Protective effects of analogs of luteinizing hormone-releasing hormone against chemotherapy-induced testicular damage in rats
(luteinizing hormone-releasing hormone agonists and antagonists/ gonadal chemoprotectors)

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ABSTRACT Possible protective effects of analogs of luteinizing hormone-releasing hormone (LH-RH) against testicular damage caused by various cytotoxic agents were investigated in rats. The agonist [d-Trp⁶]LH-RH (in which Gly-6 is replaced by d-tryptophan) and the antagonist N-Ac-[d-Phe(pCl)¹,²,D-Trp³,D-Arg⁶,D-Ala¹⁰]LH-RH were administered for 12 weeks: [d-Trp⁶]LH-RH was given once a week in the form of long-acting microcapsules liberating 25 mg of agonist per day, and the antagonist was injected s.c. at the same time. After a recovery period of 3 months, most seminiferous tubules in the antagonist-treated group showed a normal morphology, while patches of tubules in the agonist-treated group continued to show some suppression of spermatogenesis. Administration of busulfan, cisplatin, or cyclophosphamide produced only a reversible testicular injury, and pretreatment with LH-RH analogs seemed to temporarily enhance the tubular damage. Administration of procarbazine (200 mg per kg of body weight per week for 6 weeks) resulted in decreased testicular weights and increased serum LH levels 1 and 3 months after the discontinuation of treatment. The histology showed severe diffuse damage to seminiferous tubules. The germinal cells completely disappeared and the Sertoli cells were markedly degenerated. This damage was not restored even after a recovery period of 5 months. Some animals were pretreated for 6 weeks with the agonist or antagonist and then received procarbazine for 6 weeks while administration of analogs was continued. In these animals, the decrease in testicular weights and increase in serum LH levels after procarbazine were less marked than in the group not pretreated with the analogs. Three and 5 months after cessation of treatment, a large number of tubules showed a complete restoration of structural morphology in 30–45% of the animals that received procarbazine and the LH-RH agonist or antagonist. These results indicate that pretreatment with LH-RH analogs may protect testes against damage caused by some cytotoxic agents.

In recent years, cytotoxic chemotherapy has led to an increased number of sustained remissions in patients with malignant tumors. However, chemotherapy, particularly with alkylating agents, can produce damage to actively dividing tissues (such as testicular) and consequently many patients become sterile (1–4). Since dividing cells of testis are more sensitive to cytotoxic agents than resting cells (1–3), suppression of hypophysial–gonadal axis before chemotherapy might allow for return of normal spermatogenesis (5).

Chronic administration of agonists of luteinizing hormone-releasing hormone (LH-RH), after an initial period of stimulation, induces down-regulation of receptors, desensitization of pituitary gonadotrophs, and suppression of gonads (5–7). The inhibition of male reproductive functions in human beings (8–10) and rats (11–13) is reversible after treatment with LH-RH agonists is discontinued.

Whereas repeated administration of LH-RH agonists is required to reduce the levels of LH, follicle-stimulating hormone (FSH), and sex steroids, an immediate inhibition can be obtained after the first injection of LH-RH antagonists (5, 13, 14). LH-RH agonists exert their action through competition with endogenous LH-RH for receptor occupancy (6, 7). In male rats, administration of LH-RH antagonists leads to cessation of spermatogenesis, azoospernia, and total but reversible inhibition of fertility (7, 14–16).

We have previously shown that pretreatment with the agonist [d-Trp⁶]LH-RH (a LH-RH analog in which d-tryptophan replaces Gly-6) decreased the gonadal damage caused by cyclophosphamide in subhuman primates (17). We also demonstrated that pretreatment with LH-RH antagonists, and possibly agonists, might decrease the testicular damage caused by x-radiation and accelerate the recovery of reproductive functions in rats (13). In this study, we administered the agonist [d-Trp⁶]LH-RH and the antagonist N-Ac-[d-Phe(pCl)¹,²,D-Trp³,D-Arg⁶,D-Ala¹⁰]LH-RH to rats to suppress the pituitary–gonadal function and maintain inactive testes during chemotherapy. Our work was designed to obtain additional evidence that pretreatment with LH-RH analogs could reduce the testicular damage inflicted by various cytotoxic agents.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats, weighing 200 g and obtained from the Charles River Breeding Laboratory, were used in all experiments. The animals were housed at 25°C ± 1°C with a 12-hr dark/12-hr light schedule. The rats were divided into groups of 6–12 each. Some animals were pretreated for 1 or 6 weeks with LH-RH agonist or antagonist before initiation of chemotherapy. Rats not injected with cytotoxic agents or LH-RH analogs were used as controls.

