Kinetic studies and production rate of equine (e) FSH in ovariectomized pony mares. Application to the determination of a dosage regimen for eFSH in a superovulation treatment

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Abstract

The appropriate dosage regimen for equine FSH (eFSH) (dose, dosing interval) administration in a superovulation treatment in pony mares was determined by a kinetic approach using production rates and kinetic parameters of elimination of the hormone. Two dosage regimens were then tested in superovulation protocols. The eFSH production rates were determined by sampling four ovariectomized pony mares every 10 min for 8 h during the breeding season. Kinetic parameters were determined by administering four dose levels of a preparation of eFSH (4.4, 8.8, 17.6 and 35.2 µg/kg) by the i.v. route to the same mares, in a randomized 4 × 4 Latin Square protocol. The overall mean plasma clearance was $0.256 \pm 0.07 \text{ ml.kg}^{-1}.\text{min}^{-1}$, and was independent of the dose. The mean residence time ranged from 5.5 to 10.8 h and

increased with the dose. The estimated FSH production rates were 8.6 to $15.3 \text{ µg.kg}^{-1}.\text{day}^{-1}$ (i.e. 2.89 to 3.45 mg per day per mare). Two dosage regimens of eFSH were then tested in cyclic mares (ten treated mares in each trial): 3.45 mg per day (4.4 µg/kg three times a day by the i.v. route), which corresponds to the maximal daily production rate of the native hormone in ovariectomized mares, and 1.72 mg per day (2.2 µg/kg three times a day), which corresponds to half of that production rate. The dosage regimen of 2.2 µg/kg three times a day gave satisfactory results in terms of efficacy (numbers of ovulations and embryos) with minimal unwanted effects (luteinized or anovulatory follicles).

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Introduction

Superovulation treatments are less efficient in mares than in other domestic species. No commercial preparation is currently available and experimental treatments are performed with crude equine (e) pituitary extracts, or with different equine follicle-stimulating hormone (eFSH) preparations obtained from such extracts. While these treatments are generally able to increase the overall ovulation rate, the results remain very heterogeneous due to mares that either do not respond to the treatment and produce only one preovulatory follicle or present anovulatory cystic or unruptured luteinized follicles (Palmer & Hajmeli 1992, Hofferer *et al.* 1993). The therapeutic response assessed in terms of the increased number of embryos is generally poor. In most cases, equine pituitary extract or eFSH preparations are administered once daily by the i.m. route. A unitary dose corresponds to the quantity of eFSH contained in 25 mg of the extract (Hofferer *et al.* 1993), i.e. immunologically to 2.2 mg eFSH in our RIA (Guillaume *et al.* 2002). Recently, the

successful collection of two to three embryos per cycle was reported (Scoggin *et al.* 2002). In these experiments it is noteworthy that the mares received two daily i.m. injections of 25 mg equine pituitary extract, suggesting that in other trials optimal exposure to the hormone was not achieved with a single daily i.m. injection. At present there are no data reporting the plasma levels of FSH in superovulated mares. In a preliminary experiment, we tested a treatment with three daily i.v. injections of 2.2 mg eFSH (8.8 µg/kg for a 250 kg body weight pony mare) (C Briant, unpublished results). The i.v. route was selected to ensure that the systemic exposure to eFSH was higher and more reproducible. The increase in preovulatory follicles was satisfactory and relatively homogeneous but a significant number of follicles luteinized or did not ovulate. The treated mares evidenced very high levels of plasma FSH and luteinizing hormone (LH). We hypothesized that the undesirable effects resulted from too high a dose. Collectively, all these data confirm the need to determine an appropriate dosage regimen for eFSH (dose, dosing interval, route of administration) in order to optimize

superovulation treatments in mares. Rather than perform a classical dose titration trial to establish the relationship between different doses and the expected effects (increasing ovulation and embryo rates) or undesirable effects (anovulatory or luteinized follicles), we determined the dosage regimen able to reproduce a physiological FSH exposure which should then induce superovulation. As single ovulation in cyclic mares is due to the FSH depression induced by follicular dominance (Ginther 1992), our pivotal hypothesis was that achieving an FSH exposure similar to that observed in ovariectomized mares (without negative ovarian retro-control on FSH secretion), would induce superovulation. In practice, determination of the physiological production rates of eFSH only requires determination of the eFSH plasma clearance. In addition, the linearity of eFSH disposition needs to be checked in order to allow interpolation between doses. The aims of the present study were: (1) to determine the kinetic parameters of eFSH; (2) to evaluate the physiological production rate of FSH during the breeding season in ovariectomized pony mares; and accordingly (3) to analyse in clinical trials the effects on follicular growth, ovulation and embryo production of dosage regimens expected to supply a quantity of eFSH equal to approximately 100 and 50% of the FSH secreted in ovariectomized mares which also corresponds to 300 and 150% of the FSH secreted in cyclic mares (Freedman *et al.* 1979, Thompson *et al.* 1987).

