ABSTRACT

Germinal damage is 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 posttreatment 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 a direct withdrawal of gonadotrophin secretion, the testis 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 testosterone. Thus, if complete gonadotrophin deficiency were an advantage during cytotoxic exposure, then the hpg mouse should exhibit some degree of germinai 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 germinai 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 germinai 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 lutinizing 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 spermatocytes) 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//I.J.I.) and 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 spermatogenesis.
cytoprotective effects were defined as a statistically significant (P < 0.05) procarbazine, or irradiation versus control). Additional data are expressed as mean ± SE. Testis weight and spermatid numbers were analyzed by an analysis of variation using genotype as a main effect.

**RESULTS**

All data are presented in Table 1.

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4 Unpublished observations.
Effects of doxorubicin, procarbazine, and irradiation on body weight, testis weight, and testicular sperm content are shown. Data are expressed as mean ± SE with P 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>Variable</th>
<th>hpg</th>
<th>Normal</th>
<th>G</th>
<th>T</th>
<th>G×T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>22.8 ± 1.0</td>
<td>25.6 ± 1.0</td>
<td>18</td>
<td>22</td>
<td>0.011 &lt; 0.001 0.046</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 &lt;0.001 &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 &lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td>Sperm (million/testis)</td>
<td>0 ± 1.0</td>
<td>1.0 ± 0.6</td>
<td>0.7 ± 0.4</td>
<td>7.3 ± 0.4</td>
<td>&lt;0.001 &lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td>Sperm (milligram/testis)</td>
<td>0 ± 42 ± 6</td>
<td>8 ± 5</td>
<td>68 ± 4</td>
<td>0.002 &lt;0.001 0.090</td>
<td></td>
</tr>
<tr>
<td>Procarbazine</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 0.673 0.894</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 &lt;0.001 &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 &lt;0.001 0.022</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 &lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td>Sperm (milligram/testis)</td>
<td>30 ± 5</td>
<td>70 ± 5</td>
<td>46 ± 5</td>
<td>80 ± 5</td>
<td>0.021 &lt;0.001 0.534</td>
</tr>
<tr>
<td>Irradiation</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>0.629 0.373 0.173</td>
</tr>
<tr>
<td>Testis weight (mg)</td>
<td>8.5 ± 2.1</td>
<td>88.8 ± 2.3</td>
<td>39.7 ± 2.5</td>
<td>101.2 ± 2.2</td>
<td>&lt;0.001 &lt;0.001 &lt;0.001</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 &lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td>Sperm (million/testis)</td>
<td>0 ± 2.1</td>
<td>0.2 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>6.7 ± 0.2</td>
<td>&lt;0.001 &lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td>Sperm (milligram/testis)</td>
<td>72 ± 5</td>
<td>17.5 ± 5</td>
<td>67 ± 4</td>
<td>0.197 &lt;0.001 0.018</td>
<td></td>
</tr>
</tbody>
</table>

* BW, body weight.

testicular cytotoxic-induced damage (1) was also recognized to be illusory (19). Four preliminary human studies based on this hypothesis (using superactive GnRH agonist adjuvant pretreatment to suppress endogenous gonadotrophins and spermatogenesis) have demonstrated negligible cytoprotection (20–23). More recently, one report suggests that testosterone administration provides striking protection against cyclophosphamide-induced spermatogenic damage (24), whereas a previous study found no cytoprotective benefit from concurrent adjuvant treatment with a prostogen, medroxyprogesterone acetate (25). In the present study, we used three well-established gonadal toxins with different mechanisms of cytotoxic action as well as a range of severity of testicular damage. However, there was no clear evidence of cytoprotection against spermatogenic damage, because the hpg mice demonstrated no consistent amelioration of cytotoxic-induced damage as reflected by changes in testis weight, homogenization-resistant testicular spermatid numbers, and testicular histology. The hpg mouse model has particular advantages for this experiment because it has complete, congenital gonadotrophin deficiency, without requiring additional hormonal or surgical manipulations that might compromise the subsequent physiological interpretation. Yet despite the infantile state of the unstimulated testis, normal spermatogenesis (including functionally normal spermatozoa) can be readily induced so as to be ideal for testing the protective effects of gonadotrophin withdrawal. The present study used doxorubicin (a DNA intercalating agent), procarbazine (an alkylating agent), and irradiation doses based on published detailed dose-response studies showing striking, selective lethal effects on differentiating mouse spermatogonia (15–18), and such gonadal toxicity was confirmed in our study. Our finding that there was no apparent protection exhibited by the completely gonadotrophin-deficient hpg mouse is consistent with another report that the suppression of mouse spermatogenesis by a GnRH antagonist-antiandrogen combination failed to provide any protection against radiation-induced damage to the germinal epithelium (5). Notably, however, the suppression of circulating FSH and intratesticular testosterone in that study was incomplete, leaving open the possibility that more complete hormonal suppression might have been more effective. This explanation, however, is refuted by our present experimental model, which has inherently complete gonadotrophin and testicular testosterone inhibition.

The findings from this study suggest that complete gonadotrophin deficiency does not offer any protection against three characteristic testicular cytotoxins across a spectrum of severity and mechanism of action. The present findings can be most directly extrapolated to the protection of the prepubertal human testis exposed to chemotherapy drugs and/or irradiation. The failure of the present study to find any experimental evidence of cytoprotection provides scientific support to the clinical observation that the prepubertal human testis is susceptible to severe cytotoxic damage, despite the masking of the hormonal and spermatogenic manifestations until after the chronological age of puberty (19). The present findings regarding cytoprotection may not be as validly extrapolated to postpubertal cytotoxic regimes. Nevertheless, the evidence that testicular cytotoxic effects are uniform at all ages argues that the most plausible expectation would be for similar effects in men and boys. The consistently negative findings in this study regarding cytoprotection make it unlikely that the much longer studies needed to test out a more accurate replica of cytotoxic effects on once-mature regressed spermatogenesis would be fruitful.

Previous experimental studies have attempted to create a reversible inhibition of spermatogenesis by the withdrawal of pituitary gonadotrophin secretion by the administration of either sex steroids (via negative feedback) or GnRH analogues (via blockade GnRH action). Apart from limited studies in mice (3–5) and small studies in dogs (6, 7) and baboons (8), most studies have been conducted in rats that exhibit significant genetic strain variability in responsiveness (26). Rat studies have shown mostly modest, if any, cytoprotection (27), although considerable variability exists due to differences in animal species, treatment regimes, experimental designs, and biological end points (2). Some previous studies have reported hormonal suppression to be ineffective (9, 28) or even deleterious (7, 29, 30). In addition, superactive GnRH agonists, which, as partial agonists, suppress hormone secretion less effectively than GnRH antagonists, are significantly less cytoprotective than GnRH antagonists (29, 31). However, when demonstrated, benefits generally require prolonged hormonal pretreatments (32) and were most evident against relatively mild
cytotoxic 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 spermatogonia at 75 days postradiation, FSH treatment doubled the numbers of surviving spermatogonia (36). This is consistent with the observation that the most radiosensitive spermatogonial cells in the mouse testes were those in the quiescent $G_0$ 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 cytotoxin treatment (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...
pharmacological 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 pharmacological adjuvant cytoprotection regimens for efficacy, safety, and cost-effectiveness 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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Testing the Gonadal Regression-Cytoprotection Hypothesis


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