Partial Prevention of Procarbazine Induced Germinal Cell Aplasia in Rats by Sequential GnRH Antagonist and Testosterone Administration  

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ABSTRACT  

The present study examined the feasibility of using a combination of gonadotropin releasing hormone antagonist (GnRH-A) and testosterone in the prevention of procarbazine induced germinal aplasia. Daily injections of GnRH-A or vehicle were given to adult male rats for 21 days prior to procarbazine (PCB) administration and continued until 2 days after the second of two doses of procarbazine (200 mg/kg i.p.) given 1 week apart. One group of rats receiving GnRH-A and PCB was given s.c. two 5-cm testosterone capsule (TC) implants (inside diameter, 3.5 mm) immediately following the second dose of PCB.  

Eight weeks after the last PCB treatment, more than 99% of the seminiferous tubular cross-sections of rats receiving PCB alone were devoid of spermatogenic activity. Spermatogenesis in PCB injected animals receiving GnRH-A pretreatment alone was abortive but was partially preserved when exogenous testosterone was given following PCB administration. At 16 weeks, spermatogenesis was absent in all PCB treated animals and was only observed in less than 1% of the tubular cross-sections of the PCB treated rats receiving GnRH-A pretreatment alone. On the other hand, active spermatogenesis was noted in 68% of the tubular cross-sections, and complete spermatogenesis was noted in four of the five PCB treated rats receiving both GnRH-A pretreatment and subsequent TC implantation.  

At the time of sacrifice, testicular testosterone concentrations in animals receiving TC implants were below 10% of normal levels, while both serum and testicular testosterone content were increased in PCB treated animals with or without GnRH-A pretreatment. Concomitantly, testicular androgen binding protein content remained suppressed and serum androgen binding protein was elevated, indicating a prolonged defect in Sertoli cell function. These lesions were prevented by GnRH-A pretreatment.  

The present study demonstrates that GnRH-A pretreatment and subsequent TC implantation resulted in restoration of complete spermatogenesis in adult male rats given a 400-mg/kg cumulative dose of PCB. It is postulated that GnRH-A may ameliorate PCB induced Sertoli cell dysfunction and/or stimulate the number of spermatogonia to provide direction for the completion of spermatogenesis following PCB injury. The differentiation of these spermatogonias was further supported by exogenous testosterone through certain unknown local mechanisms, resulting in the completion of spermatogenesis.  

INTRODUCTION  

Now that oncology patients are living longer, the toxic effects of chemotherapeutic drugs on the health and quality of life of survivors become important issues in clinical oncology. One of the major unresolved issues is that of the deleterious effects of chemotherapy upon male reproductive function. In several cancers with a high cure rate, such as acute lymphoblastic leukemia, germinal cell tumors, and Hodgkin's disease, survivors are frequently subfertile or sterile (1). In addition, subclinical hypogonadism is a relatively common consequence (2). It is therefore important to determine whether such dysfunction can be reversed or prevented.  

Administration of gonadotropin releasing hormone analogues prior to chemotherapy has recently been proposed as a means of ameliorating chemotherapy induced testicular injury. Glode et al. (3) first reported that protective effects of a GnRH analog (d-Leu⁴-GnRH) on the seminiferous epithelium of mice receiving cyclophosphamide administration. A recent report (4) also notes preservation of the pituitary-testicular hormonal axis and spermatogenic function in baboons receiving a GnRH agonist prior to cyclophosphamide administration. In another study, Schally et al. (5) report that pretreatment of rats with a GnRH antagonist protected the spermatogenic cells against damaging effects of irradiation. On the other hand, several clinical and animal studies (6, 7) were unable to achieve a consistent beneficial effect of GnRH analogues upon testicular function after chemotherapy.  

We have previously noted (8) that administration of a GnRH antagonist to adult rats resulted in an increase in spermatogonial number and that implantation of certain lengths of testosterone filled Silastic capsules supported complete spermatogenesis despite a 95% suppression of testicular testosterone (9). It is postulated that if spermatogonial number could be stimulated by a GnRH antagonist, and their differentiation could be maintained by exogenous testosterone, then it might be possible to preserve spermatogenic function in rats treated with procarbazine, a drug known to cause irreversible germ cell aplasia.  

