Effect of GnRH conjugated to pokeweed antiviral protein on reproductive function in adult male dogs

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This study evaluated the effect of a GnRH analogue conjugated to the cytotoxin, pokeweed antiviral protein (PAP), on reproductive function in adult, male dogs. Four dogs received 0.0042 mg GnRH–PAP kg^{−1} hourly for 36 h, and four other dogs received 0.1 mg GnRH–PAP kg^{−1} as one bolus injection daily for three consecutive days. One dog received a single bolus (0.1 mg kg^{−1}). Three adult male dogs received GnRH without the PAP conjugate, as controls. Twenty-five weeks after the initial treatment, all treated dogs received 0.1 mg GnRH–PAP kg^{−1} as a single administration, whereas dogs in the control group received 0.0045 mg kg^{−1} of the GnRH analogue. Serum concentrations of testosterone and LH were determined by radioimmunoassay, and testis size was measured for 9 months after treatment. Stimulation tests (5 µg GnRH kg^{−1}) were used to evaluate LH release (−15, 0, 30, 60, 90, 120 min), which was assessed by

measuring area under the curve. Serum testosterone concentrations were significantly lower ($P < 0.05$) after treatment in the bolus and hourly groups than in the control group. Testosterone concentrations fell to less than 50 pg ml^{−1} in three of four dogs in the bolus group and one of four dogs in the hourly group by week 8–9 after treatment. Basal LH was lower ($P < 0.05$) in the bolus and hourly groups than in the control group between weeks 0 and 33 after treatment. Treatment with GnRH–PAP reduced ($P < 0.05$) LH release after GnRH stimulation in the bolus and hourly groups compared with the control group. Testis volume was lower ($P < 0.05$) in all treated versus control dogs. In conclusion, administration of the conjugate GnRH–PAP at a 25 week interval resulted in a major disruption of reproductive parameters in male dogs; this effect was maintained for 11–12 weeks after a second injection of GnRH–PAP.

Introduction

Reproductive function in mammalian species is regulated by the hypothalamic–pituitary–gonadal axis. Although there are considerable differences among species in the underlying mechanisms that regulate reproductive function, certain aspects of neuroendocrine regulation of reproduction are very highly conserved in mammals. Specifically, the pituitary production of two hormones, FSH and LH, plays a critical role in the development and regulation of gonadal functions, including the production of gametes and sex steroid hormones. Pituitary secretion of FSH and LH is in turn controlled by GnRH, which acts as the ‘master’ hormone regulating reproductive function. GnRH is a decapeptide synthesized in neuronal cell bodies distributed diffusely across the basal forebrain and is secreted from neuronal terminals in the median eminence. Once secreted, GnRH enters the portal vessels and is then transported to the anterior pituitary via the hypothalamic–pituitary portal system. GnRH has

been a useful tool for studying basic aspects of the hypothalamo–pituitary–gonadal axis as well as a target for the regulation of reproduction.

It has been recognized for many years that GnRH is a very desirable target for contraception or sterilization in humans and in animals (Ladd, 1993). Because GnRH acts through specific, high-affinity receptors on gonadotrophs, the specificity of the interaction of GnRH with its receptors on these cells has been used to target a variety of cytotoxic agents to disrupt pituitary gonadotrophs. The approach of coupling cytotoxic agents to GnRH has been used in the therapy of a number of tumours that possess GnRH receptors and thereby provide a specific targeting mechanism for the cytotoxic agent (Nagy *et al.*, 1996; Kovacs *et al.*, 1997; Nechushtan *et al.*, 1997; Paalyi *et al.*, 1999). The present study used a novel approach on the basis of GnRH conjugated to a cytotoxic protein, pokeweed antiviral protein (PAP), to directly target GnRH receptors on gonadotrophs.

