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Theriogenology
ELSEVIER
Theriogenology 60 (2003) 1153–1164

The effects of different insemination regimes on fertility in mares

H. Sieme[a,*], T. Schäfer[b], T.A.E. Stout[c], E. Klug[b], D. Waberski[d]

[a]*National Stud of Lower Saxony, Niedersaechsisches Landgestuet Celle, Spoerckenstr. 10, 29221 Celle, Germany*
[b]*Clinic for Horses, Veterinary School Hanover, Hanover, Germany*
[c]*Department of Equine Sciences, University of Utrecht, Utrecht, The Netherlands*
[d]*Institute for Reproductive Medicine, Veterinary School Hanover, Hanover, Germany*

Received 13 December 2001; accepted 3 February 2003

Abstract

This study investigated the effects of different artificial insemination (AI) regimes on the pregnancy rate in mares inseminated with either cooled or frozen-thawed semen. In essence, the influence of three different factors on fertility was examined; namely the number of inseminations per oestrus, the time interval between inseminations within an oestrus, and the proximity of insemination to ovulation.

In the first experiment, 401 warmblood mares were inseminated one to three times in an oestrus with either cooled (500×10^6 progressively motile spermatozoa, stored at $+5\,^{\circ}C$ for 2–4 h) or frozen-thawed (800×10^6 spermatozoa, of which $\geq$35% were progressively motile post-thaw) semen from fertile Hanoverian stallions, beginning −24, −12, 0, 12, 24 or 36 h after human chorionic gonadotrophin (hCG) administration. Mares were injected intravenously with 1500 IU hCG when they were in oestrus and had a pre-ovulatory follicle $\geq$40 mm in diameter. Experiment 2 was a retrospective analysis of the breeding records of 2637 mares inseminated in a total of 5305 oestrous cycles during the 1999 breeding season. In Experiment 1, follicle development was monitored by transrectal ultrasonographic examination of the ovaries every 12 h until ovulation, and pregnancy detection was performed sonographically 16–18 days after ovulation. In Experiment 2, insemination data were analysed with respect to the number of live foals registered the following year.

In Experiment 1, ovulation occurred within 48 h of hCG administration in 97.5% (391/401) of mares and the interval between hCG treatment and ovulation was significantly shorter in the second half of the breeding season (May–July) than in the first (March–April, $P \leq 0.05$). Mares inseminated with cooled stallion semen once during an oestrus had pregnancy rates comparable to those attained in mares inseminated on two (48/85, 56.5%) or three (20/28, 71.4%) occasions at 24 h intervals, as long as insemination was performed between 24 h before and 12 h after ovulation (78/140, 55.7%). Similarly, a single frozen-thawed semen insemination between 12 h before (31/75, 41.3%) and 12 h after (24/48, 50%) ovulation produced similar pregnancy rates to those attained when mares were inseminated either two (31/62, 50%) or three (3/9, 33.3%) times at 24 h intervals.

* Corresponding author. Tel.: +49-5141-929433; fax: +49-5141-909746.
E-mail address: stallions.celle@t-online.de (H. Sieme).

doi:10.1016/S0093-691X(03)00113-4

In the retrospective study (Experiment 2), mares inseminated with cooled semen only once per cycle had significantly lower per cycle foaling rates (507/1622, 31.2%) than mares inseminated two (791/1905, 41.5%), three (464/1064, 43.6%) or ≥4 times (314/714, 43.9%) in an oestrus ($P \leq 0.001$). In addition, there was a tendency for per cycle foaling rates to increase when mares were inseminated daily (619/1374, 45.5%) rather than every other day (836/2004, 42.1%, $P = 0.054$) until ovulation.

It is concluded that under conditions of frequent veterinary examination, a single insemination per cycle produces pregnancy rates as good as multiple insemination, as long as it is performed between 24 h before and 12 h after AI for cooled semen, or 12 h before and 12 h after AI for frozen-thawed semen. If frequent scanning is not possible, fertility appears to be optimised by repeating AI on a daily basis.

