

Evidence for the conservation of biological activity of ovulation-inducing factor in seminal plasma

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Abstract

An ovulation-inducing factor (OIF) in the seminal plasma of llamas and alpacas (induced ovulators) and cattle (spontaneous ovulators) suggests that OIF is a conserved constituent of seminal plasma among mammals. In this study, three experiments were designed to determine the biological effects of OIF in different species. In experiment 1, superstimulated prepubertal female CD-1 mice ($n=36$ per group) were given a single 0.1 ml i.p. dose of 1) phosphate-buffered saline (PBS), 2) 5 μ g gonadotropin-releasing hormone (GNRH), 3) 5 IU hCG, or 4) llama seminal plasma. The proportion of mice that ovulated was similar among groups treated with GNRH, hCG, or seminal plasma, and all were higher than the saline-treated group ($P<0.001$). In experiment 2, female llamas ($n=8$ or 9 per group) were intramuscularly treated with 1) 2 ml PBS, 2) 1 ml diluted llama seminal plasma, 3) 3 ml equine seminal plasma, or 4) 3 ml porcine seminal plasma. Experiment 3 was the same as experiment 2 except that the dose of equine and porcine seminal plasma was increased to 8 and 10 ml respectively. All llamas that were treated with llama seminal plasma ovulated and none that were treated with saline ovulated ($P<0.0001$). The proportion of llamas that ovulated in response to equine and porcine seminal plasma was intermediate. We conclude that the mechanism for the biological response to OIF is present in prepubertal CD-1 mice and that OIF is present in equine and porcine seminal plasma.

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Introduction

The mechanism by which ovulation is initiated has been used to classify mammals as either spontaneous or induced ovulators, based on the biological process that triggers release of gonadotropin-releasing hormone (GNRH) and initiates the ovulatory cascade. Mice, cattle, horses, and pigs are considered spontaneous ovulators because ovulation occurs at regular intervals as a result of increasing systemic concentrations of estradiol from growing dominant follicles, which stimulates GNRH secretion from neurons in the hypothalamus, which in turn elicits a surge release of LH from the anterior hypophysis (Espey & Lipner 1994). In contrast, ovulation in induced ovulators does not occur at regular intervals, but rather in response to a copulatory stimulus. Induced ovulators include camelid species (Marie & Anouassi 1986, Bravo *et al.* 1992, Bravo 1994), domestic cats (Banks & Stabenfeldt 1982), rabbits (Spies *et al.* 1997), koalas (Johnston *et al.* 2004), bushtail opossum (Crawford *et al.* 1998), voles (Clulow & Mallory 1970, Carter *et al.* 1988), bears (Boone *et al.* 2004), and ferrets (Carroll *et al.* 1985, Bakker & Baum 2000). The line that distinguishes spontaneous and induced ovulators is often blurred as copulation has been reported to influence ovarian function in some spontaneously ovulating species.

In early studies, mating hastened the onset of ovulation in sows (Signoret *et al.* 1972), and mating and/or mechanical stimulation of the vagina and cervix resulted in ovulation in rats (Zarrow & Clark 1968). Spontaneous ovulation has also been reported in induced ovulators. Spontaneous ovulation was detected by ultrasonography and rectal palpation in 5% of dromedary camels (Nagy *et al.* 2005) and 4–8% of llamas and alpacas (Adams 2007), and was detected (based on elevated progesterone concentrations) in 13/15 group-housed domestic cats isolated from copulatory stimuli (Gudermuth *et al.* 1997).

The term ‘reflex’ ovulator is often synonymously used with ‘induced’ ovulator because of the perception that ovulation occurs as a response to the stimulation of sensory nerves in the vagina and cervix by the penis during copulation (Fernandez-Baca *et al.* 1970, Bibeau *et al.* 1991). In contrast to the concept of a direct neural stimulus, there is increasing evidence for the presence of a biochemical substance in seminal plasma that acts in an endocrine fashion to elicit pituitary LH release and ovulation (Chen *et al.* 1985, Pan *et al.* 2001, Adams *et al.* 2005, Ratto *et al.* 2005). Results from one study (Adams *et al.* 2005) documented the existence of a potent factor in the seminal plasma of alpacas and llamas that elicited a surge in circulating concentrations of LH and induced an ovulatory and luteotropic response.

