GnRH and LH Challenge-Test in Male Dogs Treated with a GnRH Agonist Implants

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Abstract

Twenty mature male dogs of mixed breed were randomized divided into five groups of four animals each. Pituitary desensitization was measured by responses to GnRH and LH challenge-test. Group 1 (control): dogs received blank implants (placebo) and were injected with GnRH and LH on days 15, 25, 40 and 100 day (the interval between injection of GnRH and LH was 24 hours). Groups 2–5 received 6 mg deslorelin implants. Group 2 was given an injection on GnRH on day 15 and bovine LH on day 16 after implantation. Groups 3–5 had similar tests on days 25, 40 and 100 with GnRH and on days 16, 26, 41 and 101 with bovine LH, respectively. The level of LH and testosterone to the GnRH and LH challenge showed significant different between each level of controls and all levels at all days tested in treated dogs (P<0.001). These experiments illustrate that the implantation of GnRH agonist deslorelin as a slow release formulation in dogs, led to desensitization of the pituitary gonadotrophs as well as the testicular Leydig cells started at day 25 of deslorelin implantation.

Key words: Deslorelin, GnRH, testosterone, LH

Introduction

Chronic treatment with GnRH agonists desensitises the pituitary gonadotrophs, blocks the stimulatory effects of endogenous GnRH and suppresses gonadotrophin synthesis and release in rams (Lincoln et al. 1986), rhesus monkeys (Mann et al. 1984), rats (Ward et al. 1989), humans (Weinbauer et al. 1990) and dogs (Junaidi et al. 2003, Trigg et al. 2001). In those species in which GnRH agonists suppress the reproductive axis, there is an expected insensitivity to exogenous GnRH (Lincoln et al, 1986, Mann et al, 1984), or insensitivity of testicular to exogenous LH.

LH release response to a GnRH challenge test has been reported as the best available measure of pituitary desensitization during GnRH agonist treatment (Scheele et al. 1996, Seager 1986). This test has been investigated in agonist treated rams by Lincoln et al., (1986), who found that the injection of GnRH in rams treated with high levels of a GnRH agonist failed to stimulate the release of LH indicating desensitisation of the pituitary gonadotrophs. A similar finding has been reported by (D’Occhio and Aspden 1996) in bulls treated with GnRH agonist: the pituitary showed a classical down-regulation response and proved insensitive to endogenous and exogenous GnRH. Furthermore, rams treated with a GnRH agonist did not exhibit testicular sensitisation, as hCG administration induced a significant rise in serum testosterone (Lincoln et al., 1986). However, little is known about the degree of pituitary gonadotroph and Leydig cell desensitisation in dogs treated with long-term slow release GnRH agonists. The aim of the present study was to characterize pituitary responses to intravenous administration of a GnRH analogue and testicular responses to intravenous administration of LH in dogs treated with slow release implants containing 6 mg GnRH agonist deslorelin.

Materials and Methods

All procedures complied with the NH & MRC Code of Animal of Ethics. The Animal Ethics and Experimentation Committee of Murdoch University approved the study.
Animals
We used 20 mature male dogs (age range 2–5 years, body mass 15–22 kg) of mixed breed that were housed indoors at night and allowed outdoors for 2–6 h in large, shaded, sandy runs during the day. Semen samples were collected from each of the dogs three times before treatment to ascertain their semen quality. All dogs were fed with biscuits (about 600 g per dog daily) and canned meat (about 400 g per dog, three times per week: Pedigree® PAL®, Uncle Ben’s of Australia) and had access to water ad libitum.

GnRH agonist
Cylindrical implants (0.23 × 15.2 mm) contained 6 mg Deslorelin (D-Trp6-Pro9-des-Gly10-LHRH ethylamide) and were pre-packaged in a purpose-developed disposable implanter incorporating a 13 g needle that was sterilized by e-beam irradiation. They were injected subcutaneously in the neck of the dogs between the shoulder blades under aseptic conditions.

Exogenous GnRH and LH
GnRH analogue (Fertagyl®, Intervet) and bovine LH (prepared by Peter Stanton, Prince Henry’s Institute of Medical Research, Clayton, VIC, Australia) were used in this study. Plasma LH and testosterone responses after each challenge test were determined by administering (i.v.) 5 µg/kg body weight of GnRH and 0.5 µg/kg body weight of bovine LH.