Keywords: Horse; Artificial insemination; Spermatozoa; hCG; Fertility

1. Introduction

Numerous factors influence the pregnancy rate in horses bred by artificial insemination (AI); these include the inherent fertility of the mare and stallion, the type of semen used for insemination (i.e. fresh, cooled-transported or frozen-thawed) [1], the number of spermatozoa in the insemination dose [2–4], the concentration of extended semen [5], and the time for which liquid semen is stored prior to AI [6,7].

Previous reports have documented that the longevity of fresh equine spermatozoa in the female tract ranges from 24 h to 7 days, with considerable inter-stallion variation [8]. At the opposite pole of the gamete survival process, Woods et al. [9] and Koskinen et al. [10] reported mares to have conceived following AI as late as 24 h post-ovulation, although the former recorded a reduced pregnancy rate if AI was >18 h post-ovulation. Taking these indications of gamete longevity into consideration, the equine AI-industry has evolved a system in which mares are inseminated with fresh or cooled semen every 48 h until oestrus is no longer detected (usually 1–2 days after ovulation), or until ovulation is detected by transrectal palpation and/or ultrasonographic examination of the ovaries. In the case of frozen-thawed semen, efforts are made to inseminate closer to the time of ovulation because of the dramatic reduction in the longevity of sperm from most stallions induced by freezing and thawing. Furthermore, because the length of oestrus and the time of ovulation relative to the onset of oestrus differ markedly between mares and cycles, careful veterinary management of mares is required to identify the optimal time for insemination and thus avoid wasting commercially valuable semen or repeatedly challenging the uterus of susceptible mares. The use of human chorionic gonadotrophin (hCG), which has an LH-like action in mares, or GnRH (in the form of slow release implants) to induce ovulation can help to further optimise the time of insemination relative to ovulation [11].

The effect on the pregnancy rate of the total number of inseminations per oestrous cycle, the time interval between multiple inseminations during a single oestrus, and the proximity of insemination to ovulation have been examined previously [9,12–15], but differences in the reported insemination regimes and in the evaluation criteria have made it difficult to objectively compare between studies and, therefore, to decide on an optimal insemination strategy.

In the present study, pregnancy rates per cycle were determined in warmblood mares inseminated with either cooled or frozen-thawed stallion semen at different time intervals after, or before and after, hCG administration. These different regimes helped to create different time intervals between AI and ovulation, and the effects of the AI-ovulation interval and the other experimental variable, the number of inseminations per cycle, on the pregnancy rate were examined. In a second retrospective study, the influence on fertility of the number of inseminations and the time interval between inseminations was established by analysing the breeding records of 2637 warmblood mares.

2. Materials and methods

2.1. Animals and experimental design

In January–July 2000 (i.e. during the commercial breeding season), 401 reproductively normal Hanoverian mares were allotted randomly to one of a number of insemination regimes, which differed with respect to the number of inseminations per oestrus and the timing of AI with respect to administration of hCG. When the mares were in oestrus and awaiting insemination, they were housed individually in straw-bedded stables at the Lower Saxony State Stud Farm in Celle, Germany. During the housing period, they were fed oats and hay three times a day and provided with water ad libitum. Follicle development was monitored by transrectal ultrasonographic examination of the mares' ovaries and, when a pre-ovulatory follicle $\geq$40 mm was detected, mares were injected intravenously with 1500 IU hCG (Choriolutin®, Albrecht, Germany) to induce ovulation. For each mare and cycle, the interval between hCG administration and ovulation was recorded, and subsequently analysed with respect to mare age, reproductive status from the preceding breeding season (i.e. foaling, maiden, not covered, barren, resorbed or aborted) and time of year.