MATERIALS AND METHODS  

Animals. Mature male Sprague-Dawley rats (300–350 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were maintained in an air conditioned, light-dark controlled animal room and were given laboratory chow and water ad libitum.  

Experiment 1. Animals were given a daily s.c. injection of a GnRH antagonist, N-acetyl-3,4,1-dehydro-Pro₁'p-F-D-Phe²'p-Trp₃'p-lutering hormone releasing hormone (Bachem, Torrance, CA), in a dosage of 300 or 1000 µg/kg of body weight for 2 or 4 weeks. At the end of the experiment a portion of one testis was fixed in Bouin's solution and processed for regular histology. Four-µm-thick sections were stained with periodic acid-Schiffs reagent and counterstained with hematoxylin (10). The integrity of the seminiferous epithelium was evaluated in tubular cross-sections and the stages of the cycle of the seminiferous epithelium were determined by the periodic acid-Schiff positive acrosome according to Leblond and Clermont (11). Portions of the testis were processed for mounting the seminiferous tubules in toto (12). Type A1 spermatogonia and preleptotene spermatocytes were identified and enumerated on whole mounts of stage 7–9 seminiferous tubules as previously reported (9). The number of germ cells was expressed as cells/100 Sertoli cell nucleoli. The percentage yield of preleptotene spermatocytes was calculated as described previously (9). In each animal a total of 30–40 areas in 5–8 segments of the tubules were observed. Control 1 are animals sacrificed parallel to animals 1  

The abbreviations used are: GnRH, gonadotropin releasing hormone; GnRH-A, gonadotropin releasing hormone antagonist; FSH, follicle stimulating hormone; LH, luteinizing hormone; NIAAHD, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases; ABP, androgen binding protein.
treated with GnRH antagonists; control 2 are intact animals previously evaluated.

Experiment 2. Animals were given daily s.c. injections of either vehicle or GnRH-A (300 μg/kg) for 21 days before two weekly injections of procarbazine (200 mg/kg i.p.) were given, and administration of vehicle or GnRH-A continued until 2 days after the second procarbazine injection. Five of the GnRH-A pretreated, procarbazine injected animals were given s.c. implants of two 5-cm testosterone filled Silastic capsules immediately following the second course of procarbazine treatment. Animals were hemicastrated 8 weeks after the second procarbazine administration and were sacrificed another 8 weeks later by decapsulation. Approximately one-third of each testis was fixed in Bouin’s solution and processed for regular histology as described (10). This approach was chosen to ascertain a direct comparison of the status of spermatogenesis in individual animals after 8 and 16 weeks of recovery.

The remaining testicular tissues were stored at −30°C for subsequent testosterone and androgen binding protein assay. Trunk blood samples were collected during decapitation and sera were isolated 24 h later and stored at −30°C.

Hormone Measurement. Serum FSH and LH were measured by double antibody radioimmunoassays provided by the NIADDK. NIADDK rat FSH RP-2, rat FSH I-5, and anti-rat FSH S-7 and rat LH RP-2, rat LH I-5, and anti-rat LH S-5 were used for FSH and LH assays, respectively. The details of these assays have been described previously (13). The intraassay coefficient of variation was approximately 8% for both hormones. The sensitivity of the assays was 1.0 ng for FSH and 0.8 ng for LH/ml serum. Testosterone concentrations were determined in serum and testicular extracts without chromatography by radioimmunoassay using antibody provided by Radioassay Systems Laboratory (14). The cross-reactivity of this antibody with dihydrotestosterone was 18.7%. The intra- and interassay coefficients of variation were 11.8 and 14.4%, respectively.

Androgen Binding Protein Measurement. Decapsulated testicular tissues and epididymis were homogenized with a Polytron homogenizer for 5–10 s in ice-cold buffer containing 20 mM Tris, 50 mM CaCl₂, and 10% glycerol (pH 7.4). The tissue and buffer ratios were 1:2 for testis and 1:9 for epididymis. The homogenates were centrifuged at 40,000 x g for 5–10 s in ice-cold buffer containing 20 mM Tris, 50 mM CaCl₂, and 10% glycerol (pH 7.4). The intraassay coefficient of variation was below 5%.