The administration of GnRH–PAP offers the potential to target and disrupt the secretion of the reproductive hormones, LH and FSH, by the pituitary gland without adversely affecting the secretion of other pituitary

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hormones. The potential success of this type of approach has been demonstrated by tests conducted in sheep in which GnRH-PAP reduced the secretion of LH for a period of at least 6 months after administration (Nett *et al.*, 1999). The application of this approach in companion animals provides a unique opportunity to specifically target and eliminate reproductive function by disrupting both behavioural aspects of reproduction as well as gamete production in both males and females. The objective of the study reported herein was to evaluate the feasibility of this approach in adult male dogs by determining the effect of GnRH-PAP on testosterone secretion, LH release and testis size.

Materials and Methods

Twelve adult male dogs (mixed breed) were provided by Animal Resource Services, University of California, Davis. Animal research was conducted under a protocol approved by the University of California Animal Use and Care Administrative Advisory Committee.

The GnRH-PAP conjugate was prepared as described below. GnRH used in the stimulation test was obtained from Calbiochem (La Jolla, CA); $^3\text{[H]}$ -testosterone was obtained from New England Nuclear (Boston, MA); and testosterone antibody (S250) was kindly supplied by G. Niswender.

Purification of PAP

PAP was purified as described by Irvin (1983), with the following minor modifications. Briefly, pokeweed leaves were homogenized with 5 mmol sodium phosphate l^{-1} , pH 6.5, in a Waring blender. The extract containing PAP was filtered through a strainer and centrifuged at 10 000 *g* for 30 min. PAP in the supernatant was then purified by ammonium sulphate precipitation (40–100%), Fractogel EMD-CM (EM Science, Gibbstown, NJ) ion-exchange chromatography and DEAE Sepharose CL-6B (Sigma, St Louis, MO) chromatography. The purified protein was dialysed against water and lyophilized. Purity of PAP was assessed by SDS-PAGE (12% reducing gel).

Conjugation of D-Lys⁶-GnRH with PAP

Thiolation of D-Lys⁶-GnRH. The D-Lys⁶-GnRH analogue (9.3 mmol l^{-1}) was iminothiolated with 2-iminothiolane (Pierce, Rockford, IL; 11.3 mmol l^{-1}) in the presence of *N,N*-diisopropylethylamine to produce SH-GnRH. The yield of the SH-GnRH was 60–70%. The progress of the reaction was monitored by C18 high performance liquid chromatography. The reaction was performed in methanol for 2 h at room temperature (25°C). Methanol was evaporated under nitrogen after the reaction. The final product was analysed by mass spectroscopy.

Introduction of a maleimidobutyryl group into PAP. PAP (0.6 mmol l^{-1}) was dissolved in deoxygenated

0.05 mol sodium phosphate l^{-1} , 0.1 mol NaCl l^{-1} , 1 mmol EDTA l^{-1} , pH 7.4, and mixed with a crosslinker, *N*-(γ -maleimidobutyryloxy)sulphosuccinimide (Pierce; 1.8 mmol l^{-1}). The reaction was allowed to proceed for 60 min at room temperature. This solution (8 ml) was then mixed with the thiolated D-Lys⁶-GnRH (3.6 mmol l^{-1} , 4 ml) dissolved in the same (deoxygenated) phosphate buffer. The molar PAP:peptide ratio was 1:3. After incubation for 50 min at room temperature, Cys-SH was added and the reaction mixture was acidified to pH 4.5–5.0 with glacial acetic acid. Some precipitate was removed by centrifugation and the supernatant was applied to a BioGel P-60 column equilibrated in 0.1 mol NaCl l^{-1} . The protein fraction (with a molecular weight in the range 29 000–32 000) containing GnRH-PAP conjugate was desalted on Sephadex G-25 and lyophilized. SDS-PAGE (12% reducing gel) analysis and mass spectrometry showed that the final product (as expected) was heterogeneous and contained three major fractions: PAP with one GnRH molecule attached, PAP with two GnRH molecules attached, and unconjugated PAP. Unconjugated PAP in the final product was estimated to be in the range 25–35%.