Table 1 Ovulation-inducing effect of saline, GNRH, hCG, and llama seminal plasma in superstimulated prepubertal CD-1 mice.

End points	PBS	GNRH	hCG	Seminal plasma
Number of mice that ovulated (%)	6/36 (17%)*	31/36 (86%) [†]	31/36 (86%) [†]	28/36 (78%) [†]
Number of oocytes per mouse	6.2 ± 1.3 (n=6)*	27.4 ± 2.7 (n=31) [†]	25.8 ± 2.9 (n=31) ^{†,‡}	19.2 ± 2.8 (n=28) [‡]

Experiment 1; mean ± S.E.M. *,^{†,‡} Within rows, values with no common superscript are different ($P < 0.05$).

To determine the effect of seminal plasma of con-specific versus hetero-specific males, the ovulation-inducing effect of seminal plasma of alpacas and cattle was compared with that of the llama using female llamas as a bioassay (Ratto *et al.* 2006). Ovulation was induced by seminal plasma of all three species, providing rationale for the hypothesis that ovulation-inducing factor (OIF) is a conserved constituent of seminal plasma among mammals, and has an effect on ovarian function in females of unrelated species. The objectives of this study were to test this hypothesis by determining whether the biological response (ovulation) to llama seminal plasma is present in mice (experiment 1), and whether equine and porcine seminal plasma will induce ovulation in llamas (experiments 2 and 3).

Results

Experiment 1: effect of OIF in female mice

A greater number of mice ovulated after treatment with GNRH, hCG, or seminal plasma compared to those treated with saline ($P < 0.05$; Table 1). Correspondingly, the mean number of oocytes observed per treatment group was lower in the saline-treated group compared with other groups ($P < 0.001$). The proportion of mice that ovulated was similar in the GNRH, hCG, and seminal plasma groups. The mean number of oocytes per mouse in the seminal plasma group was lower than that in the GNRH group ($P < 0.05$), but similar to that of the hCG group.

Experiments 2 and 3: OIF in equine and porcine semen

There was no difference among treatment groups in the diameter of the pre-ovulatory follicle at the time of treatment ($P = 0.6$; Table 2). All llamas treated with

llama seminal plasma ovulated (positive control) and none treated with saline ovulated (negative control; $P < 0.0001$). The proportion of llamas that ovulated in response to equine and porcine seminal plasma was intermediate (Table 2). Compared with negative controls (PBS), the proportion of llamas that ovulated was higher ($P = 0.03$) in the equine seminal plasma group and tended to be higher ($P = 0.1$) in the porcine seminal plasma group. As a reflection of the number of llamas that ovulated, the mean circulating progesterone concentration on day 7 differed among groups ($P < 0.0001$; Table 2). Among those that ovulated, the interval from treatment to ovulation did not differ among groups (1.7 ± 0.1 , 2.0 ± 0.3 , and 1.7 ± 0.3 days after treatment in the llama, equine, and porcine seminal plasma groups, respectively, $P = 0.56$). Among those that ovulated, the diameter of the CL and plasma progesterone concentrations did not differ among groups (Table 2).

Ovulations were observed in all groups treated with seminal plasma when the dose of equine and porcine seminal plasma was increased to 8 and 10 ml respectively (experiment 2 vs 3). The proportion of llamas that ovulated in response to a low dose (3 ml) versus high dose (8 ml) of equine seminal plasma did not differ (3/8 vs 2/9; $P = 0.45$), but the proportion tended to be lower in llamas treated with a low dose (3 ml) versus high dose (10 ml) of porcine seminal (0/8 vs 3/9 respectively; $P = 0.1$).

