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Protective effects of analogs of luteinizing hormone-releasing hormone against x-radiation-induced testicular damage in rats

(luteinizing hormone-releasing hormone agonists and antagonists/gonadal radioprotectors)

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ABSTRACT **Possible protective effects of the agonist [D-Trp^6]LH-RH (analog of luteinizing hormone-releasing hormone in which Gly-6 is replaced by D-tryptophan) and antagonist *N*-Ac-[D-Phe(*p*Cl)^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LH-RH against testicular damage caused by x-radiation were investigated in rats. Three months after being subjected to x-irradiation of the testes with 415 or 622 rads, control rats showed marked reduction in the weights of the testes and elevated levels of LH and follicle-stimulating hormone (FSH), indicating tubular damage. Histological studies demonstrated that, in testes of rats given 415 rads, most seminiferous tubules had only Sertoli cells and no germinal cells, and, in the group given 622 rads, the depression of spermatogenesis was even more marked. Rats pretreated for 50 days with LH-RH antagonist (1000 μg/kg of body weight per day) showed a complete recovery of testicular weights and spermatogenesis 3 months after 415 rads and showed partial recovery after 622 rads, and LH and FSH levels returned to normal in both of these groups. Thus, pretreatment of rats with LH-RH antagonist, by reversibly inhibiting gonadal function, protected the germinal cells of the testes against damaging effects of x-rays. Three experiments were also carried out in which the rats were pretreated for 1–2 months with long-acting microcapsules of the agonist [D-Trp^6]LH-RH, liberating 25 μg of the agonist per day. Some rats were then subjected to gonadal irradiation with 415 or 622 rads and allowed a recovery period of 2–4 months. In spite of pretreatment with [D-Trp^6]LH-RH, testicular weights were significantly lower and LH or FSH levels were elevated in the irradiated groups as compared with nonirradiated controls. The recovery of spermatogenesis was incomplete, and there was a decrease in the number of germinal cells after 415 rads and especially after 622 rads. On the basis of testicular weights, histology, and gonadotropin levels, it could be concluded that the agonist [D-Trp^6]LH-RH did not protect the rat testes exposed to 622 rads and, at most, only partially protected against 415 rads. These results suggest that pretreatment with LH-RH antagonists and possibly agonists, might decrease the testicular damage caused by radiation and accelerate the recovery of reproductive functions.**

The damage inflicted by ionizing radiation is the greatest in tissues with rapid cellular turnover (1). Among the more radiosensitive are the reproductive, hematopoietic, and gastrointestinal systems (1). Most cells in the division cycle are generally more radiosensitive than are nondividing cells (2–4). These data suggest that cellular radiosensitivity could be altered by pharmacologic agents or hormones that place the cells in a resting phase (3).

The lethal effects of acute doses of radiation on fertility are well known (5). Germ cells are killed or damaged within a short time of exposure to radiation. In recent years, radiation of patients with cancer has led to an increased number of sustained remissions (6). However, among the long-term side effects of radiation, injury to the reproductive system is of particular concern (6). Treatment of patients with infradiaphragmatic radiation, particularly in the pelvic area, produces dose-dependent and sometimes irreversible gonadal failure (7), even with precautions such as gonadal shielding. This gonadal damage induced by radiation treatment is a serious long-term complication, particularly for the younger cancer patients (1, 6, 7).

Recent findings suggest that hormonal therapeutic manipulations based on analogs of luteinizing hormone (LH)-releasing hormone (LH-RH) could be tried in an attempt to reduce gonadal damage and, thus, to accelerate the recovery of normal reproductive function in cancer patients treated with radiation (8–10). LH-RH agonists and antagonists effectively inhibit the pituitary–gonadal function and fertility in animals and human beings (10–20). Chronic administration of superactive agonists of LH-RH induces down-regulation of receptors, desensitization of pituitary gonadotrophs, and suppression of gonads (10–15, 17, 20). In male animals, this is manifested by a decrease in the weights of testes, inhibition of spermatogenesis, and a reduction in plasma LH, follicle-stimulating hormone (FSH), and testosterone levels (10–15). The inhibition of reproductive functions in animals and human beings of both sexes is reversible, and fertility is restored after treatment with agonists of LH-RH is stopped (14, 15, 20). Various LH-RH agonists are clinically available and used for the treatment of patients with prostate cancer and other hormone-dependent tumors (10, 11, 17–22).

