse should exhibit some degree of germinal protection against cytotoxic-induced damage. We therefore administered three different cytotoxins (200 mg/kg procarbazine, 9 mg/kg doxorubicin, 8 Gy of X irradiation) to produce a range of severity in testicular damage and mechanism of action to either phenotypically normal or hpg mice. Testis weight and homogenization-resistant spermatid numbers were measured to evaluate the potential protective effects on spermatogenesis. Although the three cytotoxins produced a range of severity of spermatogenic damage, there was no evidence of cytoprotection in the hpg mice that were completely gonadotrophin deficient at the time of treatment. These findings cast doubt on the validity of the hypothesis that spermatogenic regression via gonadotrophin withdrawal can protect the mouse testis against cytotoxic-mediated spermatogenic damage.

INTRODUCTION

Azoospermia and infertility as a result of damage to the germinal epithelium are an almost universal accompaniment of cancer treatment. The severity, duration, and reversibility of cytotoxic-induced spermatogenic damage are influenced by the specific regimen used, depending, among other factors, on the type and dosage of drugs and ionizing radiation to which the testis is exposed. Recent decades have seen dramatic improvement in long-term survival and cure after treatment for the most common cancers of men in their reproductive years. Hodgkin’s disease, non-Hodgkin’s lymphoma, and testicular tumors are now regularly treated with curative intent, and reproductive function is an important component of the quality of life after cancer. In other situations such as the treatment of sarcoma and bone marrow transplantation, the therapeutic outlook is improving, and long-term survival after treatment is increasingly feasible. Consequently, the preservation of reproductive function for survivors of cancer treatment is increasingly important.

Strategies to reduce testicular damage from cancer treatment include sperm cryostorage, testicular shielding from irradiation, and developing less toxic therapeutic regimens. However, none are uniformly applicable, effective, or feasible, and additional measures are needed. Another approach is the development of an adjunctive treatment to provide cytoprotection for the germinal epithelium. One hypothesis has been that hormonal treatments aiming to suppress endogenous gonadotrophin secretion would cause testicular regression to an infantile state that would render the testis more resistant to gonadal toxicity. This hypothesis was supported by the supposition based on clinical observations that the immature human testis may be more resistant to damage from cancer treatments (1). Experimental animal studies using pretreatment with GnRH analogues or sex steroids to suppress endogenous gonadotrophin secretion have reported variable but mostly modest to minimal protection against cytotoxic-induced spermatogenic damage (2). Most experimental studies have evaluated cytoprotection in the rat, with a few examining other mammals such as the mouse (3–5), dog (6, 7), and baboon (8).

A major design limitation of existing experimental models to test this hypothesis has been the inability to fully suppress endogenous gonadotrophin secretion. Whereas both GnRH analogues and sex steroids can fully inhibit luteinizing hormone secretion, they may not fully inhibit FSH secretion (9). Consequently, the failure to fully eliminate all FSH action on Sertoli cells may not allow an effective test of the basic hypothesis, because it might be claimed that efficacy may require a complete rather than a partial withdrawal of gonadotrophin secretion to fully protect against gonadal cytotoxicity. To test this hypothesis more definitively, we have used the hpg mouse, a spontaneous mutant mouse strain with congenital complete gonadotropin deficiency (10) due to a major deletion in the GnRH gene (11). This mouse model is particularly suitable because we demonstrated that complete spermatogenesis (including normally fertile spermatogenesis) could be induced by the administration of testosterone alone in the absence of FSH (12), a finding that was subsequently confirmed by the similar phenotype produced by the experimental inactivation of the mouse FSHβ subunit gene (13) or a spontaneous inactivating mutation in the human FSH receptor (14). The present study was therefore based on the concept that if complete gonadotrophin suppression was protective for testis exposed to cytotoxic agents, then the hpg mouse testis that completely lacks gonadotrophin exposure should be protected against the cytotoxic effects.