In the first experiment, animals were given two injections, 1 month apart, of microcapsules of [d-Trp⁶]LH-RH, since this slow delivery system provides a continuous biological effect over a 30-day period. Seven days after the first injection of microcapsules, busulfan was given. A second injection of busulfan was administered 6 weeks later. The animals were killed 3 months after the second injection of microcapsules.

In the second experiment procarbazine, cyclophosphamide, and cisplatin were used as cytotoxic agents. Some

Abbreviations: LH-RH, luteinizing hormone-releasing hormone; FSH, follicle-stimulating hormone; [d-Trp⁶]LH-RH, analog of LH-RH in which Gly-6 is replaced by d-tryptophan.

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Table 1. Effect of the pretreatment with the agonist [D-Trp⁶]LH-RH on testicular weights and serum LH and FSH levels in normal and busulfan-injected rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total weights of both testes, g per 100 g body weight</th>
<th>Serum LH, ng/ml</th>
<th>Serum FSH, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.71 ± 0.02</td>
<td>0.75 ± 0.08</td>
<td>14.8 ± 0.9</td>
</tr>
<tr>
<td>Agonist</td>
<td>0.55 ± 0.02*</td>
<td>0.61 ± 0.04</td>
<td>14.8 ± 1.9</td>
</tr>
<tr>
<td>Busulfan</td>
<td>0.31 ± 0.02*</td>
<td>0.98 ± 0.11†</td>
<td>20.7 ± 1.6*</td>
</tr>
<tr>
<td>Busulfan plus agonist</td>
<td>0.28 ± 0.02‡</td>
<td>0.90 ± 0.07±</td>
<td>26.4 ± 1.9*</td>
</tr>
</tbody>
</table>

Results are means ± SEM and were recorded 3 months after the second injection of microcapsules. The final body weight in the control was 529 ± 9 g, and there were no differences in body weight among the four groups. There were 6–12 rats per group. Agonist was administered as microcapsules liberating 25 μg of [D-Trp⁶]LH-RH per day for 30 days, injected twice. Busulfan was injected i.p. twice at a dose of 10 mg per kg of body weight at an interval of 42 days.

*P < 0.01 vs. control.
†P < 0.05 vs. control.
‡P < 0.01 vs. agonist-treated group.

Animals were pretreated with microcapsules of [D-Trp⁶]LH-RH at monthly intervals, twice in the case of clispatin and three times in studies with procarbazine and cyclophosphamide. Two weeks after the second injection of [D-Trp⁶]LH-RH microcapsules, the chemotherapy was initiated in one-half of the animals. Other rats were pretreated with daily subcutaneous injections of LH-RH antagonist N-Ac-[D-Phe(pCl)]¹⁻³,D-Trp³,D-Arg⁶,D-Ala¹⁰]LH-RH. Six weeks later, one-half of the animals received the cytostatic agents. The administration of the LH-RH antagonist was continued during chemotherapy. Procarbazine and cyclophosphamide were administered once a week for 6 and 4 weeks, respectively, and clispatin was injected daily for 5 days.

Ten weeks after the initiation of the treatment with cytostatic agents, which corresponds to 1 month from the cessation of therapy with the analogs, one testis was removed from every animal under pentobarbital anesthesia. Most animals were decapitated 3 months after treatment while some animals in the procarbazine group were sacrificed at 5 months. Blood was collected from the trunk, and the weights of the remaining testes were recorded. Sera were separated and frozen. Serum LH and FSH levels were determined by RIA. The tests were fixed in 8% buffered formalin solution and processed for histological studies by embedding in paraffin and staining with hematoxylin and eosin. The damage to seminiferous tubules was evaluated in cross sections of the testes.

Materials. Microcapsules, prepared at Cytotech (Marigny, Switzerland), consisted of [D-Trp⁶]LH-RH (2.1% wt/wt) in poly(tet-lactide-coglycolide) (97.9% wt/wt). The microcapsules in aliquots of 33 mg, calculated to release a dose of ~25 μg per day for 30 days, were suspended in 0.7 ml of injection vehicle containing 2% CM-cellulose and 1% Tween 20 in water and were injected intramuscularly (18). The antagonist N-Ac-[D-Phe(pCl)]¹⁻³,D-Trp³,D-Arg⁶,D-Ala¹⁰]LH-RH (14, 19) was dissolved in the vehicle solution (0.5% gelatin/5% mannitol) and injected s.c. at a dose of 1000 μg per kg of body weight for the first 3 weeks and, thereafter, at a dose of 500 μg per kg of body weight.