Whereas repeated administration of LH-RH agonists is required to reduce the levels of LH, FSH, and sex steroids, an immediate inhibition can be obtained after the first injection of LH-RH antagonists (11, 16, 19, 20). Antagonistic analogs of LH-RH were developed for contraception and were tested in animals and human beings (11, 13, 16, 19, 20). Because of some side effects such as edema and erythema, no chronic clinical studies have yet been reported with LH-RH antagonists in the fields of contraception or cancer (19).

We recently demonstrated that pretreatment with the agonist [D-Trp^6]LH-RH decreased the gonadal damage caused by the chemotherapeutic agent cyclophosphamide in subhuman primates (8). Actively dividing cells are more sensitive than resting cells not only to some chemotherapeutic agents (8) but also to radiation (1–4). Therefore, in this study we decided to administer the agonist [D-Trp^6]LH-RH or the powerful LH-RH antagonist *N*-Ac-[D-Phe(*p*Cl)^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LH-RH for 4–8 weeks before radiation

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Abbreviations: LH, luteinizing hormone; FSH, follicle-stimulating hormone; LH-RH, luteinizing hormone-releasing hormone; [D-Trp^6]LH-RH, analog of luteinizing hormone-releasing hormone in which Gly-6 is replaced by D-tryptophan; H&E, hematoxylin/eosin.

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in order to suppress the pituitary-gonadal function and maintain inactive testes. Consequently, radiation might damage the testes temporarily inhibited by LH-RH analogs to a lesser extent than the unsuppressed gonads. Our work was designed to test this hypothesis.

MATERIALS AND METHODS

Animals. Young adult male rats (mean weight, 250 g) of the Sprague–Dawley strain (Charles River Breeding Laboratories) were used and divided into groups of 6–12 each. Some groups of animals were pretreated for 4–8 weeks with the LH-RH agonist or antagonist before irradiation. Rats that were not irradiated or not treated with the LH-RH analogs were used as corresponding controls. In all experiments, one testis was removed 1–2 months after the irradiation. Two to four months after irradiation or cessation of treatment with LH-RH analogs, animals were decapitated, blood was collected from the trunk, and the weights of the remaining testes were recorded. Sera were separated and frozen. FSH and LH were determined by RIAs. The testes were fixed in Bouins' solution and processed for histological studies by embedding in paraffin and staining with hematoxylin/eosin (H&E) and periodic acid/Schiff reagent. The significance of the differences between groups was determined by using Duncan's new multiple-range test. All data are expressed as the mean ± SEM.

The antagonist *N*-Ac-[D-Phe(*p*Cl)^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LH-RH, synthesized by solid-phase methods in our laboratory (16–19), was dissolved in the vehicle solution (0.5% gelatin/5% mannitol) and injected subcutaneously at a dose of 100 μg/100 g of body weight per day for 48 days before irradiation and for 3 days after irradiation.

[D-Trp^6]LH-RH (called "Decapeptyl"), a long-acting LH-RH agonist ≈100 times more potent than LH-RH, was synthesized as described (11, 13, 18). In experiment 1, the agonist was injected subcutaneously in saline solution in a dose of 50 μg twice a day for 6 days before administration of a long-acting formulation of [D-Trp^6]LH-RH (22, 23). Microcapsules, prepared at Cytotech, Martigny, Switzerland, consisted of [D-Trp^6]LH-RH (2.1% wt/wt) in poly(DL-lactide-co-glycolide) (97.9% wt/wt). The microcapsules in aliquots of 33 mg, calculated to release a dose of about 25 μg/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 through an 18-gauge needle (23). The administration was repeated at monthly intervals in experiments 3 and 4.

Irradiation. The x-ray source was a General Electric KX-10 (superficial x-ray therapy) machine. Radiation factors were: 100-kV potential at 7 mA; half-value layer of 1.3 mm of aluminum at a target–skin distance of 15.5 cm; dose rate of 419 R/min in air (1 R = 0.258 mC/kg). The rats were anesthetized with pentobarbital (Nembutal, 4 mg/100 g of body weight), and the testes were exposed to 400 R in air (415 rads at the surface) or 600 R in air (622 rads at the surface) through a cone 4.5 cm in diameter. (Conversion factor for roentgens to rads = 0.902; backscatter factor = 1.15.)

