D-Tryptophan-6 analog of luteinizing hormone-releasing hormone as a protective agent against testicular damage caused by cyclophosphamide in baboons

(chemotherapeutic agents/prevention of gonadal damage/subhuman primates)

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ABSTRACT   Possible protective effects of the agonist [D-Trp⁶]LH-RH (the D-tryptophan-6 analog of luteinizing hormone-releasing hormone) against testicular damage caused by cyclophosphamide (Cytoxan) were investigated in subhuman primates. Three adult male baboons (*Papio anubis*) were first subjected to normal semen evaluation by using electroejaculation. The average baseline count for the animals ranged from 95.7×10^6 to 585.7×10^6 sperm per ml with 90% normal forms and 85% motility with excellent rapid forward progression. After baseline evaluations, two of the animals were treated with daily subcutaneous injections of 0.5 mg of the agonist [D-Trp⁶]LH-RH. There was an initial rise in serum testosterone after 1 week, but testosterone fell to castration values at 1 month and continued at these levels during treatment with the agonist. There was also an initial rise in sperm concentration 1 month after treatment was started, but after 2 months the animals were azoospermic. After 13 weeks of therapy with [D-Trp⁶]LH-RH, these two baboons and a third untreated control animal were given cyclophosphamide at a dose of about 3 mg/kg of body weight per day for 4 months. The two animals pretreated with [D-Trp⁶]LH-RH, continued to receive this agonist until 1 week after the last dose of Cytoxan. In one of the two baboons treated with Cytoxan and the LH-RH agonist, the white blood count fell below 4000 per μl, and the dose of Cytoxan had to be reduced to 1.5 mg/kg per day for 12 days. The control animal developed azoospermia after 4 months of treatment with cyclophosphamide, and serum testosterone increased while sperm count decreased. Four weeks after the agonist was stopped, serum testosterone in both animals pretreated with [D-Trp⁶]LH-RH returned to normal levels. The control animal showed a small amount of nonmotile sperm 2.5 months after cessation of treatment, but after 9 months remains oligospermic with poor sperm motility. In one of the animals treated with LH-RH agonist, semen analysis returned to normal pretreatment values 8 months after withdrawal of treatment. The other animal remains oligospermic 10 months after therapy, but the motility is improving. These preliminary results suggest that treatment with LH-RH agonist might decrease the gonadal damage caused by some chemotherapeutic agents.

Chemotherapeutic agents have produced beneficial results in the therapy of carcinomas, particularly in some blood and lymph node malignancies, but side effects of these agents can be quite severe. One of these side effects is gonadal suppression leading to oligospermia or azoospermia (1–8). Under some conditions azoospermia appears to be permanent and results in sterility. Cyclophosphamide, a cyclic nitrogen mustard phosphamide ester and an alkylating agent,

has been used for cancer chemotherapy and treatment of the nephrotic syndrome, particularly in childhood. Oligospermia and azoospermia have been reported after the use of this agent (2, 3, 6–8).

There may be differences in the sensitivity of the prepubertal, pubertal, and adult testis to chemotherapy with alkylating agents (1–3, 8). The prepubertal testis may be more resistant to the effects of alkylating agents than is the adult gonad (2). Prepubertal boys and boys in early puberty treated with cyclophosphamide for nephrosis appear to have normal follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels after therapy as well as normal levels of testosterone. These effects seem to be also dependent on the dosage and duration of therapy (1, 3, 8). In one study cyclophosphamide, which was used for chemotherapy in a dose of 50–100 mg daily in adult males for 4 months, induced azoospermia (3). In another study with cyclophosphamide in children with nephrosis, results showed two distinct patterns. Four males who received 2–4 mg/kg of body weight per day for 49–60 days had normal semen, while four patients who received 2–5 mg/kg per day for 89–489 days showed azoospermia.