Statistics. The data were analyzed by one-way analysis of variance to detect differences between groups. When differences were detected, Scheffe’s multiple range test was used to determine the significance (16). All values are presented as the mean ± SEM.

RESULTS

Experiment 1

Spermatogenesis was maintained at least up to step 8 spermiogenesis in rats given 300 or 1000 μg/kg of GnRH antagonist for either 2 or 4 weeks (data not shown). The absence of elongated spermatids and mature spermatids in some animals suggests that the second half of spermiogenesis was affected by the treatment. Administration of GnRH-A to adult rats resulted in a 15–25% increase (P < 0.05) in the number of A₁ spermatogonia/100 Sertoli cell nucleoli (Table 1). On the other hand, the number preleptotene spermatocytes was suppressed (P < 0.05). These quantitative changes in spermatogonial proliferation apparently were not related to the dosage of GnRH-A used. Linear regression analysis of data revealed a significant negative correlation (r = −0.84) between the number of A₁ spermatogonia and the percentage yield of preleptotene spermatocytes (Fig. 1).

<table>
<thead>
<tr>
<th>A₁ spermatogonia</th>
<th>Preleptotene spermatocytes</th>
<th>Percentage yield of preleptotene spermatocytes</th>
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<tbody>
<tr>
<td>GnRH-A (300 μg/kg)</td>
<td></td>
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<tr>
<td>2 wk (n = 2)</td>
<td>17.47 ± 0.1 *</td>
<td>262 ± 2</td>
</tr>
<tr>
<td>4 wk (n = 4)</td>
<td>19.21 ± 1.2</td>
<td>266 ± 6</td>
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<tr>
<td>Total (n = 6)</td>
<td>18.46 ± 0.8</td>
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<tr>
<td>GnRH-A (1000 μg/kg)</td>
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<tr>
<td>2 wk (n = 3)</td>
<td>17.3 ± 1.1</td>
<td>256 ± 3</td>
</tr>
<tr>
<td>4 wk (n = 3)</td>
<td>21.4 ± 1.1</td>
<td>249 ± 14</td>
</tr>
<tr>
<td>Total (n = 6)</td>
<td>19.62 ± 1.1*</td>
<td>253 ± 8</td>
</tr>
<tr>
<td>Control 1 (n = 6)</td>
<td>15.2 ± 0.9</td>
<td>282 ± 5</td>
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</tbody>
</table>

* P < 0.05 versus control.

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* P < 0.05 versus control.

Fig. 1. Relationship between the number of A₁ spermatogonia and percentage yield of preleptotene spermatocytes. Linear regression analysis reveals a negative correlation between these two parameters in both control animals and intact males treated s.c. with GnRH antagonist at 300 or 1000 μg/kg for 2 or 4 weeks.

Table 2 Effects of procarbazine upon body, testicular, and epididymal weights (mean ± SE) in rats 16 weeks after procarbazine treatment

<table>
<thead>
<tr>
<th>Control (n = 5)</th>
<th>PCB (n = 5)</th>
<th>GnRH-PCB (n = 5)</th>
<th>GnRH-PCB-TC (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt (g)</td>
<td>662 ± 6</td>
<td>615 ± 14 *</td>
<td>628 ± 5 *</td>
</tr>
<tr>
<td>Testis wt (g)</td>
<td>1.70 ± 0.09</td>
<td>0.45 ± 0.18</td>
<td>0.80 ± 0.19 *</td>
</tr>
<tr>
<td>Epididymal wt (g)</td>
<td>0.64 ± 0.02</td>
<td>0.43 ± 0.2</td>
<td>0.47 ± 0.03 *</td>
</tr>
</tbody>
</table>

* PCB, procarbazine alone.

GnRH-PCB, GnRH antagonist administration prior to procarbazine administration.

GnRH-PCB-TC, testosterone Silastic capsule implantation subsequent to GnRH antagonist and procarbazine administration.

P < 0.01 versus control.

P < 0.001 versus control.

P < 0.01 versus procarbazine.

Fig. 2. Relationship between the number of A₁ spermatogonia and percentage yield of preleptotene spermatocytes. Linear regression analysis reveals a negative correlation between these two parameters in both control animals and intact males treated s.c. with GnRH antagonist at 300 or 1000 μg/kg for 2 or 4 weeks.