RESULTS

In the first experiment, two groups of 10–12 rats were pretreated with [D-Trp^6]LH-RH, and 1 month later the testes of 12 rats were pretreated with the agonist and 10 untreated rats were irradiated with 415 rads. One month after irradiation, one testis was removed, and the animals were sacrificed 1 month later. In all of the experiments, there were no significant differences in body weights among various experimental groups at the corresponding times. Testicular weights in the irradiated group were significantly decreased as compared with untreated, nonirradiated controls, at 1 and also at 2 months after irradiation. Treatment with [D-Trp^6]LH-RH also reduced testicular weights. The greatest decrease in the weights of the testes was recorded in the groups that received the analog plus the radiation. Serum levels of FSH and LH were increased in the rats that were irradiated, with or without pretreatment with [D-Trp^6]LH-RH, indicating tubular damage. One month after irradiation, testicular histology showed depopulated tubules in rats that received radiation, especially in the group that was irradiated after pretreatment with the analog. Two months after radiation, there was some recovery of spermatogenesis in the x-irradiated rats, but some patches of tubules devoid of germinal cells were present in the group subjected to both procedures.

Table 1. Effects of treatment with LH-RH antagonist and x-radiation on the testes weights and serum FSH in rats

Treatment	Testis weight after treatment, g		FSH 3 mo after treatment, ng/ml
	1 mo	3 mo	
Control	1.68 ± 0.09	1.82 ± 0.04	8.0 ± 0.7
415 rads	1.06 ± 0.10*	0.94 ± 0.14*	17.3 ± 5.6
622 rads	1.13 ± 0.04*	0.90 ± 0.11*	20.1 ± 5.6
Antagonist†	0.86 ± 0.04*	1.74 ± 0.05	7.6 ± 0.6
+ 415 rads	0.65 ± 0.03*‡	1.57 ± 0.10‡	9.8 ± 0.8
+ 622 rads	0.57 ± 0.03*§	1.06 ± 0.11*	10.3 ± 0.9¶

Results are means ± SEM. One month after irradiation, the body weights were 341–513 g, and at three months, 607–650 g, with no significant differences between the groups.
*$P < 0.01$ vs. control.
†*N*-Ac[D-Phe(*p*Cl)^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LH-RH at 100 μg/100 g of body weight for 48 days before irradiation and 3 days after irradiation.
‡$P < 0.01$ vs. 415 rads.
§$P < 0.01$ vs. 622 rads.
¶$P < 0.05$ vs. 622 rads.

Since no testicular protection against radiation was obtained in this first study, in subsequent experiments we used an LH-RH antagonist and extended the pretreatment time with [D-Trp^6]LH-RH and the recovery period after the radiation. In the second experiment, 21 rats were pretreated with the antagonist *N*-Ac-[D-Phe(*p*Cl)^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LH-RH for 48 days. The testes of 6 of these rats were irradiated with 415 rads, and those of 6 other animals, with 622 rads. The testes of untreated animals were also irradiated

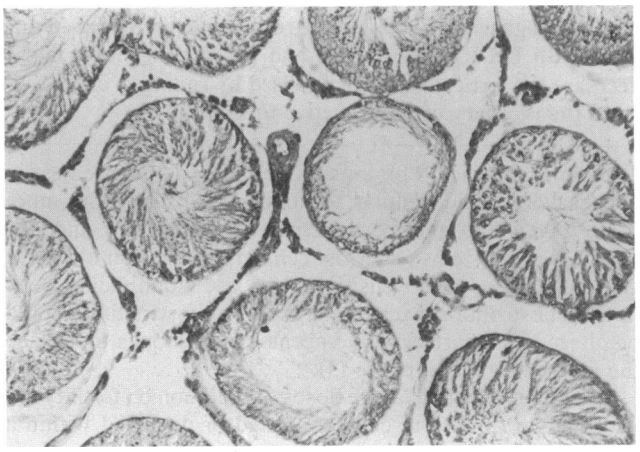

FIG. 1. A section of the rat testis 3 months after 415 rads. Germinal cells are reduced in number, and spermatides are absent. (H&E; ×100.)

