The effect of time of insemination with fresh cooled semen relative to ovulation on pregnancy and embryo loss rates in the mare

JR Newcombe[1] and J Cuervo-Arango[2]

[1]*Equine Fertility Clinic, Warren House Farm, Barracks Lane, WS8 6LS Brownhills, West midlands, UK;* [2]*Dpto. Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, 46113 Moncada, Spain*

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154 mares were inseminated with fresh semen either during the pre- or post-ovulatory periods at different intervals relative to ovulation: 36 to 24 h (n = 17) and 24 to 0 h (n = 30) before ovulation; 0 to 8 h (n = 21), 8 to 16 h (n = 24), 16 to 24 h (n = 48) and 24 to 32 h (n = 14) h after ovulation. All mares received the same routine post-mating treatment consisting of an intrauterine infusion with 1 litre of saline and antibiotics followed 8 h later by an intravenous administration of oxytocin. Inseminations from 36 h before ovulation up to 16 h post ovulation were performed with transported cooled semen, whereas mating after 16 h post ovulation were performed by natural cover. Pregnancy rate (PR) of mares inseminated 36 to 24 h (29.4%) was significantly lower ($P<0.05$) than mares inseminated 24 to 0 h before ovulation (60%), 0 to 8 h (66.7%) and 8 to 16 h (70.1%) post-ovulation. Embryo loss rate (ELR) was highest in mares mated 24 to 32 h after ovulation (75%). PR of mares mated 16 to 24 h post ovulation (54.1%) did not differ

significantly from any other group (P>0.05), however the embryo loss rate did increased markedly (34.6%) compared with inseminations before 16 h post ovulation (<12%). Good PR with acceptable ELR can result from inseminations within 16 h of ovulation, at least with this specific post-mating routine treatment.

Keywords: mare: post-ovulation insemination; pregnancy rate; post-mating treatment

Introduction

Insemination with fresh cooled transported spermatozoa is a common practice in equine reproduction. A worldwide upsurge in the use of this type of stallion semen has occurred over the past decade, especially in large land-mass countries where cooled semen is couriered by air on a regular basis over very large distances. This saves large sums on transporting mares to the stallion of choice and it greatly enhances the range of stallions available to mare owners (Allen 2005). Semen from most stallions survives slow cooling to 4 ºC and retains a good level of fertility for 48 to 72 h if maintained at this temperature (Betellier et al. 2001). However, the process of cooling definitely diminishes the ability of spermatozoa to survive within the mare's genital tract as compared with fresh spermatozoa deposited in the mare by natural cover. This reduction in longevity is more marked in frozen/thawed semen and is thought to be due to premature acrosome reaction (Thomas et al., 2006). During cooling to 4ºC, spermatozoa are also damaged and therefore its longevity in the mare's oviduct is less than that of fresh non-chilled semen. This was shown by the results of one study (Batellier et al. 2001) in which semen stored at 15 ºC for 24 h had better fertility that semen stored at 4 ºC for the same period implying a greater damage to sperm cells during the process of cooling to lower

47 temperature. While fresh semen deposited by natural cover may remain in the oviducts
48 viable for 3 to 4 days (Woods et al., 1990; Newcombe 2001) even for up to 1 week
49 (Newcombe 1994), the viability of cooled transported semen that has been stored for 24 h
50 at 5ºC is reduced dramatically after 24 h in the mare's genital tract (Sieme et al., 2003).
51 The latter study performing AI with cooled semen found a significant reduction in
52 pregnancy rate (PR) from 57.8% at 24 to 0 h before ovulation to 28.6% and 18.2% at 24
53 to 36 h and 36 to 48 h before ovulation respectively.
54 Therefore in equine practice, the time of insemination with cooled semen is intended to
55 be best performed 0 to 24 h before ovulation. Thus a common practice is to treat a mare
56 in oestrus and with a follicle of ≥35 mm with 1500 IU of hCG one day before of the
57 semen's expected arrival time so that the spermatozoa are deposited 12 to 24 h before
58 ovulation. In some occasions, however, the mare may ovulate before the expected time
59 after hCG treatment (Davies-Morel et al., 2008) or the courier transporting the semen
60 may be delayed for 24 to 48 h due to several unforeseen reasons. This will result in the
61 mare having ovulated before the semen is available for AI, leaving the veterinarian with
62 two options: a post-ovulatory insemination or a missed cycle. Usually there is pressure
63 from the client not to miss the cycle, therefore the first option is often chosen. However
64 there is the belief of the existence of two drawbacks commonly associated with post-
65 ovulatory inseminations: an increased embryo loss rate (ELR) (Woods et al., 1990) and a
66 high incidence of persistent mating-induced endometritis due to lower resistance of the
67 mare's uterine defences after the onset of the luteal phase (Gutjahr et al., 2000). This is
68 specially contraindicated in old mares or in those known to be susceptible to endometritis
69 (Newcombe and Cuervo-Arango 2008). Therefore it seems important for equine