Experiment 2

Testis Weight. The testis weights of the procarbazine treated animals remained at 25% of the control value after 16 weeks of recovery (Table 2). Pretreatment with GnRH-A provided some protection against the procarbazine induced testis weight loss. This protective effect was not affected by the subsequent implantation of testosterone capsules.

Serum and Tissue Hormone Concentrations. Serum FSH concentrations were suppressed (P < 0.05) by procarbazine treatment (Fig. 2) with or without GnRH-A pretreatment but were...
normal in those rats given both GnRH-A pretreatment and testosterone implants. On the other hand, serum LH concentrations were slightly higher ($P < 0.05$) in animals receiving procarbazine alone and in those receiving GnRH-A pretreatment and testosterone implants (Fig. 2).

Serum testosterone levels were slightly elevated in procarbazine treated animals with or without GnRH-A pretreatment (Fig. 3). This higher serum testosterone level was associated with a 2-fold increase in total testicular testosterone content ($P < 0.001$) compared with control animals. Administration of testosterone implants resulted in a 5-fold increase in serum testosterone concentration, while testicular testosterone was suppressed to below 10% of the control levels ($P < 0.001$).

Androgen Binding Protein. Testicular ABP content in rats receiving procarbazine alone remained 60% below the controls even after 16 weeks of recovery (Fig. 4) but was preserved at 75% of control value in rats which had received GnRH-A pretreatment ($P < 0.01$). Serum ABP concentration in procarbazine treated rats was 2.5-fold above the normal values ($P < 0.005$). This elevation of serum ABP was also prevented by pretreatment with GnRH-A ($P < 0.01$).

Spermatogenic Responses. In mature animals, the presence of mature spermatids at the luminal edge of stage 7–8 epithelium demonstrates the completion of spermatogenesis (Fig. 5). Eight and 16 weeks after administration of a cumulative dose of 400 mg/kg body weight of procarbazine, testicular histology revealed no evidence of spermatogenesis beyond spermatogonial proliferation (Fig. 6). This deleterious effect of procarbazine was not prevented by GnRH-A pretreatment alone, although sporadic spermatogenic activity was seen in approximately 1% of the seminiferous tubules at both 8 and 16 weeks (Figs. 7–10). On the other hand, implantation of testosterone capsules subsequent to procarbazine administration resulted in significant preservation of the seminiferous epithelium. After 8 weeks of recovery spermatogenic activity was noted in 30% of the tubular cross-sections and spermatogenesis had been restored to the second half of spermiogenesis (Figs. 11 and 12). At 16 weeks, spermatogenesis was seen in 68% of the tubular cross-sections, and complete spermatogenesis was demonstrated by
Fig. 5. Photomicrograph of testicular histology of control animal. The presence of mature spermatids (arrowhead) demonstrates the completion of spermatogenesis. Stained with periodic acid-Schiff reagent and counterstained with hematoxylin. × 30.

Fig. 6. Rat sacrificed 16 weeks after the second course of procarbazine treatment. Note the absence of spermatogenic activity in all tubules. × 30. For further information, see legend to Fig. 5.

Fig. 7. GnRH-A pretreated, procarbazine injected rats sacrificed at the end of 8 weeks of recovery. Most of the tubules were devoid of active spermatogenesis while a small number of tubules contained abortive spermatocytes or spermatids. × 30. For further information, see legend to Fig. 5.

Fig. 8. Enlarged tubule from Fig. 7 showing the degenerating spermatids and spermatocytes (arrowheads). S, Sertoli cell nuclei. × 80. For further information, see legend to Fig. 5.

the presence of step 19 spermatids at the luminal edge of stage 8 epithelium (Figs. 13 and 14).

DISCUSSION

The present study demonstrates that although spermiogenesis was suppressed, spermatogonial proliferation was maintained in intact adult rats given daily injections of GnRH-A for 2 or 4 weeks. This finding is different from that of Rivier et al. (17) but is consistent with the results of Sundaram et al. (18). These discrepancies may be attributed to variations in the nature and dosage of the compounds used, as well as differences in the duration of treatment and the methods used in the evaluation of spermatogenesis.

