Long-term reversible suppression of oestrus in bitches with nafarelin acetate, a potent LHRH agonist*

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Summary. Adult cyclic beagle bitches were treated for up to 18 months with nafarelin acetate via subcutaneously implanted osmotic pumps, starting during the first week of a pro-oestrous vaginal discharge. The imminent ovulation appeared to be unaffected by treatment, but doses of 8 or 32 µg analogue/day reduced the integrated luteal progesterone values. No new oestrus was detected in 3 bitches during 18 months of treatment with 32 µg/day, which resulted in mean plasma levels of 0.4 ng analogue/ml. A return to oestrus was observed in all 3 bitches between 3 and 18 weeks after cessation of treatment: 2 of the bitches mated at those times and produced normal litters. Another 2 bitches were similarly treated with 32 µg analogue/day; they were mated at the oestrus at start of treatment and dosing was continued for about 63 days. One of the bitches conceived and produced a normal litter. Nafarelin acetate treatment begun during anoestrus resulted in an induced heat 1–2 weeks after the start of treatment. The induced heat consisted of pro-oestrous vaginal discharge, oestrous vaginal cytology, and ovulation (judged by increased circulating levels of progesterone). Three bitches mated at the induced heat and treated for the normal duration of gestation did not litter. Nafarelin treatment of 3 bitches before puberty did not induce signs of oestrus and prevented the occurrence of oestrus through 18 months of treatment. The first oestrus in these bitches occurred 3.5–4 months after cessation of treatment, but mating at that time did not result in pregnancy. These studies have established the feasibility of and dosage requirement for the use of the LHRH agonist as a contraceptive in the bitch.

Introduction

The numbers of unwanted dogs and cats in this country and others indicate the need for methods of pet contraception. While spaying and castration are obviously effective there also appears to be a place for reversible methods. For the bitch and queen, particularly, progestational agents were at one time expected to fill this role (Harris & Wolchuk, 1963). However, unacceptable side effects, specifically of endometritis, pyometritis and mammary nodules (Anderson, Gilmore & Schnelle, 1965; Sokolowski & Zimbelman, 1974), have relegated these compounds to short courses of treatment indicated for postponement of expected oestrus (Burke & Reynolds, 1975). Mibolerone was another possible contraceptive for bitches (Sokolowski & Geng, 1977), but the inconvenience of daily oral administration, high interbreed differences in dosage requirements and the masculinizing effects of this androgenic steroid have combined to impede wide scale usage of the agent.

Luteinizing hormone releasing hormone (LHRH) (Matsuo, Baba, Nair, Arimura & Schally, 1971) and its more potent agonistic analogues can exert antireproductive effects (Vickery, 1982).

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We have been intrigued by the possibility of applying these non-steroidal agents to both male and female pet contraception. Our preliminary studies on the suppression of sexual function and reversibility in male dogs have been reported elsewhere (Vickery *et al.*, 1984a; Vickery & McRae, 1984). In the present study nafarelin acetate (Nestor *et al.*, 1982) was used to evaluate daily dosage requirement for oestrus suppression, fertility after long-term suppression, effect of timing of onset of treatment, and fertility at a drug-induced oestrus.

Materials and Methods

General procedures. Purebred beagles bred at Syntex or obtained from commercial suppliers (LRE Inc., Kalamazoo, MI, U.S.A. and Ridgeland Farms, Mount Horab, WI, U.S.A.) were used. The animals were housed 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*. Throughout the studies the bitches were examined daily for external signs of oestrus, i.e. vulvar swelling and/or sanguineous vaginal discharge. When such signs were observed, or when otherwise called for in specific studies, daily vaginal smears were taken. The smears were obtained using a cotton swab; they were stained with 1% toluidine blue and examined microscopically to identify the cytology consistent with pro-oestrus, oestrus or dioestrus (Holst & Phemister, 1975). Vaginal smears were discontinued after an oestrous period when 3 consecutive days of dioestrous smears had been obtained. The term 'vaginal oestrus' is used when vaginal smears indicated that >25% of the epithelial cells were cornified. Vaginal smears are also termed partly cornified when >25% and <100% of the epithelial cells are cornified, and termed fully cornified when 100% of the epithelial cells are cornified.

To assess fertility, bitches were mated to male beagles previously shown to be fertile. Mating was attempted when the first oestrous smear was obtained. Mating attempts were repeated daily until mating occurred and every 2 or 3 days thereafter until the bitch no longer accepted the male or until the vaginal smear indicated dioestrus. An abdominal palpation was always performed 24–30 days after mating to confirm pregnancy.

Blood samples (~5 ml) were collected from all bitches, at least 3 times weekly, throughout every study. The samples were withdrawn from the cephalic vein into heparinized tubes, placed immediately on ice, and plasma harvested by centrifugation within 2 h. Plasma was stored at –20°C until analysis of progesterone and nafarelin levels by radioimmunoassay.