Materials and Methods

Animals

The experiments were conducted on Welsh pony mares from the INRA herd in Nouzilly (latitude 47° north). Mares were housed in boxes (five per box) with a natural photoperiod, had free access to a paddock during the day and were fed twice a day with a mixture of grain during the kinetic studies and with commercial pellets during the clinical trials. Straw and water were provided *ad libitum*.

Hormone assays

Determinations of eFSH in blood plasma samples and in fractions during the different purification steps of the eFSH preparation, together with eLH and progesterone in plasma samples were carried out using specific ¹²⁵I-RIAs (Guillaume *et al.* 2002). All samples were run in duplicate. Standards for eFSH and eLH were prepared in plasma from a hypophysectomized mare, with eFSH (NHPP AFP 5022B, A F Parlow) and eLH (NHPP AFP 5130A, A F Parlow). The limits of detection were 1.6–3.1 ng/ml for eFSH, 0.4–3.12 ng/ml for eLH and 0.12 ng/ml for progesterone. The intra-assay coefficients of variation were between 11 and 20%, 5 and 17% and 4 and 14% for eFSH, eLH and progesterone respectively. The interassay coefficients of variation were less than 20%,

22% and 15% for eFSH, eLH and progesterone respectively. Samples corresponding to kinetic studies trial 1 and trial 2 were run in three different RIAs. For eFSH, due to different limits of detection between the assays and of interassay coefficients of variation higher than 10%, agreement between assays was checked from 50 samples from each experiment which were measured in a third assay. In order to bridge results of the three analytical assays, a regression line was used to express all results according to those obtained with the kinetic studies. A radioreceptor assay (RRA) using testicular porcine receptors and radioiodinated ovine FSH (CY 2178) and LH (CY 1083) (Guillou & Combarnous 1983) was also performed to determine the ratios of both FSH and LH activities in the fractions during the different purification steps of the eFSH preparation.

Purification of the eFSH preparation

Crude equine gonadotrophin (CEG 1.98) was prepared from equine pituitaries as previously described (Guillou & Combarnous 1983). This extract contained 4.8% FSH (RIA) with an FSH/LH ratio of 1.32 (RRA). Six 3 g batches from this preparation were each dissolved in 50 ml ammonium sulphate (1 M) and were successively loaded on a 200 ml phenyl sepharose hydrophobic interaction chromatography column equilibrated in ammonium sulphate (1 M) (CL4B, Amersham, Orsay, France) (Hofferer *et al.* 1993). The six eFSH fractions were eluted in ammonium bicarbonate (0.05 M) and recovered in a total volume of 2080 ml containing 649 mg FSH with an FSH/LH ratio of 6. The preparation was then directly injected into an ion exchange column (Protein-Pak DEAE-15HR, 50 × 100 mm, Waters, Milford, MA, USA), previously equilibrated with sodium acetate (10 mM, pH 6.8). The eluted fractions were then dialysed against sterile physiological saline and filtered on a 0.22 µm filter (Millex-GS, Millipore S.A., Molsheim, France). The final preparation ('eFSH preparation') contained 557 mg FSH with an FSH/LH ratio of 7.9. It was stored frozen at –20 °C in aliquots.

Kinetic studies

Animals Four pony mares (body weight ± S.D.: 275 ± 53 kg; age ± S.D.: 13.8 ± 4 years, range: 7–17 years) were ovariectomized at least 4 months before the beginning of the experiment. The mares were weighed using a mechanical weighing machine during the week before the first injection of the eFSH preparation. On sampling days they were kept for the first 8 h in their box.

Serial sampling for determination of eFSH production rates The FSH production rates were measured in July during the breeding season. On the same day, all four mares were sampled every 10 min for 8 h, from 0900 h to

1700 h through a pyrogen-free sterilized 77×1.3 mm Teflon trocar (Intraflon, Vygon, Ecouen, France) placed in the left jugular vein. The trocar was closed with a stopper between each sampling. Blood samples were collected into 5 ml heparinized tubes (Vacutainer, Becton Dickinson, Plymouth, Devon, UK), centrifuged immediately after sampling (5 min, 4000 g, room temperature) and the plasma was frozen at -20°C until assayed.