Procarbazine hydrochloride [N-isopropyl-2-(2-methylhydrzino)-p-toluuidine monohydrochloride] (Matulane; Hoffmann-La Roche) was dissolved in 20% (vol/vol) propylene glycol in saline and administered i.p. at a dose of 200 mg per kg of body weight. Busulfan [1,4-butanediol dimethanesulfonate; Fluka (Buchs, F.R.G.)] was dissolved in sesame oil and injected i.p. at a dose of 10 mg per kg of body weight. Cyclophosphamide (Cytoxan; Mead Johnson) was given i.p. at a dose of 100 mg per kg of body weight. Clispatin (cis-diaminedichloroplatinum; Bristol-Myers Laboratories) was dissolved in saline and administered i.p. at a dose of 2 mg per kg of body weight. The significance of the differences between groups was determined by Student’s t test.

RESULTS

In the first experiment, two groups of rats were injected with microcapsules of [D-Trp⁶]LH-RH. Seven and 49 days later, busulfan was given to one pretreated group and to one untreated group of rats. Three months after the second injection of [D-Trp⁶]LH-RH microcapsules, the animals were sacrificed. There were no differences in body weights among the experimental groups. Testicular weights in the

Table 2. Effect of administration of LH-RH analogs on testicular weights and serum LH and FSH levels in normal and procarbazine-injected rats 1 month and 3 months after treatment.

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Testis weight after treatment, g per 100 g of body weight</th>
<th>Serum levels 3 mo after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mo</td>
<td>3 mo</td>
</tr>
<tr>
<td>Control (6)</td>
<td>0.32 ± 0.02</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>Agonist (4)</td>
<td>0.23 ± 0.01*</td>
<td>0.23 ± 0.01†</td>
</tr>
<tr>
<td>Antagonist (7)</td>
<td>0.23 ± 0.04*</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Procarbazine (5)</td>
<td>0.11 ± 0.00*</td>
<td>0.11 ± 0.01*</td>
</tr>
<tr>
<td>Procarbazine plus agonist (4)</td>
<td>0.10 ± 0.02‡</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>Procarbazine plus antagonist (5)</td>
<td>0.11 ± 0.01†</td>
<td>0.15 ± 0.02§</td>
</tr>
</tbody>
</table>

Results are means ± SEM. There were no differences in body weight between the control groups and the analog-treated groups. Body weights of rats that received procarbazine, including analog-treated rats, were significantly smaller—i.e., 483 ± 13 g vs. 578 ± 17 g for controls 1 month after treatment (P < 0.01) and 582 ± 17 g vs. 677 ± 20 g for controls 3 months after the treatment (P < 0.01).

Agonist was administered as microcapsules liberating 25 μg of [D-Trp⁶]LH-RH per day at monthly intervals for 3 months. Procarbazine was administered at a dose of 200 mg per kg of body weight once a week for 6 weeks, starting 6 weeks after the first injection of analogs. Antagonist was N-Ac-[D-Phe(pCl)]¹⁻³,D-Trp³,D-Arg⁶,D-Ala¹⁰]LH-RH injected daily for 3 months. n, Number of animals per group.

*P < 0.01 vs. control.
†P < 0.05 vs. control.
‡P < 0.01 vs. agonist.
§P < 0.05 vs. procarbazine-treated group.
¶P < 0.01 vs. antagonist.
busulfan-treated groups were significantly decreased as compared to those in the control group (Table 1). Treatment with [D-Trp6]LH-RH also reduced testicular weights. The decrease in the weights of testis in rats that received the agonist plus busulfan was greater than in the agonist-treated group (Table 1). Serum levels of FSH and LH were increased in busulfan-treated rats, irrespective of pretreatment with [D-Trp6]LH-RH, indicating tubular damage. Testicular histology of the groups treated with agonist or busulfan showed a considerable recovery of spermatogenesis. However, some tubules were still depopulated of germinal cells, especially in the group that received busulfan and agonist. No evidence of protection of testis by pretreatment with the agonist [D-Trp6]LH-RH was obtained under these experimental conditions.