Experimental Design
Twenty male adult dogs were randomly assigned, to five groups of four animals each. Group 1 (controls): dogs received blank implants (placebo) and were injected with GnRH and LH on days 15, 25, 40 and 100 day (the interval between injection of GnRH and LH was 24 hours). Groups 2–5 received 6 mg deslorelin implants. Group 2 was given an injection on GnRH on day 15 and bovine LH on day 16 after implantation. Groups 3–5 had similar tests on days 25, 40 and 100 with GnRH and on days 16, 26, 41 and 101 with bovine LH, respectively. Full profiles of hormone concentrations, ejaculate volumes and semen quality and testicular volumes were measured over the challenge period to determine the degree of testicular dysfunction.

Blood Sampling
Before taking serial blood samples in both experiment, an indwelling intravenous cannula R 16 G (Cavafix®, B. Braun Medical, SA Barcelona) was introduced approximately 20 cm into a jugular vein and taped in position. The catheter was filled with heparin solution (10 i.u./mL ) in 0.15 M NaCl. Blood samples (4 mL) were taken by aspirating the heparin solution, collecting the blood sample, and then refilling the catheter with heparin solution after the sample had been withdrawn. To determine the effect of deslorelin, blood was collected intensively before implantation for every 20 min for 2 h, after implantation at 20 min intervals for 4 h, hourly for 6 h, daily for 5 d, then twice weekly throughout the study. In both experiments, to measure the response of exogenous GnRH and LH blood samples were taken 40, 20 and 10 min before implantation; at the time, every 10 min for 90 min, then every 20 min to 150 min. The blood was collected into sterile 5 mL heparinized tubes (Vacutainer®, Becton Dickinson Vacutainer Systems Europe, Meylan, Cedex-France). The plasma was separated and stored at −20°C until assay.

Semen Evaluation And Testicular Volume
The length, width and height of each testis were measured using callipers as described by (Love et al. 1991). The testicular volume was measured weekly, throughout the study.
Weekly semen collections were made from all dogs until the end of the experiments at 100 days. Semen was collected by hand manipulation without a teaser bitch as described by (Seager 1986).

Hormone Assays
The LH assay was based on a polyclonal antiserum (AFP 8311890) to canine LH (cLH) that had been raised in a rabbit. Canine LH was also used for radioiodination (AFP-5214B) and reference (AFP-5216B). These reagents were supplied by Pituitary Hormones and Antiserum Center (Harbor–UCLA Medical Center, Torrance, CA, USA). The limit of detection of the standard curve was 0.23 ± 0.12 ng/tube and the non-specific binding was always less than 6%. All samples were tested in the same assay run.

Plasma testosterone was measured using a non-extraction radioimmunoassay developed in our laboratory (Hötzel et al. 1995). The antiserum (R3) was raised in our laboratory against testosterone-3-CMO-HSA (carboxymethylxime-human serum albumin). The preparation 4-androsten-17β-ol-3-one (10 µg L⁻¹; Sigma Chemicals, Midland, MI, USA) was used for reference and 1,2,6,7-3H-testosterone (specific activity = 3.33 TBq mM⁻¹) (Amersham, Sydney, NSW, Australia), was used as the tracer. Cross-reactions were 100% with testosterone, 70% with dihydrotestosterone, 3.7% with androstenedione, and less than 0.05% with progesterone, oestradiol-17β, oestrone and oestriol. The limit of detection was 0.2 ± 0.1 ng/mL⁻¹. All samples were tested in the same assay run.
Statistical Analyses

'Baseline levels' are defined as the means of samples taken at –40, –20, –10, and 0 minutes before injection of GnRH or bovine LH. 'Response levels' are defined as the means of samples taken every 10 min to 80 min and 'return to baseline levels' are defined as the mean of samples taken every 20 min from 90 min to 150 min of the challenge test.

Analysis of variance for repeated measures with Tukey’s multiple comparison test was used to assess the effect of treatment over time on plasma testosterone and LH concentrations, using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, U.S.A.). Data are presented as means ± SEMs.