Administration of eFSH and serial sampling for determination of eFSH kinetic parameters In order to minimize the possible interaction between endogenous and exogenous hormones, the experiment was performed during the non-breeding season when the endogenous gonadotrophin levels are low. Additionally, to depress endogenous gonadotrophin secretion, vaginal sponges impregnated with progestagen (500 mg Altrenogest/sponge, Regumate, Hoechst Roussel Vet, SA, Pantin, France) and oestrogen (50 mg beta-oestradiol-3 benzoate/sponge, Sigma E8515) (Driancourt & Palmer 1982) were inserted in the vagina from the day before the eFSH injection until the last blood sample. The mares were also injected with a gonadotrophin releasing hormone (GnRH) antagonist (antarelix, Teverelix, Europeptides, Argenteuil, France, 0.01 mg/kg, i.v., twice a day at 0700 h and 1900 h) (Guillaume *et al.* 2002) from two days before the injection of eFSH until the last blood sample.

All four mares received each of four doses of eFSH (4.4, 8.8, 17.6 and 35.2 $\mu\text{g/kg}$) at one-week intervals in a randomized 4×4 Latin Square protocol. The reference dose, 8.8 $\mu\text{g/kg}$, corresponded to the dose usually injected in superovulation treatments: 2.2 mg FSH for a mare weighing 250 kg. The eFSH preparation (a 20 ml saline solution) was administered via the right jugular vein through a pyrogen-free sterilized Teflon trocar (Intraflon, Vygon). Blood samples were collected from the left jugular vein 60, 45, 30, 15 and 1 min before administration of eFSH and 1, 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 60, 75 and 90 min and then 2, 2.5, 3, 3.5, 4, 5, 6, 8, 14, 22 and 30 h after eFSH administration.

Pharmacokinetics analysis Plasma concentrations of eFSH after the i.v. administration of eFSH were analysed using the so-called non-compartmental approach and the statistical moments computed using commercial software (WinNonlin, version 4.01, Pharsight Corporation, Mountain View, CA, USA). Before the pharmacokinetic analysis, the pre-dose baseline of eFSH plasma concentrations (mean of five samples) was subtracted from the plasma eFSH concentrations following eFSH administration.

The area under the plasma eFSH concentration ($\text{ng} \cdot \text{min} \cdot \text{ml}^{-1}$) versus time curve ($\text{AUC}(0\text{--}\infty)$) was calculated by the trapezoidal rule with extrapolation to infinity. The extrapolated part of the curve ($\text{AUC}(\text{clast--}\infty)$) was determined using equation 1:

$$\text{AUC}(\text{clast--}\infty) = \text{Clast} / \lambda_z$$

where clast is the last considered plasma concentration for trapeze calculation and λ_z is the slope of the terminal phase obtained by log-linear regression on the last sampling points.

The plasma clearance (Cl) ($\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was calculated using equation 2:

$$\text{Cl} = \text{dose} / \text{AUC}(0\text{--}\infty)$$

where dose is the administered eFSH dose and AUC is as defined above.

The mean residence time (MRT) (min) i.e. the mean time that an eFSH molecule resides in the body was obtained using equation 3:

$$\text{MRT} = \text{AUMC}(0\text{--}\infty) / \text{AUC}(0\text{--}\infty)$$

where AUMC is the total area under the first moment curve with extrapolation to infinity (Gibaldi & Perrier 1992) and $\text{AUC}(0\text{--}\infty)$ is as defined above.

The steady-state volume of distribution (V_{ss}) (ml/kg) was obtained with equation 4:

$$V_{ss} = \text{MRT} \times \text{Cl}$$

with MRT and Cl as defined above.

The daily eFSH production rate (PR, $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) for each pony mare was calculated using equation 5:

$$\text{PR}(\text{daily}) = \text{Cl}(\text{daily}) \times \text{average plasma eFSH concentration}$$

where Cl is the plasma clearance ($\text{ml} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) calculated from the average of the four plasma clearances for the four tested doses, and the average plasma eFSH concentration is the mean plasma eFSH concentration obtained with the 48 samples collected over 8 h during the breeding season.

Statistical analysis Kinetic parameters (AUC, plasma Cl, MRT and V_{ss}) were analysed using an analysis of variance based on the SAS ANOVA procedure (SAS Institute Inc., Cary, NC, USA). The model was a Latin Square in which the main factors were week of treatment, mare, dose and the dose $\times$ mare interaction. The multiple comparisons between means were tested using the Bonferroni's test. Suitable error terms were chosen for each analysis (Dagnelie 1998). Statistical significance was considered when $P < 0.05$.

Effects of two different dose levels of eFSH on follicular growth, ovulation and embryo production ('clinical trials 1 and 2')

Animals and reproduction management Two trials were conducted in succession during the breeding season (June and July). Twenty cyclic pony mares (body weight $\pm$ S.D.: 276 ± 51 kg; age $\pm$ S.D.: 8 ± 3.9 years, range 4–20 years) were used in trial 1, and twenty cyclic pony mares (body weight $\pm$ S.D.: 303 ± 53 kg; age $\pm$ S.D.: 7 ± 4.2 years, range 3–16 years) in trial 2.