MATERIALS AND METHODS

Animals. Phenotypically normal (N/N and N/hpg) and hpg (hpg/hpg) mice were obtained from an established breeding colony maintained at the University of Sydney (Sydney, Australia) as described previously (12). Due to a major deletion in the GnRH gene, the hpg mouse has the phenotype of complete gonadotrophin deficiency with the testis remaining in the infantile state, although the administration of testosterone can fully induce spermatogonia and mediate spermatogenic regression. To overcome the cost and impracticality of animal numbers and life span, the mouse FSH/β subunit gene (13) or a spontaneous inactivating mutation in the human FSH receptor (14). The present study was therefore based on the concept that if complete gonadotrophin suppression was protective for testis exposed to cytotoxic agents, then the hpg mouse testis that completely lacks gonadotrophin exposure should be protected against the cytotoxic effects.
testis weight, respectively. Between-group differences were analyzed by unpaired i
spermatid counts are expressed in absolute terms relative to body and organ
buffered in 0.2 M sodium phosphate (pH 7.4). The right testis was then
containing 2% glutaraldehyde, 2% paraformaldehyde, and 0.1% picric acid
an infusion of 30 ml of warmed heparinized saline (10 lU/ml; Monoparin.
of testis.

a homogenizer (Potter S: Braun, Melsungen, Germany) at maximal setting.

trials Pty. Ltd.; Castle Hill, Australia) and then homogenized by 10 strokes of

testes were thawed at room temperature in an appropriate volume (0.5-1
quots of the homogenates of the testis as described previously (12). Frozen
in foil-covered aliquots for single use at —¿20°C for up to 2 weeks. In a

Doxorubicin (Pharmacia & Upjohn, Rydalmere, Australia) was prepared as a 0.01 M stock in acidified PBS (pH 4.2) and stored
in foil-covered syringes for single use at —¿20°C for up to 2 weeks. In a

Whole body perfusion was carried out in anesthetized mice who underwent
an infusion of 30 ml of warmed heparinized saline (10 IU/ml; Monoparin,
Fisons, Sydney, Australia) via the left ventricle followed by 30 ml of fixative
containing 2% glutaraldehyde, 2% paraformaldehyde, and 0.1% picric acid
buffered in 0.2 M sodium phosphate (pH 7.4). The right testis was then
dehydrated and embedded in Spurr’s resin to provide representative semi-thin
(1 μm) sections of testicular tissue for histological evaluation as described in
detail previously (12).

Data Analysis. Data are expressed as mean ± SE. Testis weight and spermatid counts are expressed in absolute terms relative to body and organ
weight, respectively. Between-group differences were analyzed by unpaired i

RESULTS

All data are presented in Table 1.

* Unpublished observations.

Body Weight. At entry, the 3-week-old mice weighed 13.2 ± 0.2 g
(n = 116). At the end of the study, neither procarbazine nor irradiation
had any significant effect (or interaction) on body weight, whereas
doxorubicin reduced the body weight of both groups of mice with
significantly greater effects in the normal (~25%) compared with hpg
(~10%) mice (interaction, P = 0.046).

Effects of Genotype. Pooling all non-cytotoxic-treated 10-week-
old mice, the hpg mice (n = 28) had significantly lower body weight
(26.1 ± 0.5 versus 29.1 ± 0.5 gm) and testis weight (0.99 ± 0.05
versus 3.57 ± 0.09 mg/gm body weight) but similar spermatid numbers
(62 ± 5 versus 72 ± 3 × 10⁶/g testis; P > 0.05) compared with
non-hpg normal controls (n = 45). Similar genotype effects observed
in all analyses were adjusted for by an analysis of variation using
genotype as a main effect.

Testsis Weight. Procarbazine treatment and irradiation reduced the
absolute and relative testis weight by ~35%, and doxorubicin reduced them by ~70%. Based on the statistical definition of cytoprotection (a
significant genotype × treatment interaction), the magnitude of the
effects was significantly greater in hpg compared with normal mice for both
doxorubicin (32 versus 28% for absolute, 34 versus 33% for
relative testis weight) and procarbazine (30 versus 26% for absolute
and 30 versus 23% for relative testis weight), whereas the effects of irradiation were less in the hpg mice (30 versus 39% for absolute, 32
versus 39% for relative testis weight).

Testicular Spermatids. Compared with saline-treated controls, the
testicular spermatid number in normal mice was severely reduced by
doxorubicin (~98% reduction) and less strikingly reduced by
irradiation (~75%), and procarbazine had only a mild effect (~43%).
Doxorubicin and irradiation completely eliminated spermatids from the
hpg mouse testis, whereas in normal mice, ~10% of untreated
control levels of homogenization-resistant spermatids remained in the
testsis. For sperm production expressed per gram of testis to adjust for
the difference in absolute testis size, using the statistical definition of
cytoprotection (a significant genotype × treatment interaction), nei
ither procarbazine nor doxorubicin (P > 0.05) showed significant
cytoprotection, whereas irradiation showed significantly greater
(P = 0.018) cytotoxic effects in the hpg mouse testis compared with
those in normal mouse testsis.