LH-releasing hormone (LH-RH) agonists and antagonists effectively but reversibly inhibit the pituitary–gonadal function and fertility in animals (9–20). Chronic administration of superactive agonists of LH-RH produces dramatic inhibitory effects through a process of down-regulation of receptors, desensitization of pituitary gonadotrophs, and suppression of gonads (9–15, 20). In male animals this is manifested by a decrease in the weights of testes, seminal vesicles, and prostate; inhibition of spermatogenesis; and a decrease in plasma LH, FSH, and testosterone levels (9–11, 13, 15, 21–27). In men, a persistent suppression of Leydig-cell function manifested by a decrease in serum testosterone and dihydrotestosterone levels and inhibition of spermatogenesis have been observed after chronic administration of the agonists [D-Trp⁶]LH-RH (the D-tryptophan-6 analog of LH-RH) or [D-Ser(Buᵗ)⁶]des-(Gly-NH₂)¹⁰-LH-RH ethylamide (9–11, 16, 21, 22, 28, 29). The inhibition of reproductive functions in animals and human beings of both sexes by agonists of LH-RH is reversible and fertility is restored after treatment is stopped (9–11, 14, 16, 19, 21, 22, 30).

Dividing cells are more sensitive to alkylating agents than are resting cells (2, 11). It is not known whether temporary suppression of spermatogenesis during chemotherapy would protect against the development of testicular failure. Our study carried out in subhuman primates was based on the assumption that administration of the agonist [D-Trp⁶]LH-RH for several weeks before therapy with an antineoplastic

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Abbreviations: LH, luteinizing hormone; FSH, follicle-stimulating hormone; [D-Trp⁶]LH-RH, D-tryptophan-6 analog of luteinizing hormone-releasing hormone; CBC, complete blood count.

agent was started, during the chemotherapy, and for 1 week after it would suppress testicular function and maintain inactive gonads. Consequently, chemotherapy might damage the testes temporarily inhibited by LH-RH analogs to a lesser extent than in the case of the unsuppressed gonads.

Recently Glode *et al.* (31) reported that temporary inhibition of the pituitary–gonadal axis by administration of [D-Leu^6]LH-RH ethylamide protected mouse testes from cyclophosphamide-induced damage (31). However, these conclusions were based on only two treated mice (31), and more experimental work is needed to confirm and extend these initial findings.

METHODS

Three adult male baboons (*Papio anubis*) were used in the study. Baseline complete blood count (CBC) and serum testosterone levels were determined, and three semen analyses were obtained by rectal probe ejaculation after ketamine injection (1 mg/kg).

After baseline studies, two animals were given daily subcutaneous injections of 0.5 mg (approximately 22.7 μg/kg) of a potent LH-RH agonist ([D-Trp^6]LH-RH). [D-Trp^6]LH-RH was synthesized by solid-phase methods and was supplied by Debiopharm SA, (Lausanne, Switzerland). Serum testosterone levels were measured by RIA at 24 hr, 1 week, and then monthly. Semen analyses were performed monthly. After 13 weeks of [D-Trp^6]LH-RH therapy, the

semen sample revealed azoospermia, and the two animals were given cyclophosphamide daily at 3 mg/kg intramuscularly in addition to daily injections of the agonist. The control animal received cyclophosphamide only. CBC and platelet counts were obtained on a weekly basis. If the leukocyte count dropped below 4000 per μl or platelets below 100,000, the cyclophosphamide dose was halved and the CBC and platelet counts were checked every other day until improved. If the leukocyte count dropped below 3000/μl or platelets below 50,000, the drug was withheld and daily blood analyses were continued until hematological findings improved. The cyclophosphamide treatment was continued for 4 months, and the [D-Trp^6]LH-RH was stopped 1 week later. After treatment, serum testosterone levels in all animals were determined every 2 weeks until normal pretherapy levels were obtained on two subsequent measurements. Semen analyses were obtained monthly. Seven months after treatment, the animals were sedated with ketamine, and bilateral testicular biopsies were obtained under general anesthesia for light and electron microscopy.