Owing to complications in catheter placement, one of the catheterized llamas in the saline-treated group was removed from the experiment after 2 h of sampling and her data were not included in LH analyses. Of the frequently sampled animals ($n = 6$ per group), only those treated with llama seminal plasma ovulated. Mean LH concentrations were numerically higher in all groups treated with seminal plasma but were statistically higher

Table 2 Effects of llama, equine, and porcine seminal plasma on ovulation and the form and function of the CL in llamas.

End points	PBS	Seminal plasma		
		Llama	Equine	Porcine
Follicle diameter on day 0 (mm)	10.1 ± 0.5*	9.9 ± 1.8*	9.6 ± 0.4*	9.5 ± 0.4*
Ovulation rate (%)	0/16 (0%)*	18/18 (100%) [†]	5/17 (29%) [†]	3/17 (18%) ^{*,†}
CL diameter on day 7 (mm)	–	10.9 ± 0.6 (n=18)*	11.0 ± 1.2 (n=5)*	10.0 ± 0.0 (n=3)*
Progesterone concentration on day 7 (ng/ml)	0.3 ± 0.05 (n=16)*	3.7 ± 0.3 (n=18) [†]	1.06 ± 0.4 (n=17)*	0.82 ± 0.3 (n=17)*
Progesterone concentration on day 7 (ng/ml) ^a	–	3.7 ± 0.3 (n=18)*	3.2 ± 0.9 (n=5)*	3.4 ± 0.5 (n=3)*

Experiments 2 and 3 combined; mean ± S.E.M.; day 0, treatment. *,^{†,‡} Within rows, values with no common superscripts are different ($P < 0.05$).

^aIncludes only those llamas that ovulated.

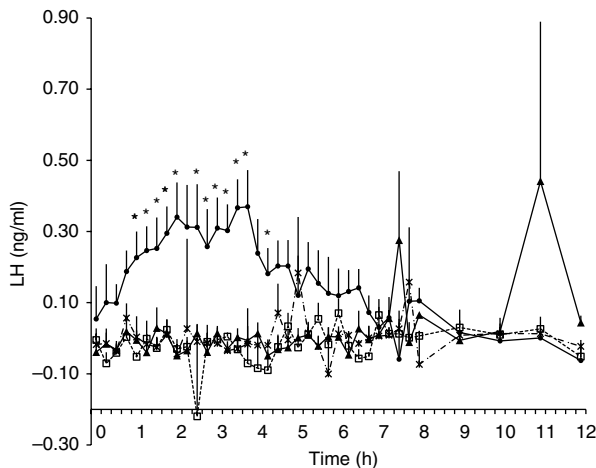


Figure 1 Plasma LH concentrations (mean \pm S.E.M.) in female llamas after intramuscular treatment with llama (filled circle; $n=6$), equine (filled triangle; $n=6$), or porcine seminal plasma (cross; $n=6$) compared with llamas treated with PBS (open square; $n=5$). Values are expressed as the difference in LH concentration in relation to basal LH concentration for each individual. Basal LH concentration for each llama was taken as the average of values for times 0, 8, 9, 10, 11, and 12 hours. *Values are higher than in the other treatment groups ($P<0.05$).

only after llama seminal plasma treatment (treatment effect, $P<0.0001$; time effect, $P=0.96$; treatment-by-time interaction, $P=0.11$; Fig. 1). Mean plasma LH concentrations began to increase within 15 min of treatment with llama seminal plasma, peaked after 2 h, and declined to basal concentrations by 7 h after treatment. Compared with the PBS-treated group, LH pulse frequency was higher in the llama and porcine seminal plasma groups ($P=0.04$), whereas LH pulse frequency in the equine seminal plasma group was intermediate (Fig. 2).