Experimental design

Nine adult male dogs were administered GnRH-PAP twice, 25 weeks apart. For the first treatment, dogs were divided into three groups according to the dose and frequency of injection. In the first group, four dogs received 0.0042 mg GnRH-PAP kg^{-1} hourly for 36 h (equivalent daily dose of 0.1 mg kg^{-1}). An i.v. catheter (20 GA, 1.88 in, Becton Dickinson Inc., Sandy, UT) was placed on the foreleg of each dog during this 36 h study. Heparinized saline was injected regularly to maintain the patency of catheters. Dogs were examined daily for 7 days after treatment to detect any local injection reactions or possible systemic effects associated with the treatment. In the second group, four dogs received 0.1 mg GnRH-PAP kg^{-1} as one i.v. bolus injection daily for three consecutive days. A single dog received one i.v. bolus injection of GnRH-PAP (0.1 mg kg^{-1}). Three adult male dogs received D-Lys⁶-GnRH analogue (0.0045 mg kg^{-1} i.v., equivalent amount of GnRH activity to that in the GnRH-PAP) without the PAP conjugate, as controls. Twenty-five weeks after the initial treatment, all treated dogs received 0.1 mg GnRH-PAP kg^{-1} as a single administration, whereas control dogs received 0.0045 mg GnRH analogue kg^{-1} .

Dogs were examined daily for 7 days after treatment to detect any local injection reactions or possible systemic effects associated with the treatment. Blood samples were taken before the primary treatment and at weekly intervals. The blood samples were always taken in the morning (09:00 h) to minimize variation. Sera were separated and stored at -20°C for determination of serum testosterone, LH, thyroxine and cortisol by immunoassay.

Testis size and development were measured with a caliper at bi-weekly intervals to determine testis length and width. Concentrations of the circulating gonadotrophin, LH, were determined periodically to evaluate the effect of GnRH-PAP treatment on pituitary function. GnRH stimulation tests ($5 \mu\text{g kg}^{-1}$) were used to evaluate the ability of the pituitary gland to respond to exogenous stimulation with GnRH. Blood samples were taken at -15, 0, 30, 60, 90 and 120 min of GnRH treatment. Blood samples were obtained via indwelling catheters placed in the cephalic vein. LH release was assessed by measuring the area under the curve. Serum testosterone, basal LH and testis size were assessed for 36 weeks after the first GnRH-PAP treatment.

Endocrine assays

Sera were separated and stored at -20°C for endocrine assays. Serum LH was measured in a standard double-antibody radioimmunoassay (Nett *et al.*, 1975). The interassay coefficient of variation (CV) for LH assays was 12.75%. The intra-assay CV was $3.21 \pm 1.16\%$. The limit of sensitivity for LH assays was 0.79 ng ml^{-1} . Serum testosterone was assessed using a radioimmunoassay described by Shille *et al.* (1979), with some modification. Briefly, testosterone concentrations were determined in diethyl ether extracts. The samples were evaporated to dryness and re-suspended in 0.1% gelatin-PBS. The tracer solution was added immediately after the antibody and samples were incubated overnight at 4°C . Separation of the antibody-bound steroids was performed by adding dextran-coated charcoal. The interassay CV for testosterone assays was 6.74%. The intra-assay CV was $10.71 \pm 9.30\%$. The limit of sensitivity for testosterone assays was 18.9 pg ml^{-1} . Cortisol was assayed using an enzymeimmunoassay (Munro and Stabenfeldt, 1985). Thyroxine assay was performed using a radioimmunoassay kit from Diagnostic Products Corporation (Los Angeles, CA).

Statistical analysis

Data were analysed by repeated-measures ANOVA (Statview; SAS Institute, Cary, NC). Data for testis volume were normalized to pre-treatment values on the basis of the mean testis volume for four monthly determinations before treatment. Data for LH release after GnRH stimulation were evaluated as area under the curve and were log-transformed before analysis. Data are expressed as mean $\pm$ SEM.