RESULTS

The average baseline sperm count for the [D-Trp^6]LH-RH-treated animals was 585.7 × 10^6 (7594) and 95.7 × 10^6 (D386) sperm per ml. The baseline count in the control animal (E028) was 195 × 10^6 sperm per ml. Each animal initially had 90% normal forms and 85% (grade 4/4) motility. The two
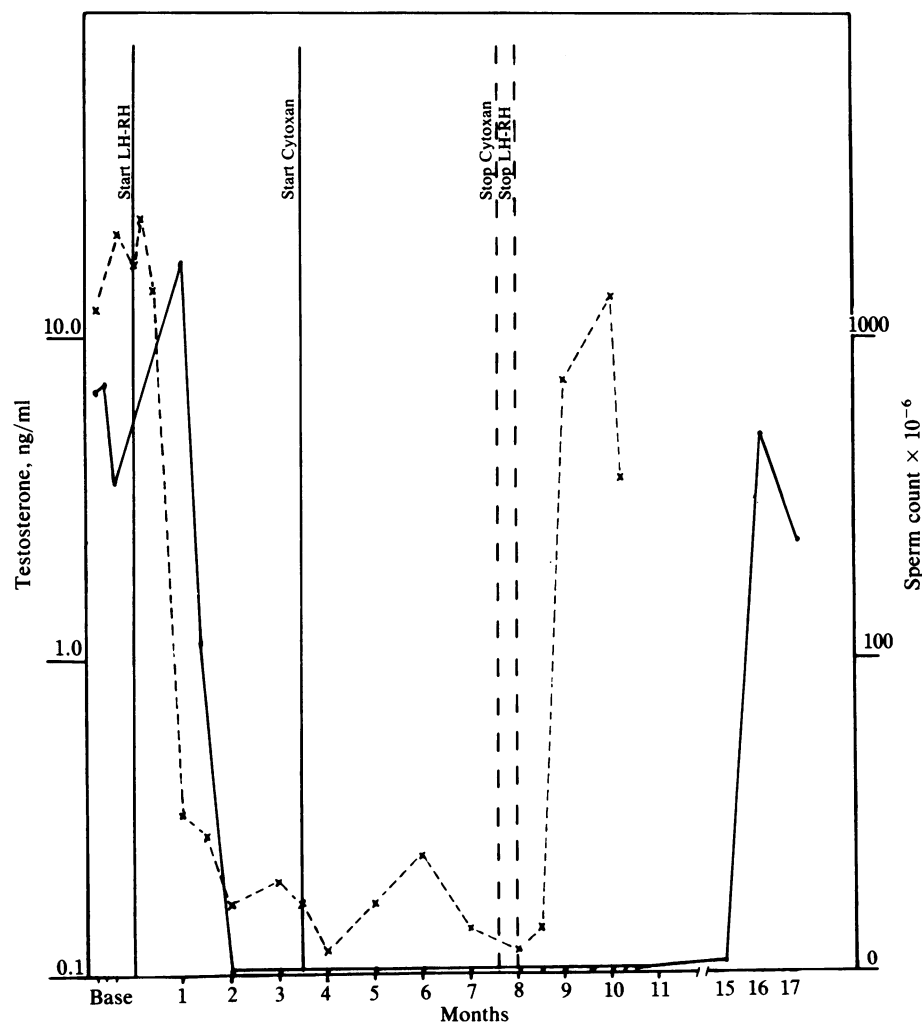

Fig. 1.   Serum testosterone (---) and sperm concentration (—) in animal no. 7594 during the period of the study. This baboon was pretreated with the agonist [D-Trp^6]LH-RH for 13 weeks prior to 4 months of therapy with cyclophosphamide.

animals receiving the LH-RH agonist showed an initial rise in the sperm count after 1 month of treatment (Figs. 1 and 2). This was followed by a precipitous decline, both animals becoming azoospermic at 2 months. The animals remained azoospermic during the remainder of the period of treatment with [D-Trp⁶]LH-RH as well as during 4 months of combined administration of cyclophosphamide and the agonist.