Discussion

Results of this study are consistent with those of a previous study of bull seminal plasma (Ratto *et al.* 2006) and collectively support the hypothesis that OIF is a conserved constituent of seminal plasma among mammals. A biological response to a single administration of OIF similar to that observed in llamas and alpacas (i.e. ovulation) was detected in prepubertal mice. Based on the results generated from camelid studies, it appears that OIF elicits an ovulatory effect by stimulating LH secretion from the pituitary gland. In experiment 1, hCG was used as an additional positive control to GnRH in the event that the hypothalamo–hypophyseal axis was not fully developed in the prepubertal mice, resulting in an inability to respond to GnRH. However, similar proportions of mice ovulated in GnRH and hCG groups, suggesting that the hypothalamo–hypophyseal axis was functional and that the ovaries were capable of responding. The proportion of mice that ovulated was similar among GnRH, hCG, and llama seminal plasma

groups, but the mean number of oocytes observed in the oviduct was lower in seminal plasma-treated mice than in mice treated with GnRH. Whether this discrepancy is the result of an incompatibility between mouse OIF receptor to llama OIF ligand or simply the result of the use of an unsuitable dose remains to be determined. Nonetheless, these findings support the hypothesis that both the OIF ligand and the physiological pathway in which OIF elicits its effect (e.g. OIF receptors) are conserved among mammals. As the structure and mechanism of action of OIF is not yet clear, it will be necessary to conduct a dose–response study in mice if this species is used as a biological model to study OIF.

Experiments 2 and 3 involved the use of a female llama bioassay (Adams *et al.* 2005) to determine the presence of OIF in equine or porcine seminal plasma. The effectiveness of this approach has been previously documented for identification of OIF in bovine seminal plasma (Ratto *et al.* 2006). The response to equine and porcine seminal plasma in this study was similar to that of bovine seminal plasma reported previously (Ratto *et al.* 2006), i.e. 29, 18, and 26% of llamas ovulated after treatment with equine, porcine, and bovine seminal plasma respectively.

Llamas were treated with two different volumes of equine and porcine seminal plasma to determine a dose effect. The initial dose of equine and porcine seminal plasma was based on total protein concentration measured before the experiment to adjust for differences in ejaculatory volume. The approximate volume of one ejaculate from a stallion is 70 ml (Suarez 2006), from a boar is 250 ml (Suarez 2006), and from a llama is 3 ml (Bravo *et al.* 2002). Ovulation rates were similar in llamas treated with 3 vs 8 ml of equine seminal plasma, equivalent to 1/23rd and 1/9th of a stallion ejaculate respectively. Ovulations did not occur in llamas treated with 1/80th of a boar ejaculate (3 ml) but were observed when the dose was increased to 1/25th of an ejaculate (10 ml) – a dose comparable to that of equine seminal plasma used in this study. These results are consistent

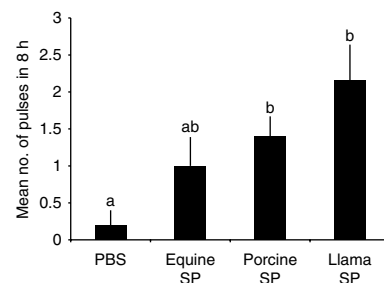


Figure 2 Plasma LH pulse frequency (mean \pm S.E.M.) determined from samples collected at 15 min intervals for 8 h starting from the time of treatment with PBS ($n=5$ llamas), or equine, porcine, or llama seminal plasma ($n=6$ llamas per group). ^{ab}Values with no common superscript are different ($P=0.04$).

with those of a previous titration study where the ovulation rate in llamas incrementally increased from 30 to 90% after treatment with the equivalent of 1/50th to 1/6th of an average llama ejaculate (Tanco *et al.* 2007). Thus, it appears that OIF behaves in a dose-dependent manner and that 1/80th of a boar ejaculate was less than the minimum dose required to induce ovulation.

The difference in ovulation rates observed following administration of seminal plasma from different species indicates that OIF is not present in equal concentrations in seminal plasma. It appears that OIF represents a greater proportion of total protein in llama seminal plasma than in the seminal plasma of horses and pigs. It is unknown whether OIF consistently constitutes a greater proportion of total seminal plasma proteins in coitus-induced ovulators compared with spontaneous ovulators.