Since there was still some suppression of the testis in the group treated with [D-Trp6]LH-RH, in subsequent experiments we allowed a longer recovery period, the duration of pretreatment with the agonist was extended to 6 weeks, and a powerful LH-RH antagonist was also tested. Because the busulfan-induced injury appeared to be reversible, this drug was thought to be unsuitable for studies on testicular damage and was replaced by procarbazine, cisplatin, and cyclophosphamide. In the second experiment, some rats were pretreated for 6 weeks with microcapsules of the agonist [D-Trp6]LH-RH or daily injections of the antagonist N-Ac-[D-Phe(pCl)1,2]-[D-Trp6]-[D-Arg6]-[D-Ala10]LH-RH. Some of the rats pretreated with the agonist and untreated control animals were injected with procarbazine once a week for 6 weeks, cisplatin daily for 5 days, or cyclophosphamide once a week for 4 weeks. The treatment with the antagonist was continued during the chemotherapy and for 3 days thereafter. Similarly, the groups that received procarbazine or cytoxan were given one more injection of [D-Trp6]LH-RH microcapsules. One month after the cessation of effective treatment with the agonist—i.e., 2 months after the last injection of microcapsules—one testis was removed from every rat. Two or 4 months later, corresponding to 3 and 5 months after treatment, the animals were sacrificed. The body weights of procarbazine-treated rats were less than those of the controls (Tables 2 and 3), but most of these rats survived throughout the experiment.

In the groups treated with the agonist and the antagonist, testicular weights were reduced 1 month after the treatment as compared to controls (Table 2). In the antagonist-treated group, the testes showed a recovery of weight 3 months after treatment, but some decrease in testicular weights was still observed in the agonist-treated group (Table 2). Treatment with procarbazine diminished testicular weights and this reduction was still evident 5 months after the treatment (Tables 2 and 3). One month after the treatment, testicular weights in groups that were given the agonist or the antagonist before procarbazine were similar to those observed in the group that received only procarbazine. However, at 3 and 5 months some recovery in testicular weights was observed in the groups that were pretreated with the analogs before procarbazine administration (Tables 2 and 3). Administration of procarbazine increased serum LH levels at 3 and 5 months and FSH levels at 5 months. In rats pretreated with the analogs before and during administration of procarbazine, no increase in LH and FSH levels was found 3 and 5 months after the cessation of treatment (Tables 2 and 3).

Histology showed normal testes in the control group (Fig. 1). One month after treatment with the agonist, some seminiferous tubules showed various degrees of damage. These damaged tubules were segmentally distributed, especially in the peripheral zone of the testis, although ~70% of tubules were intact. In most tubules, the damage to spermatogenesis, spermatides, spermatocytes, and/or spermatogonia was limited. Only a few tubules were atrophied and irregularly shaped without any germinal cells and with markedly vacuolized Sertoli cells, indicating total atrophy. One month after the treatment, tubular damage in the antagonist-treated group was not as intense as in rats that received the agonist, and there were fewer tubules with total atrophy. Three and 5 months after treatment, testicular histology showed a distinct morphological recovery of tubules in groups treated with either analog. In these groups, ~80% of the tubules showed a complete morphological restoration.

In the procarbazine-treated group, all the seminiferous tubules were totally atrophic 1 month after treatment (Fig. 2). This atrophy was still present 3 and 5 months after treatment, although the number of interstitial cells increased. There was no evidence of a spontaneous recovery (Fig. 3). On the other hand, histological improvement was seen as early as 1 month after treatment in 3 of 10 animals of the group that received LH-RH agonist and procarbazine. In these animals, the percentage of tubules with total atrophy was 10–50%, and 40–80% of the tubules contained only spermatogonia and spermatocytes. In the group that received antagonist and procarbazine, 4 of 11 rats showed a

Table 3. Effect of administration of LH-RH analogs on testicular weights and serum LH and FSH levels in the procarbazine-injected rats 1 and 5 months after treatment

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Testis weight after treatment, g per 100 g of body weight</th>
<th>5 mo after treatment, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mo</td>
<td>5 mo</td>
</tr>
<tr>
<td>Procarbazine (5)</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Procarbazine plus agonist (5)</td>
<td>0.11 ± 0.02</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Procarbazine plus antagonist (6)</td>
<td>0.12 ± 0.01</td>
<td>0.17 ± 0.03</td>
</tr>
</tbody>
</table>

Results are means ± SEM. One month after treatment, body weights of all three procarbazine-treated groups were 470 ± 11 g. Five months after treatment, body weights of rats were 658 ± 17 g and there were no differences between the groups. The treatments are the same as in Table 2. *P < 0.05 vs. control in Table 2.
histological recovery 1 month after the treatment. In these animals, the proportion of the tubules showing a total atrophy varied from 10% to 95%, and in one histological specimen, 10% of the tubules exhibited a complete morphological restoration. The spermatogonia, spermatocytes, and spermatides reappeared in 5–25%, 50–80%, and 5–10% of the tubules, respectively.