Cooled semen inseminations were performed with semen collected from fertile Hanoverian stallions used in the stud's routine AI programme. After collection, the semen was diluted with modified INRA 82 extender [16] to a total number of 500×10^6 progressively motile spermatozoa (PMS) in 20 ml seminal plasma/extender, and stored at +5 °C for 2–4 h before being used for AI. Frozen semen was derived from the same population of stallions. Semen was extended in a modified skim milk extender [16] to a final concentration of 50×10^6 spermatozoa/ml and centrifuged for 10 min at 600 g. After decantation of the supernatant, sperm pellets were suspended in a skim milk extender containing 2% egg yolk and 2.5% glycerol. The final concentration of spermatozoa was 200×10^6 spermatozoa/ml. Semen was packaged in 0.5 ml straws (Minitüb, Landshut, Germany) by the use of an automatic filling system (MRS-1™, Instruments Médicine Vétérinaire, L'Âigle, France), equilibrated for 60 min at +5 °C, and submitted to a programmed semen cryopreservation system (Minidigitcool™, Instruments Médicine Vétérinaire, L'Âigle, France) at a cooling rate of 60 °C/min from +5 °C to −140 °C and stored in liquid nitrogen. Immediately prior to use, the semen was thawed by immersion in a 37 °C water bath for 30 s and, for each insemination, 800×10^6 spermatozoa (i.e. 8 straws) of which $\geq$35% were progressively motile post-thaw, were used.

During insemination, chilled or frozen-thawed semen was deposited in the uterine body using a flexible plastic insemination pipette (Minitüb, Landshut, Germany) guided per vaginum. Mares were inseminated either one, two or three times per cycle, at different time intervals from hCG administration. The insemination regimes were as follows: one insemination at 0, 12, 24 or 36 h after hCG administration; two inseminations 24 h apart with the first at −24, 0, +12, or +24 h after hCG; and three inseminations at 24 h intervals, starting at either –24 or 0 h after hCG administration. Throughout the experimental period, mares' ovaries were examined ultrasonographically twice daily (at 06:00 and 18:00 h) until ovulation, such that the precision of ovulation detection was ±12 h. Pregnancy detection was performed ultrasonographically between days 16 and 18 after ovulation, although mares that had either failed to ovulate or had more than one ovulation were removed from subsequent data analysis. Finally, mares were assigned to analysis Groups A–L depending on the number of inseminations per oestrus and the timing of insemination relative to ovulation. Mares inseminated once in a cycle were classified in Groups A–E (AI performed 0–12 h after ovulation or 0–12, 12–24, 24–36 or 36–48 h before ovulation, respectively). Those inseminated twice in an oestrus were classified in Groups F–H (AI 12–24 h before and 0–12 h after ovulation, 24–36 and 0–12 h before ovulation; 36–48 and 12–24 before ovulation, respectively). Finally, mares inseminated three times in an oestrus were classified in Groups I–L (AI 36–48 and 12–24 h before and 0–12 h after ovulation; 48–60, 24–36 and 0–12 h before ovulation; 60–72, 36–48 and 12–24 h before ovulation, respectively).

2.2. Experiment 2

In a retrospective study, the foaling rates following insemination of 2637 warmblood mares during 5305 oestrous cycles at 11 Lower Saxony State Stud AI centres during the 1999 breeding season were examined. In all cases, mares had been inseminated with cooled semen within 12 h of collection from a similar population of stallions, and diluted in the same manner, as described in Experiment 1. For each oestrous cycle and mare, the number of inseminations per cycle (1, 2, 3, ≥4) and the time interval between inseminations within a cycle (24 or 48 h) were recorded. For the sake of analysis, time intervals between inseminations ≥48 h were pooled in a single class and, if intervals were irregular, cycles were classified further by the interval between the last two inseminations. Finally, the per cycle foaling rates were analysed with respect to the number of inseminations per oestrus and the interval between those inseminations.

2.3. Statistical analysis

Statistical analyses were performed using the statistical analysis system (SAS) statistics package. In both experiments, differences in fertility between groups were analysed using Chi-squared tests, although for groups with data from ≤5 cycles Fisher's exact test for non-parametric data was used. In Experiment 1, intervals between hCG administration and ovulation were compared using one-way analysis of variance (or Wilcoxon's test for non-parametric data). In all cases, values are given as means ± S.E.M. and differences between groups were considered statistically significant for $P \leq 0.05$ and highly significant for $P \leq 0.01$ and ≤0.001.