Osmotic mini-pumps (Alzet: Alza Corporation, Palo Alto, CA, U.S.A.), which deliver a treatment solution at a continuous rate for 14 days (Model 2002) or for 30 days (Model 2ML4) were used to deliver nafarelin acetate ([6-D-(2-naphthyl)-alanine]LHRH, synthesized by Syntex Research). The pumps were filled, under sterile conditions, with the appropriate solution of nafarelin acetate in a vehicle composed of 50% propylene glycol, 50% distilled water. The pumps were implanted subcutaneously in the flank under sterile conditions using local anaesthetic. At 2- or 4-week intervals, depending on the pump model being used, the old pump was removed and replaced with a new freshly filled pump, using similar surgical procedures.

In some studies 32 µg nafarelin were administered once daily by subcutaneous injection. The concentration of injection solution was 100 µg/ml and the injection volume was 0.32 ml. The injection vehicle was an aqueous solution containing 0.1 M-NaH₂PO₄, 0.1 M-NaCl, 0.12% methylparaben, 0.012% propylparaben and 0.9% benzyl alcohol.

Radioimmunoassays. All plasma samples were assayed for progesterone concentrations by a radioimmunoassay (Vickery & McRae, 1980) using ethanol extracts of plasma and rabbit antiserum. The limit of sensitivity for progesterone was 2 pg/tube (0.01 ng/ml plasma), cross-reactivity with 17α-hydroxyprogesterone and 20α-dihydroprogesterone was <1.0% and the inter- and intra-assay variations were 17.2% and 9.0%, respectively.

Selected plasma samples were used to measure nafarelin concentrations by radioimmunoassay (Nerenberg, Foreman, Chu, Chaplin & Kushinsky, 1984) using a rabbit antiserum generated by

immunization with a glutaric acid analogue of nafarelin conjugated to keyhole limpet haemocyanin. The antibodies did not cross-react with LHRH. The tracer was labelled with ^{125}I and charcoal was used to separate the free from bound fractions. The lower limit of the assay was 0.05 ng/ml. Linear regression analysis for the concentration range 0.05–5.00 ng/ml yielded a correlation coefficient of 0.997 and at 0.05 ng/ml the interassay CV was 11.3%. The assay was also validated by HPLC methods (Nerenberg *et al.*, 1984).

Statistics. The areas under the curves for the first luteal-phase progesterone values in Study 1 were plotted as mean $\pm$ s.e.m. The combined values for the 8 and 32 μg groups were compared to the values of the vehicle group by Student's *t* test for unpaired data.

Results

Study 1

Twelve regularly cyclic bitches were treated in groups of 3 for up to 18 months with vehicle or 2, 8 or 32 μg nafarelin/day administered from subcutaneously implanted osmotic pumps. Treatment was begun during the first week after the onset of a sanguineous vaginal discharge. Bitches in which oestrus was suppressed throughout the 18 months of treatment were mated at the first oestrus after cessation of treatment to assess the effect of treatment on subsequent fertility.

The results are presented in Text-fig. 1. The imminent ovulation was unaffected by all doses of nafarelin, as judged by a rise in plasma levels of progesterone. The integrated luteal-phase progesterone values for the bitches receiving 8 or 32 μg nafarelin/day were similar to each other and the mean $\pm$ s.e.m. value was 7.2 ± 0.7 (arbitrary units), which was significantly ($P < 0.01$) lower than the mean $\pm$ s.e.m. value of 13.5 ± 2.0 for the vehicle-treated bitches. Luteal phase progesterone values for the bitches receiving 2 $\mu\text{g}/\text{day}$ were not different from those of the vehicle-treated bitches. Continuous treatment with 2 or 8 $\mu\text{g}/\text{day}$ did not prevent the recurrence of oestrus and the intervals between oestrous periods during treatment were similar to pretreatment intervals (Table 1). However, treatment with 32 μg nafarelin/day completely suppressed the occurrence of oestrus throughout the 18-month treatment. Two bitches treated with 32 $\mu\text{g}/\text{day}$ exhibited vaginal bleeding and vaginal oestrus at 3 and 18 weeks after cessation of treatment; when mated, each conceived and produced normal litters of 5 and 6 pups, respectively. The third bitch exhibited vaginal bleeding and vaginal oestrus at 5 weeks after cessation of treatment but would not mate. Plasma progesterone did not rise at that time, indicating that ovulation did not take place. After 11 weeks, this bitch again exhibited vaginal bleeding and vaginal oestrus which was then accompanied by a normal rise in plasma progesterone. Mating was not attempted at that time. The mean plasma concentrations of nafarelin in bitches receiving 8 or 32 $\mu\text{g}/\text{day}$ during the first 6 weeks of treatment are shown in Text-fig. 2, and averaged 0.2 ng/ml and 0.4 ng/ml, respectively. In some animals changes were observed in the plasma levels of nafarelin which appear to be associated with periods of specific pump implantations.