A surge in circulating concentrations of LH is a necessary prelude to ovulation in both spontaneous and induced ovulators. In llamas and alpacas, the rise in LH occurred within 15 min and peaked after 2 h of mating or seminal plasma treatment, before returning to basal levels after 8 h (Adams *et al.* 2005). Similar patterns have been documented in other species in which coitus is necessary for ovulation. In rabbits, an LH surge began at 3 min and peaked at 15 min after copulation (Jones *et al.* 1976). In ferrets, the first significant increase in LH concentration occurred within 1 h, peaked between 6 and 8 h, and reached basal concentrations by 14 h after copulation (Carroll *et al.* 1985). In one study on cats, multiple copulations appeared necessary to progressively elevate circulating LH concentrations sufficient to elicit ovulation (Concannon *et al.* 1980). In this study, a subset of llamas was catheterized to measure the effects of treatment on LH secretion into the peripheral circulation. By necessity, the subset was chosen before the ovulatory response was known. Unfortunately, none of the llamas that ovulated in response to treatment with equine or porcine seminal plasma had been chosen for LH measurement. Hence, a distinct pre-ovulatory rise in plasma LH was not detected in the subset of catheterized animals. Despite that ovulation did not occur in this subset, treatment with porcine seminal plasma was associated with a significant increase in LH pulse frequency compared with saline-treated controls. These results support the hypothesis that OIF elicits its effect systemically, on either the hypothalamus or the pituitary, rather than at the level of the gonads.

Conclusions

OIF from llama seminal plasma induced ovulation in prepubertal mice, a species conventionally classified as a spontaneous ovulator. Results also documented the presence of OIF in the seminal plasma of horses and pigs, as evidenced by stimulation of LH release and

ovulation. Results support the hypothesis that both the OIF ligand and the receptor are conserved among mammals, and the prepubertal mouse may be a useful model for receptor/ligand studies. Although conservation of OIF among species portends functional significance, the physiological role of OIF in spontaneous ovulators remains unclear.

Materials and Methods

Experiment 1: effect of OIF in female mice

Semen was collected from male llamas ($n=4$) by artificial vagina (Bravo *et al.* 1997) twice per week over a period of 4 months at the University of Saskatchewan. Raw ejaculates were diluted 1:1 (v/v) with phosphate-buffered saline (PBS) (Gibco) and drawn back-and-forth through a 7 ml disposable transfer pipette (VWR, Mississauga, ON, Canada) to reduce semen viscosity and then centrifuged for 30 min at 1500 *g* (Adams *et al.* 2005). The supernatant was decanted to separate it from spermatozoa and a drop was microscopically evaluated to confirm the absence of cells. If spermatozoa were detected, the sample was re-centrifuged. Seminal plasma was stored at -80°C . Upon thawing, seminal plasma from different animals and different ejaculates was pooled and 1% penicillin/streptomycin (10 000 units/ml penicillin and 10 mg/ml streptomycin; Sigma-Aldrich) was added.

Prepubertal female CD-1 mice ($n=144$), 20 days of age and weighing 20–25 g, were housed at 24°C with lights on from 0500 to 1900 h and access to food and water *ad libitum*. An i.p. dose of 5 IU eCG (Pregnenol; Bioniche Animal Health, Belleville, ON, Canada) was given (day 0) for ovarian superstimulation. On day 2, mice were randomly assigned to four groups ($n=36$ per group) and given a single 0.1 ml i.p. dose of 1) PBS (negative control), 2) 5 μg GnRH (Cystorelin, Merial, Ltd, Iselin, NJ, USA), 3) 5 IU hCG (Chorulon; Intervet Canada, Ltd, Whitby, ON, Canada), or 4) llama seminal plasma. On day 3, mice were killed by administering an overdose of inhaled halothane. Oviducts were collected and oocytes were counted by oviductal trans-illumination stereomicroscopy. Ovulation was defined by the presence of oocytes in the oviduct. Thus, mice with no oocytes were considered not to have ovulated.