3. Results

3.1. Experiment 1

Of the 401 mares treated with 1500 IU hCG, 391 (97.5%) ovulated within 48 h after injection, and the time interval between hCG administration and ovulation was not affected significantly by age or reproductive status (maiden, barren, foaling, etc.). However, the time of the year did have a significant effect on the timing of ovulation; from May until July (i.e. the peak of the physiological breeding season) ovulation intervals after hCG injection were significantly shorter ($P \leq 0.05$) than they were earlier in the season (March–April, Table 1).

A comparison of the per cycle pregnancy rate between mares inseminated with cooled semen one, two or three times in a single oestrus demonstrated a significant difference ($P \leq 0.05$) between the single (84/169, 49.7%) and triple insemination groups (20/28, 71.4%), while the double insemination group (48/85, 56.5%) did not differ significantly from either of the other groups. For mares inseminated only once per cycle with cooled semen, the pregnancy rate was significantly higher if insemination was performed ≤24 h prior to ovulation (Groups B and C: 53.6 and 59.1%, respectively) than if it was performed 36–48 h before ovulation (Group E: 18.2%). Indeed, the combined pregnancy rate for AI 0–24 h before ovulation (Groups B and C: 67/116; 57.7%) was higher than for AI 24–48 h before ovulation (Groups D and E: 6/29, 20.6%, $P \leq 0.001$). Moreover, pregnancy rates resulting after a single insemination with cooled semen ≤24 h before ovulation (Groups B

Table 1
Effect of age, reproductive status and time of year on the interval from hCG administration (1500 IU, i.v.) to ovulation in oestrous mares with a pre-ovulatory follicle ≥40 mm

Interval from hCG administration to ovulation (h)

(1) Analysed as a function of mare age (years)

	Total	2–4	5–8	9–12	13–16	17–21
n	339	87	111	80	43	18
Mean	31.4	32.9	30.6	30.6	31.4	33.3
S.E.M.	12.9	11.3	12.8	14.3	13.2	14.7

(2) Analysed as a function of reproductive status

	Total	In foal	Maiden	Not covered	Barren	Resorbed	Aborted
n	329	159	61	43	54	5	7
Mean	31.2	30.5	33.3	29.7	31.7	30.0	33.4
S.E.M.	12.5	12.2	11.4	15.8	12.2	8.5	11.4

(3) Analysed as a function of time of year

	Total	Season					
		January	February	March	April	May	June	July
n	401	8	11	79	113	99	69	22
Mean	31.1	36.0	33.2	33.0 a	34.1 a	28.6 b	29.6 b	26.7 b
S.E.M.	12.5	9.1	14.3	12.5	11.1	12.9	14.1	12.9

Different letters (a, b) within a row indicate values that differ significantly ($P \leq 0.05$).

and C: 67/116, 57.7%) did not differ significantly from those for mares that were inseminated two (48/85, 56.5%) or three times (20/28, 71.4%) in a cycle.

There was a significant difference in per cycle pregnancy rates ($P \leq 0.05$) between mares inseminated with cooled (152/282, 53.9%) and those inseminated with frozen-thawed semen (97/220, 44.0%, Table 2). And while, for frozen semen too, few mares were inseminated along a triple AI regime to allow meaningful comparison (9, of which 3 became pregnant), there was no significant difference in the pregnancy rates obtained after one or two inseminations per oestrus (63/149 [42.2%] versus 31/62 [50.0%], respectively). On the other hand, when a single insemination was performed using frozen-thawed semen between 24 and 12 h prior to ovulation the resulting low pregnancy rate (Group C: 8/26,

Table 2
Effects of number of inseminations per oestrus and proximity of AI to ovulation on fertility in mares induced to ovulate using 1500 IU hCG