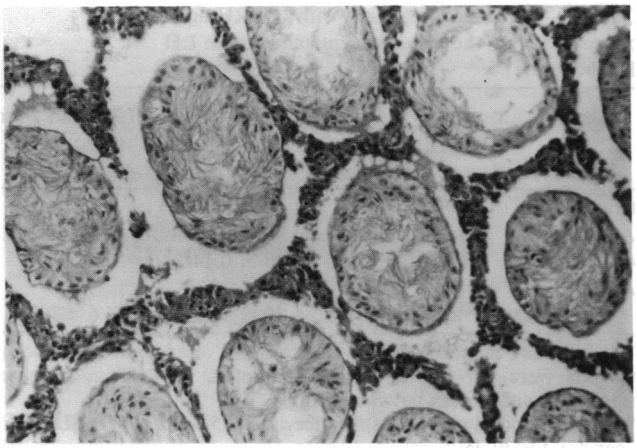

FIG. 2. A section of the rat testis 3 months after 622 rads, showing atrophy of seminiferous tubules and fibrosis. (H&E; ×100.)

with the same doses. One month after irradiation, one testis was removed from every rat. Two months later, the animals were sacrificed. Testicular weights were significantly decreased 1 month after cessation of treatment with LH-RH antagonist, but at 3 months these weights had fully recovered (Table 1). Both doses of radiation significantly diminished testicular weights, and this reduction was still found after 3 months. One month after radiation with 415 or 622 rads, the testicular weights were greatly decreased in rats pretreated with the antagonist. However, after a period of 3 months, the protective effect of the LH-RH antagonist on the testes could be seen in the group given 415 rads, while the group irradiated with 622 rads showed a partial recovery of testicular weights. Levels of FSH (Table 1) and LH were more than doubled (LH = 0.8 ± 0.1 and 0.9 ± 0.2 ng/ml, respectively) in the groups exposed to radiation, indicating tubular damage. In rats pretreated with the antagonist and irradiated, FSH values were in the normal range and LH levels corresponded to the control level (0.4 ± 0.1 ng/ml).

Histology showed normal morphology in the testes of control rats. After 1 month, the testes irradiated with 415 rads showed a small diameter of seminiferous tubules and a decrease in the number of germinal cells. Three months after the irradiation, these alterations were more evident. A few tubules showed germinal cells, but ≈60% of the tubules had only Sertoli cells, which are more resistant to radiation. The spermatogenic activity was absent (Fig. 1). After 1 month, the

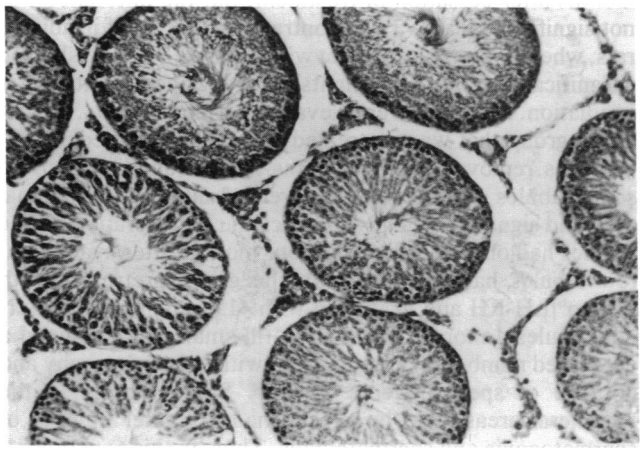

FIG. 3. A section of testis from a rat pretreated with the antagonist 3 months after 415 rads. Note recovery in a number of germinal cells and spermatogenesis. (H&E; ×100.)