70 reproductive practice to critically determine the effect of the interval from ovulation to AI
71 on PR and ELR in a commercial setting of AI.
72 The data presented here is of mares from a veterinary clinic examined at short intervals
73 during the peri-ovulatory period and inseminated with cooled semen or mated naturally at
74 different times relative to ovulation.
75
76 **Materials and methods**
77 *Animals, ultrasonography and insemination protocol*
78 This study involved data from 154 mares of mixed breeds during the 2000 to 2009
79 breeding seasons, without known reproductive problems, which were either resident or
80 visiting a veterinary clinic in the UK (northern hemisphere).
81 Internal genitalia were examined by transrectal ultrasonography with an ultrasound
82 scanner equipped with a linear-array transducer every 8 h in the peri-ovulatory period
83 such that the interval from ovulation to insemination was known to within ± 4 h.
84 The semen was sent by courier from commercial stud farms from The Netherlands and
85 Germany mainly and some (15%) from the UK. A sample of semen was evaluated at
86 37ºC before insemination. All samples included in the study had acceptable progressive
87 motility of >40 %.
88 The mares were either inseminated with cooled transported semen into the uterine body
89 (n = 92) from 36 h before to 16 h after ovulation or mated naturally (n = 62) with
90 stallions of proven fertility from 16 to 32 h after ovulation. Data from inseminations with
91 cooled semen were available only until 16 h post-ovulation since in very rare occasions
92 the insemination of client mares was delayed for longer. In contrast there was data

93 available of natural matings after that time period owing to the existence of ongoing
94 pregnancy trials.
95 Pregnancy diagnosis was performed at 12 to 14 days post-ovulation and thereafter at 30
96 and 45 days. Furthermore, in 44% of the pregnancies follow-up was available until
97 foaling. In non-pregnant mares, diagnosis of premature luteolysis was made when there
98 was an ultrasonically regressed CL, endometrial oedema with or without the presence of
99 varying amounts of free intrauterine fluid at 12 to 14 days post-ovulation which indicated
100 a premature return to oestrus.
101
102 *Post-mating routine treatment*
103 Uterine flushing with 1 liter of saline was performed in all mares 4 to 8 h post-mating
104 followed after fluid recovery by infusion of 12 ml of 1800 mg procaine penicillin (6 ml of
105 Depocillin®, Intervet/Schering-Plough Animal Health, Milton Keynes, UK) and 900 mg
106 framycetin (6 ml of Framomycin 15%®, Novartis Animal Health, Camberley, UK).
107 Intravenous oxytocin 25 IU (Oxytocin®, Leo Animal Health, Leo Laboratories Ltd,
108 Aylesbury, UK) was administered 8 h later and at further intervals as necessary (until the
109 uterus appeared free from intrauterine fluid).
110
111 *Experimental protocol and statistical analysis*
112 The mares were classified into 6 groups according to the time of insemination relative to
113 ovulation as follows:
114 - Group 1: AI with cooled semen 36 to 24 h before ovulation (n = 17).
115 - Group 2: AI with cooled semen 24 to 0 h before ovulation (n = 30).

- Group 3: AI with cooled semen 0 to 8 h after ovulation (n = 21).
- Group 4: AI with cooled semen 8 to16 h after ovulation (n = 24).
- Group 5: Mated naturally 16 to 24 h after ovulation (n = 48).
- Group 6: Mated naturally 24 to 32 h after ovulation (n = 14).

The PR and ELR were compared amongst groups. Although the last two groups (5 and 6) involved mating of mares with fresh non-cooled semen, these were analyzed along with the groups of cooled semen since all natural covers from these groups were performed during the post-ovulatory period when it is assumed that the limiting factor for a successful fertilization is the age of the oocyte and not the ability of the sperm to remain viable (longevity) within the mare's reproductive tract.

Pregnancy and embryo loss rate differences amongst groups were analyzed by Binary logistic regression. Significance was set at $\alpha = 0.05$. All data was computed in the statistical software Minitab15®.

Results

Pregnancy rate and ELR for groups 1 to 6 are shown in Table 1. Mares inseminated with cooled semen 36 to 24 h before ovulation had lower PR (29.4%) than mares inseminated within 24 h before or 0 to 16 h after ovulation ($P<0.05$). Pregnancy rate and ELR were not different ($P>0.05$) among mares inseminated from 24 h before ovulation to16 h after.

A 15.6 % (5/32) of mares that failed to become pregnant had a short cycle (n = 4) or excessive accumulation of intrauterine fluid (n = 1) detected during the first pregnancy test. All of them were mated > 16 h post-ovulation.