AI-method			Total (*n*)	Cooled semen			Frozen-thawed semen		
Group	AI (*n*)	AI → ovulation (h)	Cycles (*n*)	Cycles (*n*)	Pregnant (*n*)	Pregnant (%)	Cycles (*n*)	Pregnant (*n*)	Pregnant (%)
A	1	0 → +12	72	24	11	45.8 a,b	48	24	50.0 x,y
B	1	−12 → 0	103	28	15	53.6 a	75	31	41.3 x,y
C	1	−24 → −12	114	88	52	59.1 a	26	8	30.8 y
D	1	−36 → −24	8	7	2	28.6 a,b			
E	1	−48 → −36	22	22	4	18.2 b			
∑ 1 AI			319	169	84	49.7 A	149	63	42.2 X
F	2	−24 → −12 0 → +12	52	31	21	67.7 a	21	13	61.9 x
G	2	−36 → −24 −12 → 0	53	18	11	61.1 a	35	14	40.0 x,y
H	2	−48 → −36 −24 → −12	42	36	16	44.4 a,b	6	4	66.7 x,y
∑ 2 AI			147	85	48	56.5 A,B	62	31	50.0 X
I	3	−48 → −36 −24→ −12 0 → +12	22	13	10	76.9 a	9	3	33.3 x,y
K	3	−60 → −48 −36 → −24 −12 → 0	4	4	4	100.0 a			
L	3	−72 → −60 −48 → −36 −24 → −12	12	11	6	54.5 a			
∑ 3 AI			38	28	20	71.4 B	9	3	33.3 X
∑			504	282	152	53.9[1]	220	97	44.0[2]

Within a column values with the same letters (a,b,x,y,A,B,X) did not differ significantly ($P > 0.05$).
[1,2] Pregnancy rates resulting after cooled semen AI were significantly higher than those after AI with frozen-thawed semen ($P \leq 0.05$).

30.8%) was significantly improved by re-insemination 24 h later (i.e. 0–12 h after ovulation: Group F: 13/21, 61.9%).

3.2. Experiment 2

Of the 2637 warmblood mares inseminated with cooled semen during 5305 oestrous cycles over the course of the 1999 breeding season, 1847 (70%) produced a live foal the following year. Furthermore, insemination of mares only once in an oestrus resulted in a significantly lower per cycle foaling rate (Group 1: 507/1622, 31.2%) than insemination twice (Groups 2–4: 791/1905, 41.5%), three times (Groups 5–7c: 464/1064, 43.6%) or ≥4 times (Groups 8–10c: 314/714, 43.9%) per cycle ($P \leq 0.001$, Table 3). The highest per cycle foaling rates were achieved by three inseminations with a 24 h interval between each (Group 5: 109/215, 50.7%) or ≥4 inseminations with initial AIs at a ≥48 h interval but the last two 24 h apart (Group 10a; 131/267, 49.1%). In addition, the foaling rate for all mares inseminated more than once per cycle tended to be higher if AIs were performed at a 24 h

Table 3
Effects of number of inseminations per oestrus and time interval between inseminations on the foaling rate in 2637 warmblood mares bred with cooled semen during the 1999 breeding season

Group	AIs per oestrus (*n*)	AI-interval (h)	Cycles (*n*)	Live foals (*n*)	Per cycle foaling rate (%)
1	1	–	1622	507	31.2[1] a
2	2	24	546	229	41.9 b
3	2	48	1169	491	42.0 b
4	2	>48	190	71	37.3 b
∑ 2 AI			1905	791	41.5[2]
5	3	24	215	109	50.7 c
6	3	48	404	172	42.5 b,c
7a	3	>48, last two AI 24	223	93	41.7 b,c
7b	3	>48; last two AI 48	146	59	40.4 b,c
7c	3	>48	76	31	40.8 b,c
∑ 3 AI			1064	464	43.6[2]
8	≥4	24	123	57	46.3 b,c
9	≥4	48	103	40	38.8 b,c
10a	≥4	>48; last two AI 24	267	131	49.1 c,d
10b	≥4	>48; last two AI 48	182	74	40.6 b,d
10c	≥4	>48	39	12	30.7 a,b
∑ ≥ 4 AI			714	314	43.9[2]
∑			5305	2076	39.1

Within a column values with the same letters (a, b, c) did not differ significantly ($P > 0.05$).
[1,2] The foaling rate after a single AI per cycle was significantly lower than that after multiple AIs per cycle ($P \leq 0.001$).

(Groups 2, 5, 7a, 8 and 10a: 619/1374, 45.5%) rather than a 48 h interval (Groups 3, 6, 7b, 9 and 10b: 836/2004, 42.1%, $P = 0.054$).