The average baseline serum testosterone levels for animals 7594 and D386 were 17.0 and 12.2 ng/ml, respectively (Figs. 1 and 2). Testosterone showed an initial rise 24 hr after the LH-RH agonist was started, but after 1 week the testosterone levels began to fall, and after 1 month the values were only 0.33 and 0.28 ng/ml. Serum testosterone remained in this range throughout the cyclophosphamide-treatment period. Serum testosterone showed no change 2 weeks after therapy, but by 4 weeks the level of testosterone for both animals was back to baseline.

These two animals received a total dose of approximately 106 mg of the LH-RH agonist, 48 mg being administered prior to cyclophosphamide treatment. The animals 7594 and D386 and the control baboon E028 were treated with cyclophosphamide for 4 months. One of the animals treated with the LH-RH agonist (7594) and the control (E028) tolerated the full experimental dose of cyclophosphamide without incident, receiving 2.9 and 2.7 mg/kg per day for a total of 7 and 7.5 g, respectively. However, in one of the analog-treated animals (D386), the leukocyte count decreased to less than 4000 per μl on several occasions. This

animal received only 4 g (2.01 mg/kg per day) of cyclophosphamide, the drug being withheld for a total of 12 days during the 4-month period of treatment.

The control animal (E028) showed essentially no changes in the sperm count 1 month after the treatment with Cytoxan was started. However, with each successive month of therapy with Cytoxan, the sperm count declined, and at 4 months, when cyclophosphamide was stopped, the animal was azoospermic. The serum testosterone level increased as the sperm count decreased (Fig. 3). Semen analyses for the two LH-RH agonist-treated animals showed azoospermia until 7 months elapsed after withdrawal of treatment. At that time both animals had a minimal number of poorly motile sperm. Eight months after cessation of treatment, the sperm count in one of the animals became completely normal (588 × 10⁶ sperm per ml; 85% motile; grade 4/4). The other baboon remains oligospermic (7.4 × 10⁶ sperm per ml) 10 months after treatment, but the motility has increased to 40% of a grade 3/4. The control animal showed a small amount of nonmotile sperm 2.5 months after cyclophosphamide was stopped, but 9 months after cessation of treatment, it still remains oligospermic (4.75 × 10⁶ sperm per ml) with poorly motile sperm (Figs. 1–3).

Testicular biopsies were normal in one of the LH-RH agonist-treated animals that had normal semen analyses except for rare hypospermatogenic tubules. The other agonist-treated animal, which has remained oligospermic, shows approximately one-third of the tubules with moderate-to-
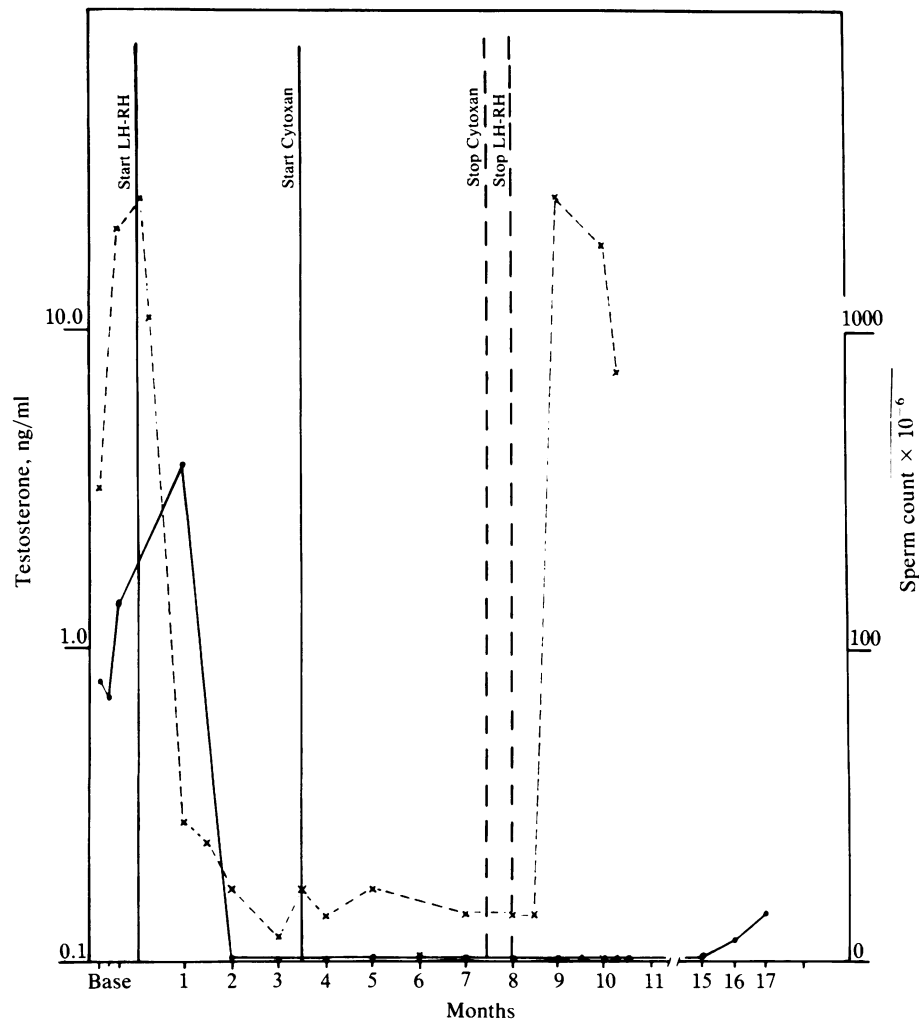