Before each trial, all mares received an intramuscular injection of prostaglandin $F_{2\alpha}$ (cloprostenol, 250 μg ,

Estrumate, i.m., Shering Plough, Levallois-Perret, France), 6 days after the previous ovulation. The ovaries and uterus were scanned by ultrasonography using a 5 MHz linear-array transducer (Aloka, Tokyo, Japan) to assess follicular growth and to detect ovulations. These examinations were carried out daily from the injection of prostaglandin until the final ovulation or luteinization when unruptured luteinized follicles were observed. According to Ginther (1992) a luteinized follicle was identified by the following successive features: observation during the ultrasound examination of echogenic particles in the lumen of a preovulatory follicle, enlargement of the follicle, organisation with apparent fibrinous bands, and then complete infiltration of the follicular lumen. On the day the dominant follicle attained 35 mm (one preovulatory follicle) with the second largest follicle reaching 33 mm (at least two preovulatory follicles), the mares received one injection of human chorionic gonadotrophin (hCG; 1600 IU, i.v.) (Chorulon, Intervet SA, Angers, France). The mares were first inseminated on the day of injection of hCG and then every other day until ovulation or luteinization. Non-ovulatory follicles were those preovulatory follicles (>30 mm) which had not ovulated 2 days after the injection of hCG. Inseminations were performed with fresh semen from two different Welsh pony stallions of proven fertility. For trial 1, spermatozoa (200×10^6 per dose) were diluted in Hank's solution (HGLL) containing 1% BSA (Sigma, Saint Quentin Fallavier, France) (Magistrini *et al.* 1992). For trial 2, the dose was increased to 1000×10^6 in accordance with the recently published protocol for superovulation treatments (Scoggin *et al.* 2002). Non-surgical embryo recoveries were performed 7 days after the last ovulation (Imel *et al.* 1981).

eFSH treatments For trial 1, the daily dose of 3.45 mg eFSH per mare was administered in three daily i.v. injections of 4.4 µg/kg (standardized on the mean weight of the treated mares). This dosage regimen corresponded to the maximal daily production rate that we determined in ovariectomized pony mares. This dose was also expected to supply approximately 300% of the amount of eFSH produced in cyclic mares (Freedman *et al.* 1979, Thompson *et al.* 1987). As the results of this first trial gave satisfactory ovarian stimulation but undesirable side effects, a second trial (trial 2) was performed with a dose divided by 2: 1.72 mg eFSH per mare (2.2 µg/kg three times a day). This second dosage regimen was expected to supply approximately 150% of the amount of eFSH produced in cyclic mares. In both trials, mares were randomly assigned to the control ($n=10$) or treated ($n=10$) group. Mares in the treated group received injections of the eFSH preparation at 0700 h, 1300 h and 1900 h from the second day after prostaglandin injection until the day of hCG injection. Jugular blood samples were collected daily just before the injection at 0700 h. Plasma was frozen until assayed for FSH, LH and progesterone.

Statistical analysis of clinical data The number of days in a cycle, the number of follicles >30 mm, the number of ovulations, the number of unruptured luteinized follicles, the number of follicles >30 mm 2 days after injection of hCG and the number of embryos per mare were compared with a non parametric exact permutation test for independent samples (StatXact5 software, Cytel Software Corporation, Cambridge, MA, USA). The number of mares with at least two pre-ovulatory follicles or two ovulations, with luteinized follicles or with premature ovulation (i.e. ovulation before injection of hCG) and the number of pregnant mares (mares in which at least one embryo was collected) were compared with a Fisher exact test (StatXact5 software).

Plasma concentrations of eFSH, eLH and progesterone were compared with analyses of variance (proc GLM in the SAS system). In the first analysis, the main factors were mare (nested under group) and group (treated or control). In the second analysis, the main factors were mare, group, period (during or after treatment), and the group $\times$ period and mare $\times$ period interactions. Suitable error terms were chosen for each analysis.

Correlations between mean endocrine values and number of follicles were made with an exact Spearman's rank-order correlation (StatXact5 software). In all cases, significance was considered when $P < 0.05$.

Results

Kinetic studies

Determination of plasma eFSH kinetics in ovariectomized pony mares The semi-logarithmic plots of eFSH plasma concentrations (ng/ml) versus time (min) after i.v. administration of each of four doses (4.4, 8.8, 17.6 and 35.2 µg/kg) for a representative mare are shown in Fig. 1. Table 1 presents the individual pharmacokinetic parameters.