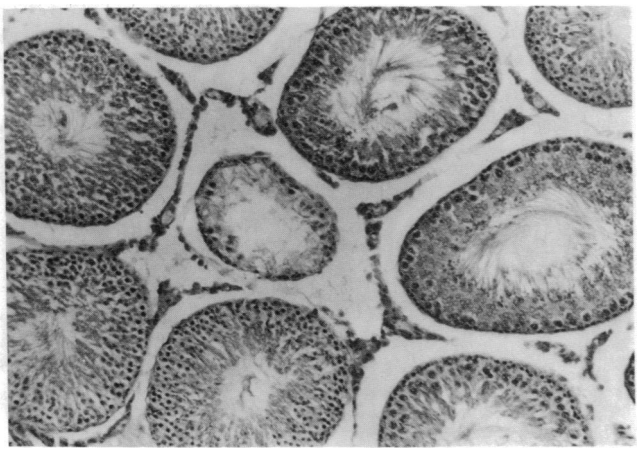

FIG. 4. A section of testis from a rat pretreated with the antagonist 3 months after 622 rads. Note an almost complete recovery in the number of germinal cells and spermatogenesis. (H&E; ×100.)

testes given 622 rads showed a greater decrease in the number of germinal cells than in the case of 415 rads. The spermatogenic activity was absent. Three months after irradiation, a high percentage of the tubules had no germinal cells and only Sertoli cells remained (Fig. 2). One month after irradiation, the testes of the rats pretreated with antagonist and then irradiated with 415 rads showed more alterations than did the testes of the group that received only radiation. However, after 3 months, there was a striking recovery in the number of germinal cells and spermatogenic activity, and many spermatozoa were observed in the lumen of the seminiferous tubules (Fig. 3). Morphologically, these testes could be considered as normal and completely recovered. One month after irradiation, the testes of the rats treated with antagonist and given 622 rads showed severe lesions and a complete absence of germinal cells and spermatogenic activity. Three months after radiation, a marked recovery in the number of germinal cells and in the spermatogenic activity was observed (Fig. 4). A small percentage of tubules showed only spermatogonia and an incomplete spermatogenesis. Thus, the germinal cells of rats pretreated with the LH-RH antagonist were completely protected against the damaging effects of 415 rads of x-radiation and partially against 622 rads.

Table 2. Effects of treatment with [D-Trp^6]LH-RH microcapsules and x-irradiation on the testes weights and serum FSH in rats

Treatment	Testis weight after treatment, g		FSH 3 mo after treatment, ng/ml
	1 mo	3 mo	
Control	1.86 ± 0.05	2.00 ± 0.05	8.1 ± 0.2
415 rads	1.21 ± 0.06*	1.04 ± 0.07*	8.4 ± 1.1
622 rads	1.12 ± 0.04*	1.02 ± 0.11*	9.8 ± 1.3
Agonist†	1.24 ± 0.08*	1.52 ± 0.08*	8.3 ± 0.7
+ 415 rads	0.84 ± 0.05*‡	1.13 ± 0.09*	12.2 ± 1.9§
+ 622 rads	0.74 ± 0.05*¶	0.82 ± 0.07*	11.8 ± 1.2

Results are means ± SEM. One month after irradiation, body weights were 509–588 g, and at three months, 607–650 g, with no significant differences between groups.

*$P < 0.01$ vs. control.

†Administered as microcapsules liberating 25 μg of [D-Trp^6]LH-RH per day for 30 days, injected twice.

‡$P < 0.01$ vs. 415 rads.

§$P < 0.05$ vs. control.

¶$P < 0.01$ vs. 622 rads.

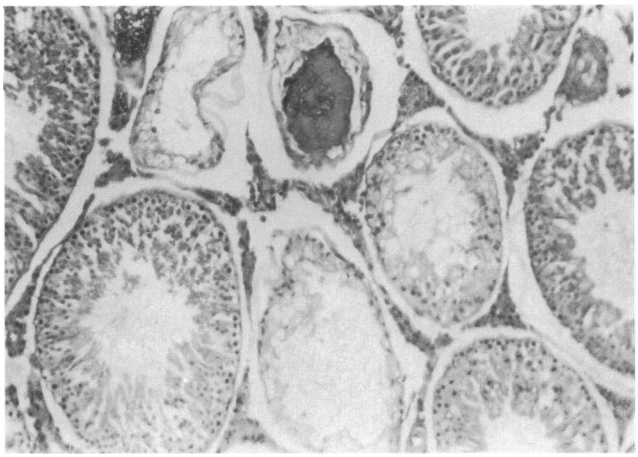

FIG. 5. A section of testis from a rat pretreated with [D-Trp^6]LH-RH 3 months after 415 rads. Some tubules show recovery in the number of germinal cells, but others remain atrophic. (H&E; ×100.)