Results

LH release

There were effects of treatment ($P=0.06$) and time ($P<0.01$) on basal LH concentrations. Basal LH concentrations were lower ($P<0.05$) in the bolus and hourly

Fig. 1. Basal serum LH concentrations in male dogs treated with GnRH-pokeweed antiviral protein (GnRH-PAP) or GnRH alone, from week 0 to weeks 35–36 after treatment. Four dogs received $0.0042 \text{ mg GnRH-PAP kg}^{-1}$ hourly for 36 h (■). Four dogs received 0.1 mg GnRH-PAP as one bolus injection daily for three consecutive days (●). One dog received a single bolus ($0.1 \text{ mg GnRH-PAP kg}^{-1}$; ▲). Three dogs received GnRH without the PAP conjugate, as controls (○). Arrow indicates time of GnRH-PAP or control injections.

Fig. 2. Serum LH concentrations in male dogs in response to a GnRH stimulation test at weeks 1, 3, 6, 12, 20, 27 and 32 after treatment. Four dogs received $0.0042 \text{ mg GnRH-PAP kg}^{-1}$ hourly for 36 h (■). Four dogs received 0.1 mg GnRH-PAP as one bolus injection daily for three consecutive days (□). One dog received a single bolus ($0.1 \text{ mg GnRH-PAP kg}^{-1}$; ▨). Three dogs received GnRH without the PAP conjugate, as controls (□).

groups than in the control group (Fig. 1). Basal LH concentrations decreased ($P<0.05$) from 3.85 ± 0.86 to $1.11 \pm 0.25 \text{ ng ml}^{-1}$ in the bolus group and from 2.37 ± 0.51 to $1.03 \pm 0.22 \text{ ng ml}^{-1}$ in the hourly group between week 0 and weeks 14–15 after treatment (Fig. 1). By week 20 after the primary treatment, basal LH concentrations had increased in the treated animals. Serum LH concentrations again declined after the administration of GnRH-PAP in treated animals at week 25 (Fig. 1).

There were treatment ($P<0.05$) and time ($P<0.01$) effects on the pituitary release of LH in response to a GnRH challenge (Fig. 2). Release of LH subsequent to

Fig. 3. Serum testosterone concentrations in male dogs treated with GnRH-pokeweed antiviral protein (GnRH-PAP) or GnRH alone, from week 0 to weeks 35–36 after treatment. Four dogs received 0.0042 mg GnRH-PAP kg⁻¹ hourly for 36 h (■). Four dogs received 0.1 mg GnRH-PAP as one bolus injection daily for three consecutive days (●). One dog received a single bolus (0.1 mg GnRH-PAP kg⁻¹; ▲). Three dogs received GnRH without the PAP conjugate, as controls (○). Arrows indicate time of GnRH-PAP or control injections.

Fig. 4. Testis volume in male dogs treated with GnRH-pokeweed antiviral protein (GnRH-PAP; ●) or GnRH alone (control; ○), from week 0 to weeks 35–36 after treatment. Four dogs received 0.0042 mg GnRH-PAP kg⁻¹ hourly for 36 h. Four dogs received 0.1 mg GnRH-PAP as one bolus injection daily for three consecutive days. One dog received a single bolus (0.1 mg GnRH-PAP kg⁻¹). Three dogs received GnRH without the PAP conjugate, as controls. Arrows indicate time of GnRH-PAP or control injections.

administration of GnRH was lower ($P < 0.05$) in the bolus group than in the control or hourly groups. Treatment with GnRH-PAP reduced ($P < 0.05$) LH release in the bolus and hourly groups after GnRH stimulation to 20% and 48%, respectively, of area under the curve values for the control group at week 12. However, at week 20, LH release after GnRH stimulation in the bolus and hourly groups was 84% and 89%, respectively, of values for the control group (Fig. 2). By week 32 after treatment (that is,

Fig. 5. Serum cortisol concentrations in male dogs treated with GnRH-pokeweed antiviral protein (GnRH-PAP; □, $n = 9$) or GnRH alone (control; ■, $n = 3$) at weeks 2, 3, 6 and 20 after treatment.