The dose had no significant effect on plasma clearance ($P=0.12$) but did have a significant effect on AUC ($P < 0.0001$), MRT ($P=0.04$) and V_{ss} ($P < 0.0001$). The overall mean plasma clearance was $0.256 \pm 0.07 \text{ ml.kg}^{-1}.\text{min}^{-1}$ (mean $\pm$ S.D.). The mean AUCs were dose-dependent. The V_{ss} (mean $\pm$ S.D.) increased dose-dependently from $67 \pm 23 \text{ ml/kg}$ (4.4 µg/kg dose) to $197 \pm 36 \text{ ml/kg}$ (35.2 µg/kg dose).

Determination of FSH production rates in ovariectomized pony mares The secretion patterns of the four mares are given in Fig. 2. The individual daily production rates, calculated from individual average plasma eFSH concentrations and mean plasma clearances, are given in Table 2. They ranged from 8.6 to $15.3 \text{ µg.kg}^{-1}.\text{day}^{-1}$ i.e. from 2.89 to 3.45 mg/day per mare.

Figure 1 Semilogarithmic plot of plasma eFSH concentrations (ng/ml) vs time (min) after an i.v. administration of the eFSH preparation at a dose of 4.4, 8.8, 17.6 and 35.2 µg/kg in a representative mare (mare D).

Clinical trials

Effects of two different dose levels of eFSH on the superovulatory response The numbers of days of treatment were (mean $\pm$ S.D.) 5.4 ± 1.1 (range: 3–7) in trial 1 and 5.7 ± 1 (range: 4–8) in trial 2. The ovarian response for cyclic mares in trials 1 and 2 is presented in Table 3. In trial 1, the mares treated with 4.4 µg/kg three times a day presented more follicles >30 mm, more ovulations and

more cycles with >2 preovulatory follicles or ovulations than control mares. However, they also presented more luteinized follicles, more cycles with luteinized follicles and more follicles >30 mm 2 days after the injection of hCG than control mares. Moreover, two treated mares (versus 0 controls) showed premature ovulations but this result was not statistically significant. Finally, the number of recovered embryos and the number of pregnant mares were lower in these treated mares than in the controls.

Table 1 Pharmacokinetic parameters of eFSH after an intravenous bolus administration of eFSH at different dose levels in four ovariectomized pony mares

	eFSH dose ($\mu\text{g/kg}$)	AUC ($\text{ng}\cdot\text{min}\cdot\text{ml}^{-1}$)	Clearance ($\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	MRT (min)	Vss (ml/kg)
Mare					
A	4.4	16 897	0.260	219	57
B	4.4	17 420	0.253	152	38
C	4.4	19 440	0.226	448	101
D	4.4	30 256	0.145	498	72
Mean $\pm$ S.D.		21 003 $\pm$ 5426	0.221 $\pm$ 0.046	329 $\pm$ 147	67 $\pm$ 23
A	8.8	33 146	0.265	343	91
B	8.8	38 458	0.229	433	99
C	8.8	31 052	0.283	465	132
D	8.8	45 745	0.192	581	112
Mean $\pm$ S.D.		37 100 $\pm$ 5674	0.243 $\pm$ 0.035	455 $\pm$ 85	108 $\pm$ 15
A	17.6	89 794	0.196	660	129
B	17.6	85 623	0.206	654	134
C	17.6	48 492	0.363	492	179
D	17.6	77 981	0.226	689	156
Mean $\pm$ S.D.		75 472 $\pm$ 1614	0.248 $\pm$ 0.067	624 $\pm$ 77	149 $\pm$ 19
A	35.2	121 295	0.290	512	149
B	35.2	143 256	0.246	934	229
C	35.2	80 581	0.437	535	234
D	35.2	124 484	0.283	620	175
Mean $\pm$ S.D.		117 404 $\pm$ 22 856	0.314 $\pm$ 0.073	650 $\pm$ 169	197 $\pm$ 36
Statistical significance of dose		<0.0001	NS	0.04	<0.0001

AUC, observed area under the plasma concentration versus time curve; Clearance, observed plasma clearance; MRT, observed mean residence time; Vss, observed steady state volume of distribution. Pharmacokinetic data were subjected to an analysis of variance (ANOVA), the model being a Latin Square in which the main factors were week of treatment, mare, dose and interaction dose $\times$ mare. NS, not significant.

In trial 2, the mares receiving 2.2 $\mu\text{g/kg}$ three times a day presented more follicles >30 mm, more ovulations and more cycles with >2 preovulatory follicles or ovulations than control mares. The numbers of luteinized follicles or of follicles >30 mm 2 days after the injection of hCG and the number of mares with luteinized follicles did not differ between treated and control mares. No premature ovulation was observed in treated cycles. The number of embryos and the number of pregnant mares did not differ between the two groups of mares. In treated mares with >2 ovulations, the number of embryos tended to be higher in those mares with bilateral ovulation (1.5 ± 1.3) ($n=6$) than in those with unilateral ovulation (0.67 ± 0.5) ($n=3$), but these results were not significantly different ($P=0.3$).