In experiment 3, we investigated whether long-term pretreatment with the agonist [D-Trp^6]LH-RH could protect the rat testes against radiation. Sixty days after starting the treatment with microcapsules of [D-Trp^6]LH-RH, two groups that received the agonist and two groups of untreated control rats had their testes irradiated with 415 and 622 rads. One testis was removed 1 month postirradiation and the other at 3 months. Testicular weights are shown in Table 2. One month after either dose of radiation, testicular weights both in the untreated and [D-Trp^6]LH-RH-pretreated rats were significantly smaller than in controls. Rats that were given microcapsules but were not irradiated also showed a decrease in testicular weights at 1 month and 3 months after discontinuation of treatment. The weights of testes exposed to either dose of x-radiation were still reduced after 3 months. In rats pretreated with [D-Trp^6]LH-RH, the testes irradiated with 415 rads showed a better recovery of weights than those given 622 rads, although the levels of FSH (Table 2) and LH (1.6 ± 0.2 and 2.1 ± 0.3 ng/ml, respectively, vs. 1.1 ± 0.2 ng/ml for controls) in both of these groups were still elevated, indicating tubular damage.

The histology of testes removed 1 month after irradiation showed that in the groups given 415 rads or 622 rads, the diameter of the seminiferous tubules was reduced and the number of germinal cells was decreased. In the rats treated

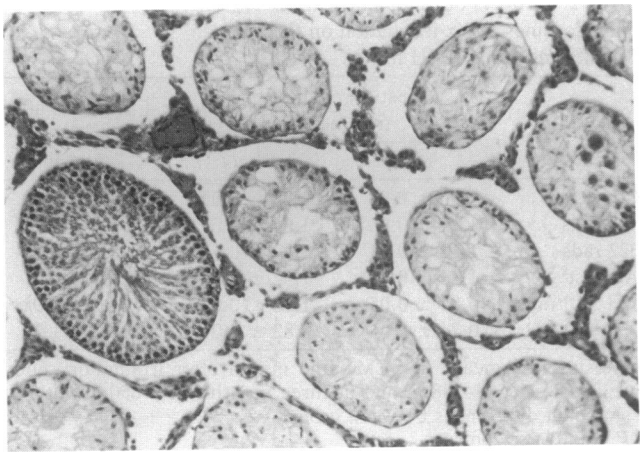

FIG. 6. A section of testis from a rat pretreated with [D-Trp^6]LH-RH 3 months after 622 rads. Note the atrophy of the seminiferous tubules and fibrosis. Some tubules show recovery in the number of spermatogonia and spermatocytes. (H&E; ×100.)

Table 3. Effects of treatment with LH-RH agonist and x-irradiation on the testes weights and serum LH levels in rats

Treatment	Testis weight after treatment, g		LH 4 mo after treatment, ng/ml
	2 mo	4 mo	
Control	1.95 ± 0.07	1.96 ± 0.04	0.6 ± 0.1
Agonist*	1.60 ± 0.07	1.73 ± 0.05	0.7 ± 0.1
415 rads	0.85 ± 0.05†	1.34 ± 0.14†	0.9 ± 0.1†
Agonist* + 415 rads	0.87 ± 0.05†	1.30 ± 0.12†	0.6 ± 0.1‡

Results are means ± SEM. Two months after irradiation, body weights were 547–621 g, and at 4 months, 598–686 g, with no significant differences between groups.

*Administered as microcapsules liberating 25 μg of [D-Trp^6]LH-RH per day for 30 days, injected twice.

†$P < 0.01$ vs. control.

‡$P < 0.01$ vs. radiation.

with [D-Trp^6]LH-RH, the diameter of the seminiferous tubules was smaller than in controls but was larger than in the irradiated groups. The germinal cells and the spermatogenic activity were preserved. The groups pretreated with LH-RH agonist and irradiated with 415 rads or 622 rads showed a decrease in the size of the tubules and in the number of germinal cells. The cells showed pyknotic nuclei and vacuolar degeneration. The spermatogenic activity was absent. Three months after irradiation, in untreated rats given 415 rads, and especially in untreated rats given 622 rads, the seminiferous tubules were small, with scarce germinal cells disorderly arranged and with marked degeneration signs. In rats treated with LH-RH agonist and irradiated with 415 rads, some tubules had recovered in size and some germinal cells were in the maturation phase (Fig. 5), but following 622 rads, the tubules were still small and contained few germinal cells (Fig. 6). The cells showed signs of atrophy.