Study 2

Two regularly cyclic bitches were treated with nafarelin on the 2nd or 3rd day of a sanguineous vaginal discharge. One bitch received once daily s.c. injections of 32 μg nafarelin and the other received continuous subcutaneous administration of 32 μg nafarelin/day via an implanted osmotic pump. Each bitch was mated at oestrus, as described earlier, and treatment was continued for 73 days or until whelping, whichever occurred first.

The bitch treated with continuous infusion of nafarelin, beginning on the 3rd day of vaginal bleeding, mated with a male on the 13th day of vaginal bleeding (Text-fig. 3). Implantation sites were palpable in the uterus 24 days after mating and 9 pups of normal appearance were born 57 days after mating. Treatment was discontinued at the time of whelping and progesterone levels

Text-fig. 2. Plasma concentrations (mean $\pm$ s.e.m.) of nafarelin acetate in beagle bitches receiving continuous subcutaneous administration of nafarelin acetate by osmotic pump.

Table 1. Intervals between oestrous periods of beagle bitches before and during continuous treatment with nafarelin by osmotic pump when treatment was begun at the onset of a spontaneous oestrus

Treatment	Bitch	Intervals between oestrous periods (months)	
		Before treatment*	During treatment†
Vehicle for 9–18 months	1	7, 8.5, 7.5, 8	8, 8
	2	7, 6.5	6
	3	5, 5, 6, 6, 6	6, 7
2 µg nafarelin/day for 7–8 months	4	7, 6, 4	4
	5	6.5, 6, 6, 6	5
8 µg nafarelin/day for 12 months	6	6.5, 6, 6, 6	5.5
	7	6, 6, 6, 6	5, 6
	8	6, 6.5	6
	9	5.5, 7, 6	5, 2.5, 4
32 µg nafarelin/day for 18 months	10	6.5, 6, 5	No oestrus occurred
	11	8	No oestrus occurred
	12	6, 6.5, 7.5, 7	No oestrus occurred

* Before treatment, occurrence of oestrus was based only on presence of vaginal bleeding.

† During treatment, occurrence of oestrus was based on the presence of vaginal bleeding and/or a rise in plasma progesterone to >1 ng/ml.

Text-fig. 1. Plasma progesterone concentrations and occurrence of vaginal bleeding and vaginal cornification during and after continuous treatment of beagle bitches with (a) 2 µg nafarelin acetate/day, (b) 8 µg nafarelin acetate/day, (c) 32 µg nafarelin acetate/day or (d) vehicle alone.

Text-fig. 3. Plasma progesterone concentrations and fertility data for 2 bitches treated with nafarelin at the onset of a spontaneous oestrous period.

showed a normal pattern for a pregnant bitch (Concannon, Hansel & Visek, 1975). The bitch treated with once daily injections of nafarelin beginning on the 2nd day of vaginal bleeding was mated on the 6th, 8th and 10th day of vaginal bleeding (Text-fig. 3). At 28 days after the first mating one uterine implantation site was palpable. However, on the Days 59–64 after mating a dark bloody vaginal discharge was produced but no fetal tissue was identified. On Day 66 an ovariohysterectomy was performed and revealed one area of apparent placentation but no evidence of retained fetuses or placentae. Plasma progesterone levels, although lower than those in the other bitch, were within the normal range for a pregnant bitch until the time of presumed abortion when they dropped to baseline levels of <0.5 ng/ml.

Study 3

Three regularly cyclic bitches were treated continuously with 32 µg nafarelin/day via subcutaneously implanted osmotic pumps, beginning at 92, 96 and 100 days after the onset of oestrus, respectively. Vaginal smears were taken daily from the start of treatment and stopped after 3 consecutive days of dioestrous smears had been obtained after an oestrus. The bitches were mated, as described earlier, at the induced oestrus, and treatment was continued for about 63 days.