Effects of two different dose levels of eFSH on endocrine parameters Mean plasma patterns of FSH, progesterone and LH for the two clinical trials are presented in Figs 3 and 4. In trial 1, plasma FSH and LH concentrations were higher in treated than in control mares during the treatment period. At this time, concentrations of progesterone tended to be higher in treated than in control mares but the difference was not significant ($P=0.15$). At the end of treatment, progesterone concen-

trations were higher in treated than in control mares due to increasing progesterone levels before and after ovulation. Examination of individual patterns (data not presented) show that, before ovulation, 7/10 mares in the treated group had concentrations of progesterone higher than 1 ng/ml compared with 0/10 in the control group. In these mares, the mean LH levels during the treatment were positively correlated with the mean levels of progesterone before ovulation ($r=0.79$; $P=0.02$) and the number of luteinized follicles ($r=0.78$; $P=0.03$). However, the numbers of preovulatory follicles or ovulations were not correlated with the FSH mean levels during the treatment. The periovulatory surge of LH was completely abolished in treated mares. In trial 2, FSH levels tended to be higher in treated mares during the period of treatment but the effect was not significant ($P=0.12$). LH levels were lower in treated mares throughout the period of sampling. Progesterone levels did not differ between the two groups during the period of treatment, as none of the treated mares exhibited progesterone levels higher than 1 ng/ml before ovulation. Progesterone concentrations were higher in treated mares after the end of treatment. As in trial 1, it is noteworthy that the FSH mean levels during the period of treatment were not correlated with the numbers of preovulatory follicles or ovulations.

Figure 2 Plasma patterns of FSH concentrations during an 8 h period in four ovariectomized mares. The sampling interval was 10 min.

Discussion

Use of kinetic studies to determine a dosage regimen for eFSH in a superovulation treatment

In the present experiment, we determined a dosage regimen of eFSH for a superovulation treatment in pony mares. The aim was to counteract the depression of FSH

induced by follicular dominance by reproducing in cyclic mares the FSH exposure of animals without negative ovarian retro-control. That is the reason why production rates and kinetic parameters of FSH were determined in ovariectomized ponies.

The selected approach required us to estimate plasma FSH clearance. Clearance is a basic pharmacokinetic parameter which expresses the body's capacity to eliminate a

Table 2 Production rates of FSH during the breeding season in four ovariectomized pony mares

	Mean plasma clearance ($\text{ml.kg}^{-1}.\text{day}^{-1}$)*	Mean plasma FSH concentration over an 8-h period ($\mu\text{g/ml}$)	Daily production rate of FSH ($\mu\text{g.kg}^{-1}.\text{day}^{-1}$)	Daily production rate of FSH per mare (mg/day)
Mare (body weight)				
A (225 kg)	364	0.042	15.31	3.45
B (276 kg)	336	0.031	10.47	2.89
C (250 kg)	471	0.029	13.79	3.45
D (348 kg)	305	0.028	8.56	2.98

*Mean of the plasma clearances for the four doses given in Table 1 $\times$ 1440 min.

Table 3 Superovulatory response in mares treated with the eFSH preparation at a dose of 4.4 µg/kg (trial 1) or 2.2 µg/kg (trial 2) i.v. three times a day. Results are means ± S.D. except where stated otherwise

Parameter	Trial 1		Trial 2	
	eFSH	Control	eFSH	Control
No. of mares	10	10	10	10
No. of days between the beginning of treatment and ovulation	7.9 ± 3	6.2 ± 1.8	8.1 ± 1.3	6.4 ± 2.1
No. of follicles ≥ 30 mm per mare	3.6 ± 1.5*	1.1 ± 0.3	3.4 ± 2.1*	1.2 ± 0.4
No. of ovulations per mare (range)	1.9 ± 1.1 (0–4)*	1 ± 0 (1)	2.9 ± 1.5 (1–6)*	1.2 ± 0.4 (1–2)
No. of mares with ≥ 2 preovulatory follicles (%)	8/9 (88%) ^{a*}	1/10 (10%)	9/10 (90%)*	2/10 (20%)
No. of mares with ≥ 2 ovulations (%)	6/9 (66%) ^{b*}	0	9/10 (90%)*	2/10 (20%)
No. of luteinized follicles per mare	1.2 ± 0.8*	0	0	0
No. of mares with luteinized follicles (%)	6/10 (60%)*	0	0	0
No. of follicles ≥ 30 mm 2 days after hCG per mare	1.9 ± 1.7*	0.2 ± 0.6	0.3 ± 0.4	0
No. of mares with premature ovulation (%)	2/10 (20%)	0	0	1/10 (10%)
No. of embryos per mare (range)	0 (0)*	0.7 ± 0.5 (0–1)	1.1 ± 1.1 (0–4)	0.75 ± 0.8 (0–2)
No. of pregnant mares ^c	0/7 (0%) ^{d*}	6/9 (67%) ^e	7/10 (70%)	4/8 ^f (50%)

^aOne mare had no follicle of preovulatory size; ^bone mare did not ovulate; ^ca mare was considered pregnant when at least one embryo was recovered 7 days after ovulation; ^dtwo mares were not inseminated because they had ovulated before and one mare did not ovulate; ^eone mare was not collected; ^fone mare was not inseminated and one mare had metritis.