Fig. 6. Serum thyroxine concentrations in male dogs treated with GnRH-pokeweed antiviral protein GnRH-PAP; □, $n = 9$) or GnRH alone (control; ■, $n = 3$) from week 0 to week 10 after treatment.

7 weeks after the second treatment with GnRH-PAP), LH release after GnRH stimulation in the bolus and hourly groups was 22% and 30%, respectively, of values for the control group.

Serum testosterone

There were effects ($P < 0.05$) of treatment on serum testosterone concentrations, and bolus and hourly groups had lower ($P < 0.05$) serum testosterone concentrations than the control group (Fig. 3). Serum testosterone concentrations decreased by the third week after GnRH-PAP treatment and were less than 50 pg ml⁻¹ in three of four dogs in the bolus group and in one of four dogs in the hourly group by week 8–9 after treatment. Testosterone concentrations at week 14–15 after treatment were 11-fold and fivefold lower in the bolus and hourly groups, respectively, than in the control group (Fig. 3). However, by weeks 19–20, most treated dogs showed a partial recovery of testosterone secretion (Fig. 3), although testosterone concentrations remained lower than in the control group. Serum testosterone concentrations decreased again after the second treatment with GnRH-PAP

and were lower at $291 \pm 141 \text{ pg ml}^{-1}$ versus $5396 \pm 276 \text{ pg ml}^{-1}$, 2 months after the second injection.

Testis size

There were effects of treatment ($P < 0.05$) and time ($P < 0.01$) on testis volume (Fig. 4). Testis volume was reduced to 56% of baseline by weeks 14–15 in treated animals and 46% of baseline by weeks 35–36 after initial treatment.

Changes in peripheral cortisol and thyroxine

There was no significant effect of GnRH-PAP treatment on either serum cortisol or thyroxine concentrations, indicating that GnRH-PAP treatment was specific and did not adversely affect corticotroph or thyrotroph functions (Figs 5 and 6).

Discussion

The high specificity and affinity of GnRH for its receptor provides a unique method for delivery of agents to pituitary gonadotrophs that express GnRH receptors. GnRH acts via G-protein coupled receptors on gonadotrophs to stimulate the synthesis and the exocytotic secretion of gonadotrophins. Sustained exposure to GnRH is known to reduce GnRH-stimulated gonadotrophin secretion probably through GnRH receptor internalization and downregulation or uncoupling of signalling and alterations in gonadotrophins (Nett *et al.*, 1981; Conn *et al.*, 1987). GnRH receptors are known to be sequestered from the plasma membrane and internalized via clathrin-coated vesicles after stimulation and binding. The internalization of GnRH receptors in this case allows delivery of the GnRH-PAP conjugate into the gonadotrophs, where the cytotoxin disrupts protein synthesis and results in cell death. PAP, a 30 kDa plant protein isolated from the leaves or seeds of *Phytolacca americana*, displays a broad spectrum of antiviral activity against different plant and mammalian viruses (Tomlinson *et al.*, 1974; Aron and Irvin, 1980), including the human immunodeficiency virus HIV-1 (Zarling *et al.*, 1990; Uckun *et al.*, 1998, 1999). Conjugates of PAP and anti-CD7 have been used in human and primate trials for therapy of HIV, and the safety of such preparations has been established (Uckun *et al.*, 1999). PAP is an RNA *N*-glycosidase, which specifically removes an adenine residue from a highly conserved loop in the large ribosomal RNA (Endo *et al.*, 1988). This depurination of the SR loop results in irreversible inhibition at the translocation step (Kurinov *et al.*, 1999) by impairing both the elongation factor 1-dependent binding of aminoacyl tRNA and the GTP-dependent binding of elongation factor-2 to the affected ribosome. This inactivates the ribosomes and inhibits protein synthesis, resulting in cell death.