Results and Discussion

Plasma Concentrations of LH and Testosterone During Treatment with Deslorelin

The mean plasma LH and testosterone concentrations of the dogs during the deslorelin implantation are shown in Figure 1. Twenty minutes after implantation with deslorelin, plasma LH concentration increased and reached a peak at 40 min. The concentrations started declining thereafter and were very low (0.06 ± 0.03 ng/ml) by the second week of treatment. Thereafter, plasma LH became undetectable (< 0.06 ng/ml) until the last challenge trials (100 days of implantation).

Plasma testosterone concentrations followed patterns parallel to those of LH concentrations, showing an initial rise, followed by a gradual decline. The testosterone concentrations were significantly suppressed by 25 days of implantation and from 30 to 100 days of implantation the concentrations were undetectable.

Semen Characteristics

Semen parameters changed rapidly and no ejaculate could be obtained within 25 days after implantation in two dogs, and within 40 days in the other eight. The mean testicular volume decreased progressively starting from day 25 of implantation. The lowest volume was reached at 100 day of implantation when mean testis volume dropped to 30% of the pre-treatment value.

Release of LH and testosterone in response to exogenous GnRH in control dogs and in dogs treated with deslorelin implants after 15, 25, 40 and 100 days of implantation

The patterns of LH and testosterone release after i.v administration of 5 µg/kg of GnRH to control dogs that received placebo implants were used to compare treatment groups and are shown in Figure 2. The summary data together with statistical analyses are included in Table 1.
Fig. 2. Comparison of mean (± SEM) responses of LH and testosterone to 5 μg/kg of GnRH analogue (Fertagyl®, Intervet) between control dogs (placebo implants, n = 4) and implanted dogs (n = 4) at 15, 25, 40 and 100 days after implantation.

Table 1. Hormonal Responses to GnRH Challenge-Test in the Dogs Treated with 6 mg Deslorelin Implants (means ± SEM)

<table>
<thead>
<tr>
<th>Days</th>
<th>LH (ng/mL)</th>
<th>Testosterone (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline level</td>
<td>Response level</td>
</tr>
<tr>
<td>Control #)</td>
<td>0.73 ± 0.03a</td>
<td>2.25 ± 0.45a</td>
</tr>
<tr>
<td>15 day</td>
<td>0.06 ± 0.03b</td>
<td>0.08 ± 0.03b</td>
</tr>
<tr>
<td>25 day</td>
<td>0.01 ± 0.01b</td>
<td>0.01 ± 0.01b</td>
</tr>
<tr>
<td>40 day</td>
<td>0.03 ± 0.01b</td>
<td>0.01 ± 0.01b</td>
</tr>
<tr>
<td>100 day</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Results were significantly different between each level of controls and all levels at all days tested in treated dogs (P < 0.001); # Control (mean ± SEM) dogs in all day tested; * Undetectable

An LH peak occurred 20 minutes after the i.v injection of GnRH in control dogs. A corresponding testosterone peak was observed at 40 minutes. The response of LH and testosterone after i.v injection of GnRH in the dogs after 15 days of implantation was significantly reduced (P < 0.05) compared with control dogs. There was no response of LH and testosterone in the dogs challenged with GnRH at 25, 40 and 100 days of deslorelin implantation.

Release of testosterone in response to LH injection in control dogs and in the dogs treated with deslorelin implant after 15, 25, 40 and 100 days of deslorelin implantation

Changes in plasma concentrations of testosterone after i.v. injection with 0.5 μg/kg bovine LH at day 16, 26, 41 and 101 in control dogs and in dogs received deslorelin at are shown in Fig. 3. Summary data with statistical analyses are shown in Table 2.
Fig. 3. Comparison of mean (± SEM) responses of testosterone to 0.5 μg/kg bw of bovine LH between control dogs (placebo implant, n = 4) and dogs implanted with the GnRH agonist deslorelin at 16, 26, 41 and 101 days after implantation.

A testosterone peak occurred 40 minutes after i.v. injection of bovine LH in the dogs without implants. The increase in the plasma concentration of testosterone following the i.v. injection of bovine LH was significantly lower in the deslorelin implanted dogs on all days tested (P < 0.001).