FIG. 2.   Serum testosterone (---) and sperm concentration (—) in animal no. D386 during the period of the study. This baboon was pretreated with the agonist [D-Trp⁶]LH-RH for 13 weeks prior to 4 months of therapy with cyclophosphamide.
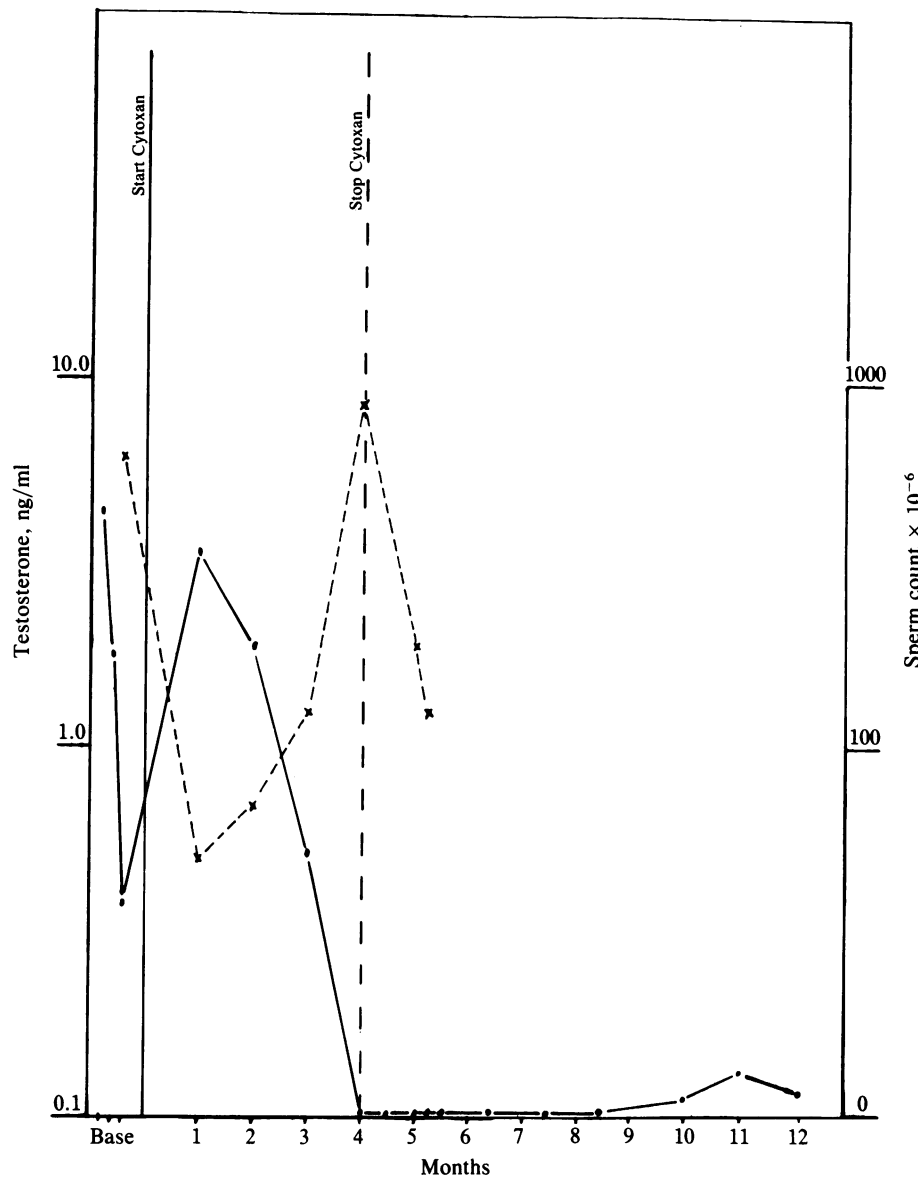
FIG. 3.   Serum testosterone (---) and sperm concentration (—) in animal no. E028 during the period of the study. This baboon received cyclophosphamide alone for 4 months.