Histology. As described previously (12), hpg mice treated with
testosterone for 6 weeks demonstrated histologically normal spermatogenesis equivalent to that of normal control mice. Doxorubicin (Fig.
1) and irradiation treatment produced marked testicular damage of a
similar degree in both hpg and normal mouse testsis. In most tubules,
germ cells were obliterated, leaving only atrophic tubules lined by a
single basal layer of mainly Sertoli cells. The remaining tubules were
less severely damaged, but only the early stages of spermatogenesis
were present. Procarbazine treatment produced a similar but less
severe and more variable (between tubules) pattern of spermatogenic
damage in both hpg and normal mouse testsis. Areas of vacuolated
cytoplasm within the germinal epithelium representing early germinal
cell degeneration alternated with more normal-appearing tubules,
some of which appeared to retain completely normal spermatogenesis.

DISCUSSION

This study tested the hypothesis that complete gonadotrophin defi
ciency protects the germinal epithelium against severe spermatogenic damage caused by cytotoxic drugs or irradiation. The first experimental study to suggest this approach claimed that pretreatment of mice with a superactive GnRH agonist prevents spermatogenic damage due to an alkylating agent, cyclophosphamide (3). Subse
quently, however, these experimental findings were not reproducible
(4), and the clinical impression of prepubertal protection against
Effects of doxorubicin, procarbazine, and irradiation on body weight, testis weight, and testicular sperm content are shown. Data are expressed as mean ± SE with Ps from two-way ANOVA with the main effects being G (genotype, hpg versus normal), T (treatment, drug or vehicle, irradiation versus no treatment), and G × T [the statistical interaction term representing the potential protective (or aggravating) effects of genotype on treatment effects]. For this experiment, genotype also represents functionally complete genetic gonadotropin deficiency in the hpg strain.

Table 1 Effects of cytotoxic drugs and irradiation on the testis of hpg and control mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>hpg</th>
<th>Normal</th>
<th>G</th>
<th>T</th>
<th>G × T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>10</td>
<td>9</td>
<td>18</td>
<td>22</td>
<td>0.011</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>22.8 ± 1.0</td>
<td>25.6 ± 1.0</td>
<td>23.3 ± 0.7</td>
<td>29.7 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Testis weight (mg)</td>
<td>7.0 ± 6.5</td>
<td>22.2 ± 6.9</td>
<td>28.7 ± 4.9</td>
<td>103.7 ± 4.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Testis weight (mg/g BW)</td>
<td>0.30 ± 0.21</td>
<td>0.87 ± 0.22</td>
<td>1.16 ± 0.16</td>
<td>3.50 ± 0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm (million/testis)</td>
<td>0</td>
<td>1.0 ± 0.6</td>
<td>0.7 ± 0.4</td>
<td>7.3 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm (millions/g testis)</td>
<td>0</td>
<td>8 ± 5</td>
<td>68 ± 4</td>
<td>0.002</td>
<td>0.090</td>
</tr>
<tr>
<td>Procarbazine</td>
<td>14</td>
<td>12</td>
<td>16</td>
<td>15</td>
<td>0.001</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>24.9 ± 1.0</td>
<td>25.2 ± 1.0</td>
<td>28.4 ± 0.9</td>
<td>29.0 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Testis weight (mg)</td>
<td>18.5 ± 2.7</td>
<td>26.3 ± 2.9</td>
<td>76.9 ± 2.5</td>
<td>103.3 ± 2.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Testis weight (mg/g BW)</td>
<td>0.74 ± 0.11</td>
<td>1.06 ± 0.11</td>
<td>2.77 ± 0.10</td>
<td>3.58 ± 0.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm (million/testis)</td>
<td>0.6 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>3.4 ± 0.3</td>
<td>8.1 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm (millions/g testis)</td>
<td>30 ± 5</td>
<td>70 ± 5</td>
<td>46 ± 5</td>
<td>80 ± 5</td>
<td>0.021</td>
</tr>
<tr>
<td>Irradiation</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>0.069</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>26.1 ± 1.0</td>
<td>28.5 ± 1.1</td>
<td>28.1 ± 1.2</td>
<td>27.5 ± 1.0</td>
<td>0.373</td>
</tr>
<tr>
<td>Testis weight (mg)</td>
<td>8.5 ± 2.1</td>
<td>28.8 ± 2.3</td>
<td>39.7 ± 2.5</td>
<td>101.2 ± 2.2</td>
<td>0.173</td>
</tr>
<tr>
<td>Testis weight (mg/g BW)</td>
<td>0.33 ± 0.09</td>
<td>1.02 ± 0.10</td>
<td>1.43 ± 0.11</td>
<td>3.70 ± 0.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm (million/testis)</td>
<td>0</td>
<td>2.1 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>6.7 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm (millions/g testis)</td>
<td>72 ± 5</td>
<td>17 ± 5</td>
<td>67 ± 4</td>
<td>0.197</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

