Potentiation of the Gonadotoxicity of Cytoxin in the Dog by Adjuvant Treatment with a Luteinizing Hormone-releasing Hormone Agonist

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ABSTRACT
This study evaluates the effect on spermatogenesis of coadministration of Cytoxin (cyclophosphamide) and nafarelin, a luteinizing hormone-releasing hormone agonist. Nafarelin causes complete aspermatogenesis in dogs by interrupting the hypothalamic-pituitary-gonadal axis, which might protect against the testicular cytotoxicity associated with cyclophosphamide. The four treatment groups, each consisting of 2 mature male beagle dogs, were (a) no drug; (b) cyclophosphamide (p.o. 3x weekly for 43 and 48 wk for a total dose of 582 and 709 mg/kg, with dose varying according to weekly hematological profile); (c) nafarelin (2 μg/kg s.c. daily for 48 and 52 wk); and (d) cyclophosphamide plus nafarelin [same schedule as above with cyclophosphamide (570 and 698 mg/kg total dose) starting 7 wk after beginning nafarelin]. Plasma testosterone, spermatogenesis, and ejaculate volume were completely suppressed by nafarelin prior to starting cyclophosphamide. By 2 wk after cessation of treatment (posttreatment, PT), plasma testosterone reached normal levels, and at 5 wk PT ejaculates appeared which reached normal volumes 2 to 3 wk later. Normally motile ejaculated spermatozoa were noted at 6 to 8 wk PT in nafarelin-only-treated animals; normal sperm numbers were reached at 14 wk PT. The animals receiving cyclophosphamide plus nafarelin were azoospermic for the entire 65-wk PT period, and at 65 wk PT no germinal cells were found upon evaluation of testicular histology. Sperm numbers in cyclophosphamide-only-treated animals began to rise 10-11 wk PT and reached 150 × 10⁶ sperm/ejaculate at approximately 65 wk PT (contemporary control dogs had sperm numbers of approximately 300-600 × 10⁶/mL/ejaculate). Spermatogenesis in these cyclophosphamide-only-treated animals was normal in most seminiferous tubules at this time. The addition of nafarelin to cyclophosphamide treatment thus exacerbated the deleterious effects of cyclophosphamide on the testes, suggesting caution for use of such a protocol clinically.

INTRODUCTION
There has been considerable interest in the possibility of using an LHRH² analogue treatment to protect against the testicular cytotoxicity, and resulting depression of sperm count and infertility, associated with cancer chemotherapy (1-3). This possibility is based upon three observations, (a) The cytotoxicity of chemotherapeutic agents is expressed preferentially in rapidly dividing tissues (4, 5); (b) it has been claimed that a better prognosis for subsequent fertility is present with chemotherapy in prepubertal boys than in sexually mature men (6); and (c) LHRH analogues are able to disrupt the pituitary-testicular axis resulting in aspermatogenesis in some species (7).

First report evaluated this possibility in mice using concomitant treatment with cyclophosphamide and the LHRH agonist (n-Leu⁶, Pro³-NHET) LHRH, and concluded that protection was obtained (8, 9). A subsequent study has not confirmed this finding (10), and it has been noted that mice are particularly insensitive to the pituitary down-regulatory effects of LHRH agonists (11, 12). Indeed, review of these early studies shows that the mice were not rendered infertile, much less returned to a prepubertal state. However, small-scale studies in other species continue to be reported which tend to support the concept (13, 14).

To evaluate properly this concept it is important to select a species in which total suppression of spermatogenesis can be achieved with the selected LHRH agonist. The rat is unsuitable for such studies since LHRH agonists cause only focal suppression of spermatogenesis (15) and complete suppression of fertility is not achievable (16). Male macaques are poorly sensitive to the LHRH receptor down-regulation and desensitization effects of LHRH agonists (17, 18) requiring continuous infusion of these agents for full suppression (7). In the dog, however, daily injection of relatively low doses of an LHRH agonist rapidly induces complete azoospermatogenesis through aspermatogenesis (7).

The present study therefore readdresses in the dog the concept that returning the testis to a prepubescent quiescent state with an LHRH agonist will protect against the testicular cytotoxic effects of a chemotherapeutic agent.