severe hypospermatogenesis. Tubular basement membranes are normal in both animals. The biopsies of the control animal showed tubules with hypospermatogenesis and no basement-membrane abnormalities.

DISCUSSION

Although the results in this pilot study are not conclusive, they suggest that the agonist [D-Trp⁶]LH-RH may exert a protective effect against testicular failure caused by the chemotherapeutic agent cyclophosphamide. In one of the two animals treated daily with 0.5 mg of the agonist, administration of cyclophosphamide for 4 months did not damage the testicles, since sperm counts have returned to normal pretherapy levels. The other animal pretreated with [D-Trp⁶]LH-RH is oligospermic 10 months after both agents were stopped, but the motility is improving, and there has been a steady increase in sperm concentration. In this animal bone marrow was affected by cyclophosphamide because the leukocyte count declined to less than 4000 per μl. This may indicate increased sensitivity of this particular animal to cyclophosphamide, accounting for greater testicular damage. It should be noted that sperm were not found in either

of these animals until the month 7 after withdrawal of treatment with the agonist. This period is somewhat longer than has been reported previously for the subhuman primates (27, 32). However, our doses of agonist were higher and the duration of treatment was more extended than in those studies (27, 32). The control animal developed sperm in the ejaculate 2.5 months after cyclophosphamide was stopped but remains oligospermic after 9 months, and the sperm has shown poor motility. An interesting observation in the two animals treated with the LH-RH agonist was an initial increase in sperm concentration. This factor should be further investigated in the subhuman primate model as a possible treatment for oligospermia in infertility.

Our work shows that the subhuman primate is a suitable model for such a study and can be treated with therapeutic doses of the antineoplastic agent equivalent to those that produce testicular failure in humans. The response of the animals to the LH-RH agonist is reversible and similar to that observed in man.

Although our results are encouraging, the hypothesis of achieving a possible protection against testicular failure induced by chemotherapeutic agents by means of chronic treatment with an LH-RH agonist has to be tested again in a

larger group of animals and with lower doses of the agonist, similar to those used in clinical regimens for patients with prostate and mammary cancer.

Note Added in Proof. One year after cyclophosphamide was stopped, semen analyses in the two baboons treated with [D-Trp^6]-LH-RH indicate that animal 7594 has remained completely normal with an average sperm concentration of 761.5×10^6 per ml with a 75% (grade 4) motility. Animal D386 has now an average sperm concentration of 223×10^6 per ml with a low 1% (grade 1) motility. The control animal E028 not treated with [D-Trp^6]LH-RH, 9 months after cyclophosphamide was stopped, has an average sperm concentration of 45.8×10^6 per ml with no motility. These additional findings reinforce our hypothesis on the protective effect of [D-Trp^6]LH-RH against testicular damage inflicted by chemotherapy.

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