Discussion

The results of insemination of mares within 16 h after ovulation showed that acceptable pregnancy rates (PR) can be achieved and were superior (68.9%) to the 24 h period before ovulation (60%). An acceptable PR (54.1%) was achieved when mating was delayed until 16 to 24 h after ovulation. However ELR increased to 35.4%. Mating more than 24 h after ovulation resulted in seriously reduced PR and increased ELR of 28.6 and 75% respectively.

In the pre-ovulatory insemination groups, the PR was reduced by half when the spermatozoa remained longer than 24 h in the mare's uterus before the oocyte was released. This fall in PR at that time interval are in agreement with those of the study from Sieme and co-workers (2003) which also showed that 24 h was the threshold time after which spermatozoa from most stallions lost significantly their fertilizing ability. It appears that during the process of cooling to 5ºC, sperm membranes can be seriously damaged which results in decreased sperm lifespan within the mare's reproductive tracts once it warms up to body temperature. Nonetheless, approximately a third of mares inseminated 24 to 36 h before ovulation (29.4%) became pregnant. This variation in sperm's longevity from different stallions after cooling and insemination may result from individual variation in the ability of some stallions' spermatozoa to better withstand the process of cooling. The variation in freezability of semen from individual stallions is well documented and appears to be unrelated to the fertility of stallions when covering mares naturally (Allen 2005).

Good early PR is of no value if subsequent foaling rates are poor. Detected pre- and post-ovulatory losses after insemination with cooled semen up to 16 h were low (4.3% and

9.7% respectively) and not significantly different ($P > 0.05$). Only after 16 h post-ovulation, the ELR rose significantly.

In contrast with the study of Woods and co-workers (1990) in which the level of embryonic loss (up to 40 days post-ovulation) rose from 14% in the pre-ovulatory period, to 27%, 23% and 43% in 0 to 6, 6 to12 and 12 to 18 h post-ovulation respectively. In the current study, known embryonic and foetal losses were 4.3% in the pre-ovulatory period and 7.1 and 11.8% in 0 to 8 and 8 to16 h (mean 9.7%) respectively, not only substantially lower and not significantly different from pre-ovulatory inseminations, but not showing either any marked increase until the interval was more than 16 h. The differences in ELR between both studies at comparable post-ovulatory intervals are surprisingly high. Other studies reporting ELR after post-ovulatory inseminations seem to be in agreement with the ELR observed in the current study. Barbacini and co-workers (1999) reported a PR of 38% and an ELR of 9.3% after inseminating 351 mares with frozen/thawed spermatozoa 0 to 6 h after ovulation. Newcombe (2005) reported an ELR of 12.8% (11/86) in pregnancies conceived 0 to 24 h after ovulation. In both studies and in most veterinary clinics mare received some sort of post-insemination treatment consisting of intrauterine antibiotics, ecbolic drugs, uterine lavage with large volume of saline or a combination of them specially in post-ovulatory inseminations. In contrast, in the study of Woods et al. (1990) no post-insemination treatment was used at all. Whether this lack of post-insemination treatment accounted for the increased embryonic losses is not known but it is widely assumed that embryo viability is incompatible with an inflamed uterine environment.

Some mares, not recognized as “problem mares”, are still susceptible to persistent mating induced endometritis although they show no evident sign of susceptibility (Pycock and Newcombe 1996). Therefore many practitioners prefer to use routinely some form of

post-mating preventative treatment. In a large field trial, significant improvements in PR over controls showed the value of a single routine treatment with either antibiotics or oxytocin or both combined (Pycock and Newcombe 1996). After ovulation post-mating treatment becomes even more important since the mares' natural resistance to infection becomes less effective once the cervix begins constrict and progesterone concentrations begin to rise after ovulation.

The results of this study showed evidence that mares mated after ovulation were at increased risk of endometritis. The only mares with premature luteolysis (n = 4) or mares with excessive intrauterine fluid accumulation (n = 1) seen in this study at the time of the pregnancy test (12 to 14 days post-ovulation) resulted from inseminations in the period from 16 to 32 h post-ovulation (15.6% of 32 non pregnant mares in that period). Some types of bacterial endometritis have been shown to cause premature luteolysis (Newcombe, personal observation).

In conclusion**,** and contrary to popular opinion, good pregnancy rates with acceptable pregnancy losses can result from insemination with cooled transported semen within 16 h of ovulation, at least in an insemination regime using the reported preventative post-mating treatment protocol. After 16 h post-ovulation, although the PR may be acceptable, the high incidence of embryonic death and short-cycles renders insemination inadvisable.

Author contributions

JR. Newcombe and J. Cuervo-Arango contributed equally.

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Author's address (for correspondence): J Cuervo-Arango, Departamento Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad CEU-Cardenal Herrera, 46113 Moncada, Spain. E-mail: juan.cuervo@uch.ceu.es.