MATERIALS AND METHODS
Mature male beagle dogs were housed in segregated quarters, quarantined from the remainder of the dog colony, but were otherwise kept under normal laboratory conditions in 12 h light (06:00-18:00 h)/24 h. They were fed commercial dog food and given water ad libitum. Blood was drawn weekly from the cephalic vein, using sterile technique, for creatinine and BUN determination to monitor renal function, and for hematological evaluation (complete blood count and platelet count) to monitor bone marrow function.

Testicular volumes were determined and ejaculates were collected at weekly intervals (7). Duration and volume of ejaculation were determined and recorded. Sperm motility was assessed visually, using a light microscope at 400X, within 30 min after ejaculation was complete, having kept the semen at 37°C. Motility was expressed as the percentage of motile spermatozoa in a minimum of five microscope fields. A minimum of 10 spermatozoa was observed when very low sperm numbers were present. Sperm numbers were determined by counting diluted or concentrated aliquots in a white blood cell hemacytometer. A small aliquot of undiluted or concentrated semen was smeared onto a microscope slide, air dried, fixed in absolute ethanol, and stained with Papanicolaou stain. The slide was allowed to dry and a coverslip was permanently affixed to the slide with Preservslide (Matheson Coleman & Bell, Norwood, OH). Sperm head length and width were measured directly from a screen onto which the slide was projected using a bright field microscope and video camera (19). Sperm heads were traced with the cursor of a digitizer, the data from which were analyzed by computer.

There were four experimental groups. Two dogs were untreated (group 1), receiving only gelatin capsules. Two dogs were treated with cyclophosphamide (Cytoxin; Mead Johnson, Evansville, IN) p.o. in gelatin capsules (3.5-6.5 mg/kg 3 times weekly, with dose regulated according to the hematological profile) for 43 and 48 wk for a total of 582 and 709 mg/kg cyclophosphamide, respectively (group 2). Nine and 5 wk after cessation of treatment, respectively, the animals were necropsied from the remainder of the dog colony, but were otherwise kept under normal laboratory conditions in 12 h light (06:00-18:00 h)/24 h. They were fed commercial dog food and given water ad libitum.

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1 To whom requests for reprints should be addressed.
2 The abbreviations used are: LHRH, luteinizing hormone-releasing hormone; BUN, blood urea nitrogen; WBC, white blood cell.

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presses spermatogenesis in dogs (7). Four dogs received nafarelin daily as s.c. injections (2 µg/kg/day), two for 48 wk and two for 52 wk. Seven wk after starting nafarelin treatment, two of the dogs (one from each group) also received cyclophosphamide treatment, as described above, for 42 and 45 wk (570 mg/kg and 698 mg/kg total cyclophosphamide). Cyclophosphamide and nafarelin therapy were stopped on the same day. Hemicastrations were performed 9 and 5 wk after cessation of treatment, respectively. The remaining testis of all animals was removed at 65–69 wk after cessation of treatment and fixed in Bouin’s solution, sectioned at 4–6 µm, stained with hematoxylin and eosin, and evaluated under light microscopy.

RESULTS

Total sperm numbers per ejaculate, percent sperm motility and testicular volume as they varied with time are summarized for each of the four treatment groups in Figs. 1 to 3.

Sperm numbers and motility were in the same range in all animals prior to treatment (Figs. 1–3) and continued in this range for control animals. At the end of cyclophosphamide treatment alone, total sperm numbers per ejaculate for the two dogs had declined to $1.9 \times 10^5$ and $7.9 \times 10^4$, at which point the sperm were immotile (Fig. 1). Total sperm numbers reached a nadir of approximately $2 \times 10^5$ at 7 wk after stopping treatment and rose slowly starting 11–13 wk after the last drug dose [approximately 1½ spermatogenic cycles in the dog (20)]. Motile sperm were not observed until 22–24 wk after cessation of treatment. In one of the dogs a normal level of sperm motility was achieved by 45 wk and normal forward progression at about 48 wk postdosing. The other dog had approximately 50% sperm with forward progression by 62 wk after stopping treatment. Total sperm numbers in these 2 animals were 20–50% of the pretreatment values or values in control animals at approximately 65 wk after dosing was stopped.