Table 2. Testosterone Responses to Bovine LH Challenge-Test in Dogs Treated with 6 mg Deslorelin Implant (mean ± SEM)

<table>
<thead>
<tr>
<th>Days of Challenge</th>
<th>Testosterone (ng/ml)</th>
<th>Baseline level</th>
<th>Response level</th>
<th>Return to baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control #</td>
<td></td>
<td>0.72 ±0.18a</td>
<td>2.58 ±0.38a</td>
<td>2.26 ±0.2a</td>
</tr>
<tr>
<td>15 days</td>
<td></td>
<td>0.12±0.02b</td>
<td>0.73 ±0.16b</td>
<td>0.22 ±0.04b</td>
</tr>
<tr>
<td>25 days</td>
<td>*</td>
<td>0.10 ±0.03c</td>
<td>0.06 ±0.03b</td>
<td></td>
</tr>
<tr>
<td>40 days</td>
<td>*</td>
<td>0.13 ±0.04c</td>
<td>0.16 ±0.04b</td>
<td></td>
</tr>
<tr>
<td>100 days</td>
<td>*</td>
<td>0.03 ±0.02c</td>
<td>0.04±0.01b</td>
<td></td>
</tr>
</tbody>
</table>

Results were significantly different between each level of control and all levels at all day tested in the treated dogs (P < 0.001); # Control (means ± SEM) dogs in all day tested; * Undetectable

Implantation of the GnRH agonist deslorelin in dogs led to an initial stimulation followed by an inhibition of LH and testosterone secretion. This confirms previous finding that deslorelin can be used effectively to suppress testicular function in dogs (Trigg et al., 2001; Junaidi, et al, 2003). These data were also consistent with those reported by (Vickery et al. 1984) and (Inaba et al. 1996) in dogs using nafarelin and leuprolide acetate, respectively.

On Day 15, plasma concentrations of both LH and testosterone were low (0.06 ± 0.01 ng/ml and 0.25 ± 0.01 ng/ml, respectively). Testicular volumes were slightly decreased and the number of sperm abnormalities had doubled. However, the semen volume, sperm concentration and sperm motility were not changed. The injection of 5 μg/kg exogenous GnRH on Day 15 after implantation with 6 mg deslorelin resulted in a small LH response that was accompanied by a significant rise in testosterone. However, the peak hormone values in these responses were significantly lower than in the control dogs. The injection of 0.5 μg/kg exogenous bovine LH on Day 16 cause testosterone to rise from 0.25 ± 0.01 ng/ml to 1.13 ± 0.2 ng/ml. Thus deslorelin had partially desensitized the pituitary gonadotrophs without markedly affecting the responsiveness of the testicular Leydig cells at Day 15 after implantation with deslorelin.

On Day 25, plasma concentrations of LH became undetectable while the mean plasma concentration of testosterone dropped to 0.08 ± 0.01 ng/ml. These hormonal changes were accompanied by decreased ejaculate volume, sperm concentration and sperm motility. The injection of 5 μg/kg of exogenous GnRH failed to caused an LH response, and plasma testosterone concentrations also remained low. Similar findings have been reported for agonist treated rams by Lincoln et al., (1986). The injection of a physiological dose of GnRH failed to stimulate the release of LH, and the injection of 0.5 μg/kg bovine LH produced no testosterone response on Day 26, whereas control dogs (blank implant) showed an acute testosterone response (2.4 ± 0.3 ng/ml). This indicates that the testicular Leydig cells had no capacity to respond to the injection of bovine LH in the deslorelin-treated dogs after 26 days. This was in agreement with that reported by (Fraser and Lincoln 1980) for rams.

On Days 40 and 100, both plasma LH and testosterone concentrations became undetectable. At this stage, testicular volume dropped to 40% and 30% of pretreatment value, respectively. No semen could be obtained from the dogs. On Days 40 and 100, all treated dogs failed to show an LH response to exogenous GnRH, and plasma testosterone concentrations also remained low and unchanged. Thus by 40 days the gonadotrophs of the anterior pituitary gland had been down-regulated by the constant, slow release of deslorelin. The injection of 0.5 μg/kg of bovine LH at 40 days after deslorelin implantation resulted in no measurable increase in plasma testosterone concentration. By contrast
Lincoln (1986) showed a normal increment in the secretion of testosterone in rams infused with the agonist whereas in this study we found complete desensitization of Leydig cells to LH.

**Conclusion**
These experiments illustrate that the implantation of GnRH agonist deslorelin as a slow release formulation in dogs, led to desensitization of the pituitary gonadotrophs as well as the testicular Leydig cells by 25 days after deslorelin implantation.

**Acknowledgments**
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**References**