In experiment 4, we investigated whether the possible protective effects of the agonist [D-Trp^6]LH-RH could be seen better if we allowed more time for testicular repair after exposure to 415 rads. Sixteen rats were pretreated with [D-Trp^6]LH-RH for 60 days. The testes of 9 rats pretreated with the agonist and of 8 untreated rats were then irradiated with 415 rads. Two months later, one testis was removed from every rat and 4 months after the irradiation, all the animals were sacrificed. The testes of rats pretreated with [D-Trp^6]LH-RH weighed less than those of controls, 2 and 4 months after cessation of treatment, but the difference was not significant (Table 3). In contrast, the testes of irradiated rats, whether pretreated or not with [D-Trp^6]LH-RH, showed a significant decrease in weights, both at 2 and 4 months after irradiation. LH levels were elevated in irradiated rats, but not in the group that was pretreated and then irradiated.

Testes removed 2 or 4 months after irradiation showed small tubules with a greatly decreased number of germinal cells and signs of pyknosis and vacuolar degeneration. Some tubules had only Sertoli cells. Four months after irradiation, few tubules had recovered. In the group pretreated with [D-Trp^6]LH-RH and irradiated with 415 rads, the seminiferous tubules 4 months later were still small and contained a decreased number of germinal cells with signs of atrophy and absence of spermatogenic activity. Some tubules in the peripheral areas were larger and had a greater number of spermatogonia and spermatocytes. Thus, in this experiment, as in the previous experiment, pretreatment with agonist [D-Trp^6]LH-RH provided only a partial gonadal protection against x-radiation.

DISCUSSION

In the studies described here, we investigated whether a prolonged pretreatment with the agonists or the antagonists of LH-RH would protect the testes from injury inflicted by x-radiation. We found that the testes of rats pretreated with the antagonistic analog showed a full recovery of spermatogenesis 3 months after exposure to 415 rads of x-radiation. These testes could be considered as essentially normal and completely recovered. The pretreatment with LH-RH antagonist also protected the germinal cells of the testes against the dose of 622 rads, since in this group, too, there was a striking recovery of spermatogenesis. Only a small percentage of tubules was still depopulated, but these tubules contained at least spermatogonia, suggesting that it was only a matter of time for a complete recovery. The dose of antagonist used, 1000 μg/kg, was based on our studies on the depression of spermatogenesis in male rats (19). Thus, these histological examinations conclusively demonstrated that the rats pretreated with the LH-RH antagonist were protected against the deleterious effects of the x-rays on the germinal cells.

The agonist [D-Trp^6]LH-RH could provide, at most, only a partial protection against radiation under conditions used by us. Although the testicular damage 1–2 months after radiation in the group pretreated with [D-Trp^6]LH-RH was greater than in rats that received only the radiation, in time the recovery of the testes was better in rats that were pretreated with the agonist.

Although the LH-RH antagonists act on the same LH-RH receptors as do the LH-RH agonists and reversibly suppress fertility, their action does not lead to down-regulation of receptors (20). The mechanisms of the paradoxical antigonadal function of the LH-RH agonists varies with the species and duration of treatment (10–20). The inhibition of testicular cells obtained in this study after 60 days of treatment with microcapsules of [D-Trp^6]LH-RH appeared to be less complete than that seen in rats with Dunning R3327H prostate cancer treated for 3 months or longer. Thus, it is possible that after a longer time of pretreatment with LH-RH agonists and an extended period of recovery, a protection against radiation-induced testicular damage could be demonstrated. There are also marked differences among species in the sensitivity to radiation and the rate of recovery (1, 3, 5, 9). Nseyo *et al.* (9) reported a recovery of spermatogenesis in a dog pretreated with the agonist Buserelin 6 months after exposure to 150 rads.

Continued research with LH-RH antagonists and possibly even the agonists could lead to a new class of radiation protectors (24). Radioprotective agents used at present are toxic and have side effects (24, 25). Since LH-RH agonists are already used clinically, and soon LH-RH antagonists may also be utilized, our findings suggest the merit of continued exploration of LH-RH analogs as possible gonadal radioprotectors.

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