Testicular volumes began to decline at approximately 20 wk of treatment in cyclophosphamide-treated animals (Fig. 1) and continued to decrease until, at cessation of treatment, they were approximately 40% of pretreatment and control values. Testicular volume was approximately 60% that of the control dogs at 65 wk after cessation of dosing.

After 4 wk of nafarelin treatment, none of the 4 animals produced an ejaculate (Figs. 2 and 3, as shown by absence of data points during most of the treatment and early recovery periods) although erection was achieved and maintained, and anal contractions (normally associated with ejaculation) were evident. All 4 animals did produce ejaculates, which were azoospermic, by the fifth wk after cessation of treatment. The two animals receiving nafarelin alone had spermatooza, which were normally motile, in their ejaculates starting 6–8 wk after withdrawal of therapy. No sperm were found over the 65- to 69-wk observation period after cessation of treatment in the ejaculates of animals which had received cyclophosphamide in addition to nafarelin.

Testes volumes declined over the first few wk of nafarelin treatment (Figs. 2 and 3) and reached a nadir after 8 wk of dosing, at approximately 30% of pretreatment values. Within 6 wk after cessation of treatment, testes volumes began to increase in all animals, and at the time of hemicastration (5 and 9 wk), testes volumes were 50–75% of pretreatment values. The remaining testes of nafarelin-treated animals continued to increase in size until approximately 12 wk after cessation of treatment at which time they had reached pretreatment values. Animals receiving combination treatment did not return to more than 60% of pretreatment values.

No effect of cyclophosphamide on ejaculated sperm morphology was detected using sperm morphometry. Mean sperm...
length, mean sperm width, length times width, and length over width were unchanged compared to animals not receiving cyclophosphamide.

Plasma testosterone levels remained within the normal range throughout the entire study in control animals and those receiving cyclophosphamide alone (data not shown). Semen volume was also unchanged (data not shown), reflecting these normal testosterone levels. Plasma testosterone decreased to approximately 0.3 ng/ml by day 2 of nafarelin treatment in 3 of 4 animals. After some variability during the following several wk, testosterone was suppressed to castrate levels for the rest of the treatment period in all 4 animals. Following cessation of treatment, plasma testosterone rose to normal levels within 12 days (Fig. 4).

Normal testicular histology was observed in control animals as shown in Fig. 5, A and B. In the case of the cyclophosphamide-treated animals, the dog that was hemicastrated 5 wk after the last drug dose had atrophic seminiferous tubules containing Sertoli cells and occasional spermatogonia. In the other dog, hemicastrated 9 wk after the cessation of treatment, there was also extensive seminiferous tubule atrophy (Fig. 5C), but in approximately 10% of the tubules there were germinal cells present at each stage of spermatogenesis up to spermatid. Leydig cells were present in both dogs. At 67 wk after stopping treatment, all spermatogenic elements were present in more than 50% of tubules (Fig. 5D).

The two animals treated with nafarelin alone had all stages of spermatogenesis present in most seminiferous tubules at 5 and 9 wk after cessation of treatment, and Sertoli and Leydig cells were present (Fig. 5E). Normal testicular histology was present in these animals at 65 wk after stopping treatment (Fig. 5F).

Mostly atrophic seminiferous tubules were observed at 5 and 9 wk after cessation of combination treatment with cyclophosphamide and nafarelin (Fig. 5G), although there were numerous tubules with mitotic activity and occasional tubules with all stages of spermatogenesis. However, no germinal cells could be found in the testes of these animals at 65 to 69 wk, although Sertoli and Leydig cells were present (Fig. 5H).

BUN and creatinine remained normal in all animals receiving cyclophosphamide, indicating the absence of renal damage. On the other hand, bone marrow function was significantly affected. WBC counts were decreased after 2-3 wk of treatment and were at leukopenic levels (<6000 x 10^3/mm³) after approximately 5 wk and at severely leukopenic levels (<2000 x 10^3/mm³) after about 34 wk. WBC counts of ≥6000 x 10^3/mm³ were regained 9 wk after cessation of treatment. Platelet counts decreased more slowly than WBC counts, reached levels of <150 x 10^3/mm³ at approximately 20 wk of treatment, and went as low as 30 x 10^3/mm³. At 43 wk after stopping cyclophosphamide treatment, platelet counts were 203 and 267 x 10^3/mm³ in cyclophosphamide-treated animals, and 205 and 222 x 10^3/mm³ in cyclophosphamide + nafarelin-treated animals. Those dogs receiving empty capsules or nafarelin alone had platelet counts at this time of 265 and 293 x 10^3/mm³, and 244 and 261 x 10^3/mm³, respectively. None of these 4 animals showed a decrease in platelet count during empty capsule or nafarelin treatment.