* $P \leq 0.05$ compared with respective control value.

molecule. In the present experiment the estimated plasma clearances were between 0.22 and 0.31 ml.kg⁻¹.min⁻¹. These values are in the same range as those previously found in cyclic mares injected with an iodinated fraction of FSH/LH, which were between 0.19 and 0.27 ml.kg⁻¹.min⁻¹ (Irvine 1979). As expected, this result shows that clearance does not change with ovarian status, age or weight of the mares. These clearance values are rather low and consistent with the fact that sialylated glycoproteins, such as eCG, do not bind to liver cells (Klett *et al.* 2003) and are only eliminated by renal glomerular filtration (Combarrous *et al.* 2001). In horses, the glomerular filtration rate is about 1–2 ml.kg⁻¹.min⁻¹ (Reece 1993), suggesting that eFSH is not freely filtered by the glomerulus. This is consistent with the molecular weight of eFSH (around 33 kDa), the ultrafiltrate-to-plasma concentration ratio for protein of this molecular weight being about 0.33 (Rowland & Tozer 1995). The plasma clearance did not significantly vary with the dose, indicating the non-saturability of the elimination mechanisms within the range of tested doses. This property should guarantee that the overall eFSH exposure (i.e. eFSH AUC) is proportional to the administered dose. The low clearance of the molecule explains the rather high values of MRTs (from 5.5 to 10.8 h).

The Vss ranged from 67 to 197 ml/kg, which is in the same order of magnitude as previously published values (between 87 and 147 ml/kg) (Irvine 1979). The low value of this parameter suggests that eFSH remained located mainly in the extracellular space. The progressive increase of Vss with the dose could be attributed to an underestimation of this parameter after administration of lower eFSH doses, the terminal part of the curve being not

properly characterized due to the limit of quantification of the analytical technique.

In the present experiment the eFSH production rates over 24 h in ovariectomized mares ranged from 8.6 to 15.3 µg.kg⁻¹.day⁻¹ (i.e. 2.89 to 3.45 mg/day per mare). In 400–500 kg body weight cyclic mares, these rates were lower, ranging from 0.84 to 2.6 mg/day (Irvine 1979) which is consistent with the fact that plasma concentrations of FSH are from three to four times lower in cyclic than in ovariectomized mares (Freedman *et al.* 1979, Thompson *et al.* 1987).

The determination of an efficient dosage regimen of eFSH for mares requires calculating a dose, a route of injection (which determines the bioavailability of the product) and a dosage interval (which determines plasma concentration fluctuations). The objective in trial 1 was to administer daily the maximal quantity of eFSH produced in ovariectomized mares, i.e. 3.45 mg (without taking into account the basal levels of FSH in cyclic mares). The i.v. route (bioavailability of 100%) was selected in this experiment to reduce between-animal variability in the plasma concentration patterns, potentially associated with erratic extra-vascular bioavailability. Considering the MRTs of the molecule (between 5.5 and 10.8 h), the total dose was administered in three daily injections of 4.4 µg/kg, standardized on the mean weight of the treated mares. In trial 2, half of the total dose was administered in three daily i.v. injections.

Effect of two different dose levels of eFSH in a superovulation treatment

In both clinical trials, the two tested doses were able to induce ovarian stimulation, as evidenced by the numbers

Figure 3 Plasma concentrations of FSH and progesterone (ng/ml, mean $\pm$ S.E.M.) in mares treated (open triangles) ($n=10$) with a daily dose of 4.4 µg/kg eFSH in trial 1 (a) or 2.2 µg/kg eFSH in trial 2 (b) and in control mares (closed circles) ($n=10$). Data are presented from the day of prostaglandin injection until 3 days after ovulation. D0 is the first day of eFSH treatment. The mean duration of treatment is shown by the solid bar. The intervals between ovulations are represented by a dotted (treated) or a continuous (controls) arrow.