Because GnRH binds with high affinity (K_d value about $10^{-9} \text{ mol l}^{-1}$, Srkalovic *et al.*, 1990) to its receptor on the gonadotrophs, the interaction of GnRH with its receptors has been used to target cytotoxic agents to disrupt these cells within the pituitary. The approach of coupling cytotoxins to GnRH has been used in the therapy of a number of tumours that possess GnRH receptors and thereby provide a specific target for the linked cytotoxin (Nagy *et al.*, 1996; Kovacs *et al.*, 1997; Nechushtan *et al.*, 1997; Paalyi *et al.*, 1999). The administration of an analogue of doxorubicin, linked to GnRH, transiently reduced the pituitary secretion of LH with minimal effects on the secretion of growth hormone or thyroid stimulating hormone from the pituitary (Kovacs *et al.*, 1997).

In vitro studies of a recombinant GnRH-PAP fusion protein demonstrate that the cytotoxicity of this preparation is limited to tumour cell lines that express the GnRH receptor (Schlick *et al.*, 2000). Treatment with either GnRH alone or PAP alone does not induce cytotoxicity in cells expressing the GnRH receptor. Specificity of the cytotoxic response with GnRH-PAP was confirmed by the competitive inhibition of cytotoxicity between free GnRH and GnRH-PAP (Schlick *et al.*, 2000). The report of Schlick *et al.* (2000) confirms that the effects of GnRH-PAP are mediated via GnRH receptors.

The present study demonstrates that GnRH conjugated to PAP disrupts the hypothalamo-pituitary-gonadal axis in adult male dogs. By the third week after treatment, there was a reduction in testis size, serum testosterone and LH concentrations, as well as a decreased release of LH in response to GnRH stimulation. There was no significant difference between the two regimens used (bolus versus hourly), although the dogs that received the bolus injection appeared to show a better response. The dog that received a single bolus also had the lowest LH and testosterone concentrations after GnRH-PAP treatment.

The suppression of LH release from the pituitary gland appeared to persist for approximately 5 months after the initial treatment. After that time, there was an increase in basal LH, LH response to GnRH stimulation and serum testosterone concentrations. Administration of a second GnRH-PAP treatment at week 25 again resulted in a suppression of the pituitary release of LH, which persisted to the end of the study at week 36. A subset of treated animals was examined beyond the end of the study at week 36 to week 52 (data not presented). In these animals, basal LH, serum testosterone concentrations and testis size increased to values comparable to the control group by week 52. These data indicate that administration of GnRH-PAP to intact male dogs suppressed pituitary gonadotrophs for approximately 5–6 months after administration.

In the present study, no adverse side effects were noted subsequent to treatment of adult male dogs with GnRH-PAP other than a transient ($< 24 \text{ h}$) arthralgia in

some animals. In addition, there was no significant effect on either serum cortisol or thyroxine concentrations, indicating that GnRH–PAP treatment was specific and did not affect corticotroph or thyrotroph functions. Further studies need to address whether the GnRH–PAP-induced gonadotrophin inhibition has any effects on other pituitary cells such as somatotrophs or lactotrophs.

In conclusion, the administration of GnRH–PAP to adult male dogs in this study resulted in a major disruption of reproductive parameters by the third week after treatment. Administration of GnRH–PAP once per day for 3 days appeared to result in a greater suppression of pituitary LH release than did hourly administration for 36 h of an equivalent total dosage. Although GnRH–PAP suppressed pituitary release of LH with a concomitant reduction in serum testosterone concentrations and testis size, there was an apparent recovery of pituitary function at approximately 5 months after the first and second administration of GnRH–PAP. It should be emphasized that this trial utilized a single dosage of GnRH–PAP that constituted the best estimate of an effective dosage. Further studies are required to determine whether this approach may be useful to disrupt reproductive function in this species permanently and should include a more thorough dose-ranging study.

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