4. Discussion

Within the German horse breeding industry, it has become commonplace to administer 1500 IU hCG intravenously to oestrous mares once their pre-ovulatory follicle reaches 40 mm in diameter, and to inseminate them 24 h later. In the present study, mares were treated with hCG along the same protocol and inseminated at pre-determined times before or after hCG, to examine the effect of the insemination–ovulation interval on the pregnancy rate. In accordance with previous studies [11,17], a very high proportion of mares (97.5%) ovulated within 48 h of hCG administration, confirming the efficacy of hCG as an ovulation 'ensuring' agent. The interval from hCG administration to ovulation was not significantly affected by either mare age or reproductive status but was significantly affected by the time of year, being considerably shorter during the second half (May–July) than the first half (March–April) of the breeding season. This finding corresponds with the shortened oestrus and earlier ovulation characteristic of the peak of the physiological breeding season and, as such, presumably resulted in large part from mares ovulating spontaneously before hCG could exert its effect. Nevertheless, the possibility that hCG has a more rapid effect at the peak of the season cannot be ruled out and, in this respect, it has previously been reported that the interval between hCG administration and ovulation is shorter in mares treated during the breeding season than in the spring transitional period [18]. Clearly, hCG provides the equine stud practitioner with an excellent tool for ensuring ovulation within a given time interval, thus reducing the number of examinations and inseminations necessary per cycle and preventing excess use of commercially valuable semen. However, in the summer months, the greater likelihood of ovulation sooner after hCG injection than normally expected (36–42 h: [11,17]) should be borne in mind when deciding when to inseminate.

Relatively poor pregnancy rates after AI with frozen-thawed compared to fresh or chilled stallion semen are common [1,13], and are generally accepted to result from damage suffered by sperm during the freezing and thawing process [19]. It was thus no surprise that, in the current study, per cycle pregnancy rates were significantly higher for mares inseminated with cooled (152/282, 53.9%) than with frozen-thawed semen (97/220, 44.0%). On the other hand, Vidament et al. [20] reported similar pregnancy rates for mares inseminated at the French National Studs with cooled (56%; 2050 cycles, 1999 breeding season) or frozen-thawed semen (49%; 4190 cycles, 1996–99 breeding seasons). In the current study, pregnancy results after frozen semen AI did not differ whether mares were inseminated one (63/149, 42.2%), two (31/62, 50%) or three (3/9, 33.3%) times in an oestrus. However, as the time interval between insemination and ovulation decreased, pregnancy rates increased. For example, the highest pregnancy rates with a single insemination were attained when AI was performed up to 12 h before (Group B: 31/75, 41.3%) or after (Group A: 24/48, 50%) ovulation. In this respect, other authors have reported satisfactory pregnancy rates after post-ovulatory breeding with frozen-thawed stallion semen, including Newcombe (40% per cycle for AI 0–12 h post-ovulation; [21]),

Kloppe et al. (70% per cycle for AI 0–6 h post-ovulation; [22]) and Darenius and Darenius (43% per cycle for AI 0–8 h post-ovulation; [23]). With regard to the temporal extreme for post-ovulation AI, in an excellent study with fresh stallion semen Woods et al. [9] demonstrated that AI up to 12 h post-ovulation resulted in pregnancy rates equivalent to those from pre-ovulation AI, but that mares inseminated ≥30 h after ovulation never got pregnant. On the other hand, the embryonic loss rate was greater for mares inseminated after ovulation [9,10]. Although it has not been demonstrated exactly how long after ovulation insemination with frozen-thawed semen will still result in acceptable pregnancy rates, because of the likelihood of falling pregnancy rates coupled to increasing embryonic loss rates, it is usually recommended that AI with frozen-thawed semen is performed no more than 6–8 h after ovulation [12,22,23].