**DISCUSSION**

Long-term therapy with cyclophosphamide in male beagles caused severe oligozoospermia, which was associated with virtually complete suppression of spermatogenesis and approximately 50% reduction in testicular volume. In addition, the declining sperm count was associated with a decline in sperm motility to zero. The lack of sperm motility was not due to androgen deprivation as the circulating testosterone levels were unchanged by treatment. Following cessation of treatment, severe oligozoospermia and absent sperm motility persisted for 2-3 spermatogenic cycles, followed by a slow, progressive return to slightly oligospermic sperm counts and normal sperm motility.

These findings are in keeping with those in young men. In retrospective studies of men treated with cyclophosphamide there is a reported decrease in testicular volume, severe oligozoospermia, and infertility (21). Abnormal sperm morphology and motility have been noted (22), and there is also depletion of the testicular germinal epithelium (22-25).
Fig. 5. Photomicrographs of testes from dogs at time of hemicastration at 5 to 9 wk after stopping treatment (left) and at removal of the remaining testis at 62 to 67 wk (right); hematoxylin and eosin stain. Animals received no drug (A and B), cyclophosphamide (C and D), nafarelin (E and F), or cyclophosphamide and nafarelin (G and H) as detailed in text. Internal scale marker in H is for all panels.
Severe depression of myeloid function was observed in the dogs, with greatly reduced WBC and platelet counts. In addition, there was evidence of cyclophosphamide-induced cystitis (bloody urine). These effects are also noted in men. However, renal function appeared unaffected in the present study, as measured by BUN and creatinine levels.

Nafarelin treatment completely suppressed both gametogenesis and steroidogenesis in the testis as previously published (7). Since ejaculates were not obtainable from the nafarelin-treated dogs immediately prior to start of chemotherapy, additive effects on sperm count could not be monitored. However, the slope of the decline in testicular volumes in the combination-treated animals resembled that seen in the nafarelin-treated dogs rather than the animals receiving chemotherapy alone.

Return of testicular function following cessation of treatment with nafarelin was prompt. This confirms and extends a previous report which evaluated effects of 42 days of treatment (7) and underscores the reversibility and lack of toxicity of long-term treatment with this LHRH agonist. Concomitant treatment with nafarelin and cyclophosphamide on the other hand resulted in azoospermia which persisted for at least 65 wk after stopping treatment, at which time the animals were castrated. No germ cells could be found at histological evaluation. The gonadotoxic effects of cyclophosphamide were thus clearly potentiated in the presence of nafarelin.

Other studies report various and conflicting results regarding the possible use of LHRH agonists for gonadal protection during chemotherapy. Whereas two groups (13, 14) have presented results which confirm the original reports of Gloyd et al. (8, 9), Handelsman et al. showed an exacerbation of gonadotoxicity (26). The attempt of Da Cunha et al. to repeat the original results in mice was not successful (10). Clinically, the application of this concept has not met with success as yet (27-29).

The finding in the present study of a repopulation of the spermatogenic elements at the end of combined treatment with cyclophosphamide and nafarelin, followed by a rededication of the germinal epithelium later in the posttreatment phase, suggests that the treatment regimen might be at fault. It is possible that there was accumulation of the chemotherapeutic agent or its metabolites in testicular or other tissues, which subsequently exerted cytotoxic effects at the gonad. Protocols involving continuation of the LHRH analogue treatment until any tissue stores of the cytotoxins are depleted might be more successful. Alternatively, cessation of long-term LHRH antagonist, as opposed to agonist, treatment has been associated with a significant rebound of gonadotropins and testicular function in primates (30). Therefore, use of an LHRH antagonist in conjunction with chemotherapy may better restore or preserve gonadal function.

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REFERENCES

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