BW, body weight.
cytotoxin regimens (33–35). A major limitation of these studies has been the incomplete suppression of gonadotrophin secretion and spermatogenesis, because GnRH analogues and sex steroids effectively inhibit luteinizing hormone but not necessarily FSH secretion. In addition, mice are significantly more resistant than rats to the desensitizing effects of GnRH analogues (5). Clinical studies of adjuvant cytoprotective therapy based on gonadotrophin suppression by using GnRH agonists have also shown negligible promise thus far. These studies include only one randomized study (21), two nonrandomized controlled studies (22, 23), and one uncontrolled study (20). In addition to their design limitations (lacking randomization, adequate length of follow-up), the inability to obtain rapid, complete gonadotrophin suppression and spermatogenic regression have left the hypothesis unable to be tested definitively. Although the cytoprotective efficacy of pure GnRH antagonists has not been tested in humans, the present study militates against the likelihood that a pure GnRH antagonists would be effective clinically. A recent study has shown surprising efficacy for testosterone in protecting against cyclophosphamide-induced spermatogenic damage (24). Another clinical study using high-dose oral medroxyprogesterone acetate concurrently with chemotherapy for testis cancer found no cytoprotective efficacy (25). It remains unclear whether the findings of Masala et al. (24) are reproducible, and whether this regimen is feasible or effective against the more intensive combination chemotherapy that is regularly used for the treatment of malignancy. In this context, it is notable that the procarbazine effects, the least-damaging regimen in this study, gave the closest approximation to cytoprotective effects, consistent with the results of Masala et al., in which only modest doses of a single testicular toxin were used.

Alternative approaches to cytoprotection may be feasible. One study has suggested a reversal of the original hypothesis as increased proliferative activity of spermatogenic stem cells may provide resistance to subsequent cytotoxic damage. In this study, rhesus monkeys pretreated for 14 days with FSH before testicular irradiation showed that despite severe depletion of spermatagonia at 75 days postradiation, FSH treatment doubled the numbers of surviving spermatagonia (36). This is consistent with the observation that the most radiosensitive spermatogonial cells in the mouse testes were those in the quiescent G0 phase of the cell cycle, whereas the proliferating cells were more radioresistant. Nevertheless, the suggestion that proliferating cells are more resistant to cytotoxic effects is counterintuitive and conflicts with a large body of evidence demonstrating that cytotoxic damage occurs primarily to rapidly dividing cells. Consistent with this different perspective are observations that gonadotrophin suppression by adjuvant GnRH analogue treatment may be deleterious (rather than protective) during concomitant cytotoxic chemotherapy (7, 29), and that superactive GnRH agonists (which remain partial agonists) are significantly more protective than GnRH antagonists (29, 31). In addition, one experimental report has shown that hCG treatment protects against the testicular effects of an atypical alkylating agent (37). Other nonhormonal cytoprotective strategies including testicular hypothermia (38), reduced testicular blood flow (39), and nonhormonal chemicals (40) might also be feasible. Recent developments in reproductive medicine relevant to the potential future role of
pharmaceutical cytoprotection include the ability to produce fertility with a single spermatozoa through an intracytoplasmic sperm injection as well as the development of autologous germ cell transplantation (41). Ultimately, long-term studies comparing pharmaceutical adjuvant cytoprotection regimens for efficacy, safety, and cost-effectiveness as compared with sperm cryopreservation plus artificial reproductive technologies or germ cell transplantation would be needed to determine the optimal cost-effective strategy for the increasing numbers of men in the reproductive age group exposed to treatments for cancer and other serious diseases that compromise subsequent male fertility.

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