GnRH-A prevents the interaction between endogenous GnRH and the pituitary and thereby inhibits the secretion of FSH and LH (19). Consequently, since Leydig cell testosterone production may be inhibited, the mechanisms responsible for the spermatogenic effects in GnRH-A treated rats may mimic those in rats given exogenous androgen (9). Recently, Huang and Boccabella (20) reported the presence of a local feedback mechanism in the regulation of the number of A1 spermatogonia which was sensitive to the change in the efficiency of spermatogonial proliferation. Thus, the increase in the number of A1 spermatogonia following GnRH-A administration probably can be accounted for by the decrease in the efficiency of spermatogonial proliferation due to the suppression of testicular testosterone.

The present study demonstrates that the combination of GnRH-A pretreatment and subsequent implantation of testosterone capsules prevented the procarbazine induced regression of spermatogenesis. This preservation occurred in the presence of less than 10% of normal testicular testosterone concentration and thus is apparently not related to the status of testicular
PREVENTION OF CHEMOTHERAPY INDUCED TESTICULAR INJURY

androgen. This is consistent with the notion that a high intratesticular testosterone concentration is not a prerequisite for the completion of spermatogenesis (9, 21).

The mechanisms by which the combination of GnRH-A and testosterone support spermatogenesis in procarbazine treated rats remain unknown. Since the cumulative dose of procarbazine used in the present study (400 mg/kg) was not sufficient to abolish the entire spermatogonial population (22), and the number of A1 spermatogonia is expected to increase following GnRH-A pretreatment, the number of germ cells that survived procarbazine treatment should be higher in rats receiving GnRH-A prior to procarbazine administration as compared to animals given procarbazine alone. Continuous differentiation of these surviving cells should result in complete spermatogenesis. Failure to achieve this with GnRH-A pretreatment alone suggests that other factors are involved.

Although procarbazine preferentially kills spermatogonia (23), the drug is also likely to affect Sertoli cells since they are metabolically active and are undergoing cyclic modification of their cellular activities (24). The persistence of the abnormal distribution of a Sertoli cell marker protein, ABP, in serum and in testis 16 weeks after procarbazine treatment supports this speculation. Prior reports have noted morphological effects of procarbazine upon Sertoli cells (25–27). Consequently, the procarbazine induced damage of Sertoli cells may have been so severe that these cells were unable to support germ cell differentiation even though some of the germ cells may have survived the procarbazine insult and were able to differentiate to a certain extent.

The concurrence of complete spermatogenesis and the maintenance of 70% of normal testicular ABP content in intact adult rats given 5-cm testosterone capsules suggests that the exoge-
nous testosterone may benefit spermatogenesis through specific Sertoli cell mechanisms (20). Thus, implantation of testosterone capsules following procarbazine administration may enable Sertoli cells to counteract the effects of the drug and to support the differentiation of surviving spermatogenic cells to completion. This is consistent with the recent work of Delic et al. (28) that testosterone enanthate pretreatment can prevent procarbazine induced germinal regression in rats.

Results of the present study illustrate the feasibility of using combinations of endocrine therapies to preserve the spermatogenic function of procarbazine treated rats, although the applicability of these findings to patient care remains to be determined. GnRH antagonists have not been used clinically in the prevention of chemotherapy induced testicular injury. However, the marginal beneficial effect of a GnRH agonist upon the spermatogenic function of one of six lymphoma patients primarily receiving mustargen-Oncovin-prednisone-procarbazine therapy (6), and the more substantial effects of a similar agonist in two of three nonhuman primates receiving cyclophosphamide (4), warrant the further investigation of the usefulness of GnRH analogues in the protection of reproductive function in cancer patients.

Ultimately, the number of germ cells and the normalcy of Sertoli cell function probably dictate the change of spermatogenic recovery postchemotherapy. However, since both the cumulative dose and the frequency of administration of cytotoxic agents are the major determinants that dictate the extent of testicular injury (22, 23), these factors must be considered in future animal or clinical studies. Nonetheless, the recent successful attempts in animals (29) and humans (30) to accelerate myeloid recovery subsequent to chemotherapy by the administration of a recombinant granulocyte stimulating factor support our effort to stimulate spermatogonial number and support germ cell differentiation as a means of preserving the germinal epithelium subsequent to chemotherapy.

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