Three or 5 months after therapy, a further recovery was seen in both groups that were pretreated with analogs. In the group receiving LH-RH agonist and procarbazine, 3 of 10 animals showed 55–90% completely normal tubular structures (Fig. 4). An even more marked protective effect was seen in the group that received LH-RH antagonist and procarbazine; 5 of 11 animals displayed normal tubules, with the recovery varying from 30% to 90% in each specimen (Figs. 5 and 6).

In the group treated with cyclophosphamide or cisplatin, body weights were decreased but testicular weights were not, and serum LH and FSH levels were unchanged 1 and 3 months after treatment. Histologically, 5–10% of tubules in the cisplatin-treated group showed moderate damage without total atrophy at 1 month and complete recovery occurred 2 months later. In the cyclophosphamide-treated group, 1 month after the treatment, ~30% of tubules exhibited some injury, but a total atrophy was not found and recovery from

this damage took place 2 months later. In the groups that received cytoxan or cisplatin, the pretreatment with LH-RH agonist or the antagonist enhanced the damage induced by these cytostatic agents, as ascertained 1 month after the treatment, and no protective effects were seen at that time.

DISCUSSION

In the studies described here, we investigated whether a prolonged pretreatment with the agonist [d-Trp5]LH-RH or a powerful antagonist would protect the testes from injuries inflicted by cytotoxic agents. We used four different cytostatic agents and found that procarbazine, a hydrazine-derived antineoplastic agent, produced the greatest testicular damage in rats, which continued up to 5 months after the treatment, in agreement with previous reports (20, 21). On the other hand, although cisplatin (22, §) and cyclophosphamide (20, 22, 23) were reported to cause injuries to testis, in various species, these two agents, as well as busulfan, produced only reversible damage to seminiferous tubules. Some reports (4, 6, §) indicated that LH-RH analogs might potentiate the toxic effects of chemotherapeutic agents on the testes. In our studies, LH-RH analogs seemed to temporarily enhance the testicular damage induced by some cytotoxic agents. Although this deleterious effect of analogs on the testes is transient and reversible, it complicated the interpretation of the results when the protective action of LH-RH agonists and antagonist against chemotherapy and radiation-induced damage is studied. Therefore, only the agents that produce a permanent or more protracted damage to the testis in animal models should be used for evaluating the protective effects of the LH-RH analogs. Procarbazine may be one of such effective agents that induce "permanent" damage to the testes of experimental animals.

We found that the testes of rats pretreated with LH-RH agonist or antagonist showed a marked recovery of spermatogenesis 3 and 5 months after the treatment with procarbazine. Glode et al. (23) reported that the agonist [d-Leu9]LH-RH ethylamide protected mouse testes from damage induced by cyclophosphamide. Attempts to confirm this result have been unsuccessful (24). Nseyo et al. (22) reported a recovery of spermatogenesis in a dog pretreated with the agonist [d-Ser(Bu)5]-des-Gly10-LH-RH ethylamide (Buserelin) 6 months after exposure to radiation or chemotherapy with

cytoxan or cisplatin. Although in rats androgen treatment partially protected spermatogenesis from procarbazine-induced damage (21), clinical implementation of this procedure would be connected with various problems.

The successful use of LH-RH antagonists in rats for prevention of testicular damage induced by chemotherapy or radiation may have clinical implications. We observed that a LH-RH antagonist protected rat testes from the permanent damage inflicted by x-irradiation more effectively than the LH-RH agonist (13). Despite inhibition of gonadal function in many species (6–12), LH-RH agonists do not produce complete azoospermia (12, 25) in contrast to the antagonist (14, 15). LH-RH antagonists should be more effective than LH-RH agonist in inhibiting the gonads, thus producing a more efficient protection. In our study, LH-RH antagonist appeared to protect the testes against chemotherapy better than the agonist. Nevertheless, the treatment with the agonist [d-Trp6]LH-RH for long periods exerted a protective effect against procarbazine-induced damage to seminiferous epithelium. A report that administration of the agonist Buserelin failed to preserve fertility in patients subjected to chemotherapy may have been due to inadequate doses and short duration of treatment (26).

In view of our findings (13, 17) and those of others (22, 23) that LH-RH analogs can protect testes and ovaries (27) from chemotherapy and/or irradiation, additional studies are required to determine optimal dosages and duration of treatment for possible clinical applications. Since in patients afflicted with various malignancies, chemotherapy or radiation may have to be started soon after the diagnosis is made, LH-RH antagonists may be preferred over the agonists for induction of gonadal inhibition considering their rapidity of suppressive action.

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