Experiments 2 and 3: OIF in equine and porcine semen

Semen was collected from six stallions (Samper 2007; four ejaculates per stallion) by artificial vagina over a period of 2 months at the University of Saskatchewan. Immediately after collection, the semen was filtered to remove the gel fraction. Porcine semen was collected by the gloved-hand method (Althouse 2007) from four boars (four ejaculates per boar) at the Prairie Swine Center, University of Saskatchewan. The gel fraction was separated at the time of collection using a gauze filter. Seminal plasma was decanted from spermatozoa by centrifugation in the same fashion described for llama semen (experiment 1), but was not diluted. The seminal plasma was stored at -80°C . Upon thawing, the seminal plasma from different animals within species was pooled and 1% penicillin/

streptomycin (v/v; 10 000 units/ml penicillin and 10 mg/ml streptomycin, Sigma–Aldrich) was added. Llama seminal plasma was that used in experiment 1.

Experiment 2 was conducted during May to June at the University of Saskatchewan (52°N, 106°W, and 500 m above sea level) using a herd of mature non-lactating female llamas ≥ 5 years of age and weighing from 90 to 120 kg ($n=36$). To facilitate data collection in experiment 2, ovarian follicular development among females was synchronized by administering a single i.m. dose of 5 mg pLH (Lutropin-V, Bioniche Animal Health) to induce ovulation. We expected 80–90% of the animals to ovulate in response to pLH treatment resulting in synchronous emergence of a new follicular wave 2 days after treatment, and for those that did not ovulate to be temporally near natural wave emergence (Ratto *et al.* 2003). Llamas were examined daily by transrectal ultrasonography (Aloka SSD900, Tokyo, Japan with a 7.5 MHz linear array probe) for 3 days, and those that ovulated were given 250 μ g cloprostenol (Estrumate; Schering-Plough Animal Health, Kirkland, QC, Canada) i.m. 8 days after pLH treatment to ensure luteolysis. At 10–12 days after pLH treatment, llamas with a follicle ≥ 7 mm in diameter were assigned randomly to four groups ($n=8$ or 9 per group) and given: 1) 2 ml of PBS (negative control), 2) 3 ml equine seminal plasma, 3) 3 ml porcine seminal plasma, or 4) 1 ml diluted (1:1/v:v) llama seminal plasma (positive control) by i.m. injection. The initial dose of equine and porcine seminal plasma was largely arbitrary. On the basis of an average ejaculatory volume of 70 ml for stallions, 250 ml for boars, and 3 ml for llamas, the dose represented $\sim 1/20$ th, $1/80$ th, and $1/6$ th of the ejaculate respectively. On the basis of total protein concentrations measured in ejaculates collected for this study (30.8 ± 3.4 mg/ml for stallion semen, 39.2 ± 12.6 mg/ml for boar semen, 5.9 ± 1.8 mg/ml for llama semen; mean \pm S.E.M.; NanoDrop 2000c Spectrophotometer; Thermo Fisher Scientific, Inc., Waltham, MA, USA), the dose was ~ 90 , 120, and 3 mg respectively. Injection sites were monitored after treatment. No inflammation or abscessation was detected.

Llamas were subsequently examined by transrectal ultrasonography daily for 3 days to detect ovulation and again on day 7 for corpus luteum (CL) formation (day 0, day of treatment). Ovulation was defined as the sudden disappearance of a dominant follicle from one day to the next and was confirmed by later detection of a CL (Adams *et al.* 1989). A blood sample was collected into a heparinized tube by jugular venipuncture immediately before treatment on day 0 and once more on day 7 for measurement of progesterone concentration (BD Vacutainer Systems; Becton Dickinson, Franklin Lakes, NJ, USA).