In the current study, pregnancy results were similar for all frozen semen AI protocols examined, with the exception that a single AI ≥12 h before ovulation yielded poor results (Group C: 8/26, 30.8%) that could be improved significantly by a second AI 24 h later, and thus shortly (<12 h) after ovulation (Group F: 13/21, 61.9%). This suggests that if a mare fails to ovulate within 12 h of frozen-thawed semen AI, insemination should be repeated 24 h after the first AI. It also emphasises the reduced longevity of frozen-thawed as compared to cooled semen. The results of this study therefore suggest that with frozen-thawed semen it should be aimed to inseminate between 12 h before and 12 h after ovulation, and that AI should then be repeated at 24 h intervals until ovulation is detected. How exactly this is organised will depend largely on the balance between the cost of semen per dose versus the costs of insemination and veterinary attention. Thus, when dealing with expensive semen or a mare prone to post-mating endometritis, the economics are likely to favour more intense veterinary management and a single peri-ovulation insemination whereas for abundant cheap semen, it may be more cost-effective to choose for less frequent examination and AI at 24 h intervals until ovulation. This latter approach was championed by Vidament et al. [13,20] who found a clear increase in the per cycle pregnancy rate with the number of frozen semen inseminations (at 24 h intervals) before ovulation, and good pregnancy rates (49% of 4190 cycles) despite infrequent (never more than every 24 h) examination of the mares' ovaries.

In a previous 5-year survey (1991–1995), Vidament et al. reported per cycle fertility rates of between 32 and 41% (total of 1473 cycles) when mares were inseminated two to three times per cycle with either 150×10^6 or 300×10^6 frozen-thawed spermatozoa at 24 h intervals [13]. During the 1996–1999, the AI dose was increased to 400×10^6 frozen-thawed spermatozoa and the per cycle pregnancy rate rose to 49% (4190 cycles; [20]), suggesting that 400×10^6 approaches the average minimum sperm dose required for optimal fertility, assuming of course that there were no coincident confounding changes in other management or treatment practices that may have improved fertility. The present study demonstrated that inseminating with 800×10^6 spermatozoa, of which ≥35% were progressively motile, once within the peri-ovulatory period (12 h pre- to 12 h post-ovulation) yields similar pregnancy rates to repeated insemination at 24 h intervals, but with more economical use of expensive semen or a busy stallion.

For cooled semen, fertility in Experiment 1 tended to decline for mares inseminated only once per cycle if that insemination was performed more than 24 h before ovulation (Group D: 2/7, 28.6%) and was significantly lower if AI was ≥36 h before ovulation

(Group E: 4/22, 18.2%). Indeed, pregnancy rates from these early inseminations tended to be less successful even than post-ovulation breeding (Group A: 11/24, 45.8%; $P = 0.062$). This contrasts with the findings of Woods et al. [9] who reported that mares inseminated once per cycle 1–3 days before ovulation had pregnancy rates significantly higher than those inseminated either ≥4 days before ovulation or on the day that ovulation was detected. The window for optimal AI timing of 1–3 days before ovulation thus implied by Woods et al. contrasts markedly to the 24 h before to 12 h after ovulation window suggested from our data. Reasons for this difference probably include the frequency of examination; certainly the twice daily examinations in the current study would have allowed more accurate determination of the time of ovulation. In addition, the fact that Woods et al. used fresh, as opposed to chilled, semen and pony or pony-cross, rather than warmblood, horses may have been factors. Indeed, it is likely that semen subjected to cooling has both a reduced longevity and fertility, while pony-type animals may be intrinsically more fertile than warmbloods. Finally, while multiple inseminations per cycle tended to improve pregnancy rates in the current study (three AIs in a cycle gave a 71.4% [20/28] pregnancy rate compared to 49.7% [84/169] for a single AI per cycle, $P \leq 0.05$), this difference was distorted by the poor results obtained after inseminating ≥24 h before ovulation, and a single AI between 24 h before and 12 h after ovulation gave similar results to two or three AIs per cycle (78/140, 55.7%; 48/85, 56.5%; and 20/28, 71.4%, respectively).