of preovulatory follicles, ovulations and progesterone concentrations after ovulation. In trial 1, during the period of treatment with the daily dose of 3.45 mg eFSH, the concentrations of FSH 12 h after the previous administration of eFSH were still more than twice those of control mares. These high FSH concentrations were associated with high levels of LH, which were probably the cause of premature ovulations, formation of luteinized follicles which secreted progesterone before ovulation

and decreased fertility. At the end of the treatment period, a periovulatory LH surge was initiated in control mares, whereas it was suppressed in treated ones. These observations are consistent with those reported in other species, namely in cows superovulated with porcine FSH (Callesen *et al.* 1986, Roberge *et al.* 1995, Greve & Callesen 2001). This type of endocrine pattern is associated with a loss of transferable embryos (Roberge *et al.* 1995). Premature ovulations (Callesen *et al.* 1987), anovulatory

(a)

(b)

Day (D0 = first day of eFSH treatment)

Day (D0 = day of ovulation)

Figure 4 Plasma concentrations of LH (ng/ml, mean $\pm$ S.E.M.) in mares treated (open triangles) ($n=10$) with a daily dose of 4.4 μ g/kg eFSH in trial 1 (a) or 2.2 μ g/kg eFSH in trial 2 (b) and in control mares (closed circles) ($n=10$). The mean duration of treatment is shown by the solid bar. Data are presented from the day of prostaglandin injection until 3 days after ovulation. D0 is the first day of eFSH treatment (left panels) or the day of ovulation (right panels).

and luteinized follicles are also observed (Callesen *et al.* 1986).

In trial 2, throughout the period of treatment the concentrations of FSH, 12 h after injection in mares

treated with the daily dose of 1.72 mg eFSH, were no higher than in control mares and the progesterone levels were not different. Nine out of ten mares had more than one preovulatory follicle and no adverse effect was

observed on follicular growth, ovulation or fertility. This suggests that the injected dose was too high in trial 1, but appropriate in trial 2, to induce ovarian stimulation without triggering adverse effects. The suppression of the periovulatory surge of LH in treated mares, also reported in superovulated cows (Gosselin *et al.* 1995, Price 1995, Price *et al.* 1998), did not seem to have any adverse effect on ovulation, progesterone secretion or embryo production. This is consistent with the fact that the suppression of the LH surge in mares with a GnRH antagonist associated with hCG has no detrimental effect on ovulation, corpus luteum formation or embryo production (Briant *et al.* 2003, 2004). The fact that ovarian stimulation was not correlated to the levels of FSH during treatment in either trial could suggest that a minimal FSH threshold level exists in mares above which ovarian stimulation is obtained for all the mares, but that individual responses in terms of preovulatory follicles are regulated by other factors. In the present experiment the number of collected embryos was not significantly different between treated and control mares. However, results obtained in mares with bilateral ovulations, which produced twofold more embryos than control mares, seem very encouraging. As the number of mares (ten) is not enough to appreciate the effects of a treatment on fertility, this treatment should be tested in a larger number of mares.

There is no guarantee that the nominal dose that we calculated and tested will be the same with a different eFSH preparation. Indeed, different isoforms of eFSH with different kinetic parameters can be produced depending on the preparation protocol. However, a therapeutically equivalent dose for a new eFSH batch could easily be bridged with that of the present experiment, by titrating this new batch against the physiological eFSH production rate in ovariectomized mares which, in the present context, can be seen as an internal *in vivo* standard.

On the basis of the present results we can propose some explanations for the high variability observed in classical superovulation treatments in mares. The selection of dosage regimens which induce insufficient levels of eFSH is probably responsible for some non-responses of mares which develop only a single preovulatory follicle. Considering that the daily i.v. eFSH dose of 1.72 mg per mare gave a satisfactory ovarian stimulation in nearly all pony mares, it is likely that the observed treatment failures following a single daily i.m. dose of 2.2 mg eFSH (as used in conventional superovulation treatments) were due to a low bioavailability associated with the i.m. route of administration (Gaillot *et al.* 1994). Conversely, the administration of dosage regimens which supply too high a dose of gonadotrophins and induce excessively elevated plasma LH peaks may lead to the formation of anovulatory and luteinized follicles or decreased fertility. For example, the administration of a single dose of 50 mg equine pituitary extract (equivalent to 4.4 mg eFSH) gave more

anovulatory and luteinized follicles than two daily doses of 25 mg (Scoggin *et al.* 2002).

In conclusion, we used a pharmacokinetics approach to document the two components (i.e. dose and dosing interval) of an efficient dosage regimen of eFSH for superovulation treatment in pony mares. The daily dose was equivalent to half the production rate of the native hormone in ovariectomized pony mares, i.e. 1.72 mg to be administered three times a day by the i.v. route. Every effort should be made in future experiments on a larger number of mares to confirm the effects of this dosage regimen on embryo production, to determine the therapeutic window which brackets this dosage regimen and to carry out studies to understand the reasons for individual ovarian responses.

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