A subset of three llamas per group was catheterized to determine the effects of seminal plasma treatment on LH secretion. Catheters were inserted into the jugular vein the day before treatment to minimize the effect of stress at the time of sampling (Adams *et al.* 2005). Beginning immediately before treatment, samples were taken every 15 min for 8 h and then every hour until 12 h. Blood samples were collected in heparinized glass tubes (Hepalean, Organon Canada Ltd, Toronto, ON, Canada) and centrifuged within 1 h of collection at 1700 g for 20 min, and the plasma was stored at -20°C until the time of assay.

Experiment 3 was done immediately after experiment 2 (i.e. during July to August) at the University of Saskatchewan using the same herd of female llamas and experimental design as that of experiment 2 except that the dose of equine and porcine seminal plasma was increased to 8 and 10 ml respectively. Llamas assigned to a given treatment group in experiment 2 were randomly reassigned to a new treatment group for experiment 3, that is, they were not given the same treatment as in the previous experiment. The llamas catheterized for frequent blood sampling ($n=3$ per group) were not the same as those used in Experiment 2.

Hormone assay

Plasma progesterone concentrations were determined using a commercial, double-antibody RIA Kit (Coat-a-Count progesterone; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). All samples were analyzed in a single assay. The intraassay coefficients of variation (CVs) for the low, medium, and high reference plasma progesterone concentrations (1.78, 3.59, and 14.77 ng/ml) were 7.6, 6.1, and 3.6% respectively.

Plasma LH concentrations were measured using a double-antibody RIA (Evans *et al.* 1993). The displacement curves (percent of LH bound relative to the concentration of LH present in the sample) for bovine and llama plasma samples were parallel. The slopes of the linear portion of the displacement curves were -0.2447 and -0.2491 for bovine and llama plasma respectively. Concentrations of LH are expressed in terms of NIAMDD-bLH-24. The minimum detectable limit of the assay was 0.1 ng/ml. The range of the standard curve was from 0.063 ng/ml (80% ligand labeled LH) to 8.0 ng/ml (20% ligand labeled LH). The intra- and interassay CVs were 9.4 and 10.6% respectively, for the high reference plasma LH concentration (2.73 ng/ml). The intra- and inter-assay CVs were 13.3 and 12.3% respectively for the low reference plasma LH concentration (1.20 ng/ml). The PC-Pulsar program was used to assess LH pulse frequency in blood samples collected every 15 min for 8 h (Merriam & Wachter 1982, Gitzen & Ramirez 1986). Pulses of LH were defined by pre-set G-parameters, that is, the number of s.d. above baseline that LH values must consecutively exceed to be categorized as a pulse (Merriam & Wachter 1982). The Pulsar parameters were set at 2.00, 1.50, 1.30, 1.20, and 1.10 for G_1 – G_5 respectively. Owing to high individual variation, LH concentrations taken from frequent blood sampling are expressed as the difference between the LH concentration of the sample and the basal LH concentration for the same individual. Basal LH concentration was calculated as the mean of pretreatment (time 0) and late post-treatment values (times 8, 9, 10, 11, and 12), that is, values not expected to be influenced by treatment (Adams *et al.* 2005). The mean change in LH concentration and standard error was then calculated within each treatment group (Fig. 1).

Statistical analyses

Single-point measurements (i.e. number of oocytes, follicle size at the time of treatment, LH pulse frequency, corpus luteum diameter, and progesterone concentration) were compared

among groups by ANOVA. Serial data (i.e. LH concentrations) were compared among groups by ANOVA for repeated measures using the Mixed Procedures of SAS (Statistical Analysis System Institute, Inc., Cary, NC, USA) to determine the effects of treatment, time, and treatment-by-time interaction. In the absence of an experiment-effect, data from experiments 2 and 3 were combined and analyzed as a total data set. When main effects or interactions were significant (i.e. $P \leq 0.05$), means were compared using Tukey's multiple comparison as a *post hoc* test. Ovulation rates were compared among groups by χ^2 analysis (experiment 1) and the Fisher's exact test (experiments 2 and 3; Proc Genmod, SAS).

The experimental protocols were approved by the University Committee on Animal Care and Supply in accordance with the guidelines of the Canadian Council on Animal Care.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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