Overall pregnancy results obtained under carefully controlled conditions using cooled semen in Experiment 1 (152/282, 53.9%) were much higher than the foaling rates obtained using field data in Experiment 2 (2076/5305, 39.1%) and the pregnancy rates for cooled semen AI published by Vidament et al. (56% of 2050 cycles; [20]) were much closer to those attained in Experiment 1. Of course, one important difference is that the outcome in Experiment 2 was the percentage of live, registered foals per cycle and thus, unlike the other two studies, the figures took account of subsequent embryo, fetal and peripartum losses. If this was the only source of difference it would suggest that approximately 30% of pregnancies failed before foal registration, a figure that is not out of the question given that early pregnancy losses alone run at 5–17% in young healthy mares and up to 60–70% in older problem mares [24]. It has also been reported that time in storage of cooled semen can affect pregnancy rates [1], and in the two experiments there was a difference in semen storage time from 2–4 h in Experiment 1 to up to 12 h in Experiment 2. On the other hand, Heiskanen et al. [6] demonstrated that the detrimental effect of sperm storage on pregnancy rates can, at least in part, be compensated by increasing the total number of sperm and inseminating within 12 h post-ovulation. In the retrospective study (Experiment 2), it was also clear that mares inseminated only once per cycle with cooled semen (Group 1: 507/1622, 31.2%) had significantly lower foaling rates than those inseminated twice (791/1905, 41.5%), three times (464/1064, 43.6%) or ≥4 times (314/714, 43.9%) per cycle, while the latter groups did not differ, just as reported by Vidament et al. [20]. However, it is not clear whether the apparent inadequacy of the single AI per oestrus regime was because of inaccurate timing of that insemination or because there was a positive effect of repeated insemination in establishing a healthy oviductal sperm reservoir.

In our field study, the highest foaling rates were obtained when mares were inseminated either three times in a cycle with a 24 h inter-insemination interval (Group 5) or ≥4 times per cycle, with the last two inseminations 24 h apart (Groups 8 and 10a). Furthermore, per

cycle foaling rates after repeated insemination with at least the last two inseminations 24 h apart tended to result in higher foaling rates than every other day AI (619/1374, 45.5% versus 836/2004, 42.1%, $P = 0.054$). This suggests an advantage of breeding mares with cooled semen daily rather than every other day presumably, at least in part, because daily insemination increases the likelihood of AI close to ovulation. Previous studies vary in their recommendations for the most appropriate insemination interval for cooled stallion semen between daily [14,25] and every other day [26]. In the United States, however, transported stallion semen is often supplied as a single shipment containing two AI doses and Shore et al. [15] found no advantage of holding the second dose for insemination on the following day, suggesting that it was better to inseminate with both immediately. By contrast, Squires et al. [14] reported significantly better pregnancy rates after inseminating mares with one dose on each of two consecutive days [14]. In both studies, mares were injected with hCG simultaneous to initial insemination and, while the results of Squires et al. [14] may suggest an advantage of repeated AI or of AI closer to ovulation, it needs to be confirmed that this effects holds when the choice is prolonged storage of semen in vitro as opposed to earlier insemination with a double dose of semen.

A widely accepted prerequisite for good fertility is the availability of sufficient viable spermatozoa within the oviduct to fertilise the oocyte when it arrives after ovulation. One of the hypotheses guiding our study, was that either repeating AI (particularly with a shorter inter-AI interval) or inseminating closer to the time of ovulation may promote a more healthy oviductal sperm reservoir and positively influence pregnancy rates. In summary, our findings were equivocal because although repeated insemination seemed to help in the field, as they did in Vidament et al.'s survey [20], under more carefully controlled conditions a single insemination during the optimum period (12 h before to 12 h after ovulation for frozen-thawed semen and 24 h before to 12 h after ovulation for cooled semen) was just as successful as repeated AI. This suggests that a large part of the apparent beneficial effect of repeated AI is an increase in the likelihood that AI is performed close to ovulation; this would also explain why a 24 h AI interval appears better than 48 h.

In conclusion, the present data suggest that the optimum insemination regime for mares is a single AI within a restricted period close to ovulation, namely 12 h before to 12 h after ovulation for frozen-thawed semen and 24 h before to 12 h after ovulation for cooled semen. Re-insemination 24 h later would be advised if ovulation did not occur within the specified period after AI. The major effect of multiple inseminations per cycle seems to be to increase the likelihood of AI within the optimal 'window' and this needs thus to be played off against the extra costs of semen or semen transportation. Finally, hCG was confirmed as a valuable and reliable way for increasing the chance of correctly timing insemination.

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