At the start of treatment, plasma progesterone concentrations were <0.5 ng/ml in two of the bitches (A and C) and 5.4 ng/ml in the third bitch, B (Text-fig. 4). All 3 bitches had cornified vaginal smears for 8–18 days beginning 4–11 days after the start of treatment. Bitches B and C had a sanguineous vaginal discharge for 10 and 14 days beginning 9 and 12 days after start of treatment. In Bitches A and C plasma progesterone concentrations rose at the time of vaginal oestrus in a fashion similar to that at normal oestrus (Concannon *et al.*, 1975), suggestive of the occurrence of ovulation. In both bitches, however, the luteal phases were markedly shorter than would be expected in an untreated bitch, with plasma progesterone levels falling to <0.5 ng/ml at 30–36 days after the initial rise in plasma progesterone levels. In Bitch B plasma progesterone began to rise about 1 week after vaginal oestrus and rose more gradually than in the other two, also showing an atypical hormone profile for a normal oestrous cycle. All 3 bitches were mated during the time of vaginal oestrus. Uterine palpation at 3.5 to 4 weeks after mating identified two implantation sites in Bitch C and none in Bitches A and B. Treatment was continued in all 3 bitches until Day 54–60

Text-fig. 4. Plasma progesterone concentrations and fertility data for 3 bitches at an oestrus induced by treatment with nafarelin beginning between 92 and 100 days after the onset of a previous oestrus.

after mating. None of the 3 bitches showed any signs of whelping or abortion. It is assumed that conception occurred in only one bitch and that the resulting fetal and placental tissue subsequently resorbed.

Study 4

Three prepubertal bitches were treated with 32 µg nafarelin/day beginning at 4 months of age. Treatment was continued for 18 months. During the first month and the last 6 months nafarelin was given via s.c. osmotic pumps; the remainder of treatment was by daily s.c. injection. Vaginal smears were taken daily for the first 14 days of treatment and once weekly until the 12th month of treatment. The bitches were mated at the first oestrus after cessation of treatment to assess fertility at that time.

No evidence of oestrus (i.e. vulvar swelling, vaginal discharge or cornified vaginal smears) was observed throughout the entire 18 months of treatment in the 3 bitches in which treatment was begun at 4 months of age. Plasma progesterone concentrations remained consistently <0.5 ng/ml throughout treatment. Oestrus occurred in all bitches 3–5 months after cessation of treatment and all 3 bitches mated. Plasma progesterone levels rose rapidly to peak values of 38–60 ng/ml, suggesting that ovulation occurred. No uterine implantation sites were identified by uterine palpation at 3–5 weeks after mating and none of the bitches littered.

Discussion

These studies have shown that it is possible to achieve long-term suppression of oestrus and of ovulation in bitches with an agonistic analogue of LHRH. While this is the first such report for bitches it is not unexpected in relation to the effects previously observed in other species. The high potency of nafarelin was first established in a screening assay based upon suppression of oestrous cyclicity in female rats (Nestor *et al.*, 1982) and is, as are other analogues, being intensively evaluated as an ovulation inhibitor for women (Bergquist, Nillius & Wide, 1982; Nillius, Bergquist, Gudmundsson & Wide, 1984).

Nafarelin appears not to interfere with an already entrained ovulation in the bitch but to prevent further ovulation. A similar observation was made for heifers (Herschler & Vickery, 1981). The induced oestrus at initiation of treatment in anoestrous bitches is probably a result of the initial, albeit transitory, stimulation of gonadotrophin release which precedes the suppressive effect of these agents.

The finding of a significant reduction in integrated levels of progesterone in the luteal phase and/or the shortening of the luteal phase of non-pregnant bitches treated with $\geq 8 \mu\text{g}$ nafarelin/day but not in pregnant bitches also has its parallel in other species. For example, in non-pregnant women, a dramatic shortening of the luteal phase can be achieved with as few as two doses of these agents (Lemay, Labrie, Belanger & Raynaud, 1979). However, they are without effect on the course of pregnancy (Casper, Sheehan & Yen, 1980). It has been established that the luteal suppressive effect of the LHRH agonists in women is overridden by the luteotrophic effect of chorionic gonadotrophin. Whether a similar principle lies behind the difference in response between the pregnant and non-pregnant dog is unknown.

The lack of fertility associated with the induced oestrus in the anoestrous bitch also has its parallel in other species. While it must be noted that, in the present studies, diagnosis of ovulation was based upon circulating levels of progesterone only and not direct evidence, induction of ovulation with small doses of LHRH in anoestrous sheep was also followed by very poor luteal function and low fertility (McLeod, Haresign & Lamming, 1982).

Similar to the original observations of delay of puberty in rats with these agents (Johnson, Gendrich & White, 1976) we have shown a prevention of oestrus in prepubertal bitches. Although pregnancy was not achieved at the first oestrus after cessation of treatment, it is possible that normal fertility may occur at the next oestrus. It has been reported that fertility is often low in the pubertal bitch (Olson, Husted, Allen & Nett, 1984). The long-term suppression of oestrus without any initial overt stimulation may make this timing of onset of treatment the one of choice.

Neither daily injection nor devices needing replacement on a monthly basis are acceptable for anything other than the research environment. However, the low daily dosage requirement, calculated as only 11.7 mg per dog per year, together with our studies in controlled release technology (Vickery *et al.*, 1984b), indicate the feasibility of developing a controlled-release system for use as a contraceptive for the bitch.

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