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Recovery Rate and Quality of Embryos from Mares Inseminated at the First Post-Partum Oestrus

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Huhtinen, M., T. Reilas and T. Katila: Recovery rate and quality of embryos from mares inseminated at the first post-partum oestrus. Acta vet. scand. 1996, 37, 343-350. – The pregnancy rate is lower in mares inseminated at the first post-partum (p.p.) oestrus (40-50%) compared with pregnancy rates in subsequent oestrous cycles (55-65%). The causes of the lowered pregnancy rate are not fully understood. The aim of the present study was to examine if embryonic defects could be one of the reasons for lowered pregnancy rate. A total of 23 p.p. and 14 non-lactating control mares were flushed 7 days after detection of ovulation. Embryo recovery rate was 48% and 71% in p.p. and control mares, respectively ($p=0.16$). Embryos were photographed, measured, graded and stained with fluorescein diacetate to assess their viability. Thereafter embryos were bisected and stained with Hoechst 33342 to count the cell nuclei. Embryos in both groups were equally viable and the cell numbers were not significantly different. According to morphological evaluation all embryos were classified as excellent or good. Embryos aged 7.3 to 7.6 days (± 0.25 days) were smaller in the p.p. group than in the control group ($p<0.05$). Forty-seven (9/19) and 8% (1/13) of the uterine swabs, taken before the first insemination, yielded bacteria and neutrophils in p.p. and control mares, respectively. The amount of neutrophils and/or bacteria had no statistically significant effect on embryo recovery rate ($p>0.10$). Recovery of embryos was not related to histological findings in uterine biopsies taken after embryo recovery. Embryo recovery rate in p.p. mares (48%) was similar to previously reported foal heat pregnancy rates (40-50%). Hence, early embryonic death in utero would not be the most likely reason for lowered pregnancy rate in mares inseminated at the first p.p. oestrus. Sperm transport and oviductal conditions by the time of the first p.p. oestrus would need to be studied to clarify the role of fertilisation failure as the cause of lower pregnancy rate in mares inseminated at foal heat.

bacteria; neutrophils; viability; FDA- staining; cell number; Hoechst 33342.

Introduction

Most studies indicate that the pregnancy rate is at least 10% to 15% lower when mares are bred at the first post-partum (p.p.) oestrus as compared with subsequent oestrous periods *(Caslick* 1937, *Sullivan et al.* 1975, *Lieux* 1980, *Badi et al.* 1981, *Loy* 1988). Managemental factors greatly contribute to the variation in conception rates between stud farms *(Loy* 1980, *Lowis & Hyland* 1991). The majority of mares have various degrees of bacterial contamination of the uterus following parturition *(Katila* 1988), but most mares are able to eliminate bacteria by the time of foal heat *(Saltiel et al.* 1987, *Purswell et al.* 1989). Because uterine involution is not completed by the time of the first p.p. oestrus *(Andrews & McKenzie* 1941, *Katila et al.* 1988, *McKinnon et al.* 1988), inappropriate

uterine environment may prevent spermatozoa from reaching the site of fertilisation or hinder normal embryonic development *(Fischer & Beier* 1986).

Some studies have shown that the incidence of pregnancy loss has been higher in mares bred at the first p.p. oestrus than in mares bred at subsequent oestrus periods *(Merkt* 1966, *Platt* 1973, *Lieux* 1980). However, in many other studies the pregnancy loss rates have been about the same in mares bred at foal heat as compared with mares bred later *(Bain* 1969, *Loy* 1980, *Scherbarth* 1980, *Woods et al.* 1987, *Torp et al.* 1989).

Embryo recovery is a mean to examine conceivable reasons for lowered pregnancy rate. In the mare, normal embryos enter the uterus by day 6 post ovulation *(Oguri & Tsutsumi* 1974), whereas unfertilised ova are retained in the oviducts *(Van Niekerk & Gerneke* 1966). Several studies have shown that embryo recovery rates on days 6 to 8 are significantly lower in subfertile than in normal mares, and the suggested reasons for this include: lack of fertilisation, morphological and chromosomal abnormalities of the embryo, oviductal problems, and a hostile uterine environment *(Douglas* 1982, *Squires et al.* 1982, *Pascoe et al.* 1985, *Woods et al.* 1985, *Ball et al.* 1986). Fertilisation failure is probably not the major cause of low pregnancy rates in subfertile mares, since no significant difference in day 2 pregnancy rates was found between subfertile and normal mares *(Ball et al.* 1986). Embryo abnormalities, other than those that are morphologically apparent, appear to be an important cause of pregnancy loss in subfertile mares, since fewer fair-to-good quality day 4 embryos from subfertile mares versus normal donor mares survived, when transferred to the uterus of normal recipient mares *(Ball et al.* 1989).

To our knowledge, no reports have been published on embryo recovery from mares bred at the first p.p. oestrus. The objectives of the present study were to investigate if the lowered pregnancy rates after foal heat breeding would be expressed as a decrease in embryo quality and/or recovery rate, and to compare the results with those from non-lactating control mares.

Materials and methods

The study was carried out from March to May using 23 p.p. mares and 14 non-lactating control mares, and one stallion of known fertility. The mares were brood mares of different breeds (weight 450-750 kg) owned by the Equine Research Station. The average age of p.p. mares was 10.8 years (range 4 to 18 years) and they had foaled 1 to 7 times prior to this experiment. The control mares were on average 9.6 years old (range 4 to 15 years), had foaled 1 to 5 times previously, were clinically normal and had no history of reproductive failure. All mares were exposed to artificially prolonged day length (14.5 h of light) from the beginning of December.

Rectal palpation and ultrasonography of the ovaries and uterus were started 5 days after parturition in p.p mares, and in the beginning of oestrus in control mares. The examination was repeated every other day until occurrence of a follicle of 35 to 40 mm in diameter. At this point ovarian activity was monitored every 12 h, and mares were inseminated with 500×10^6 progressively motile spermatozoa every other day until ovulation (day 0). The actual time of ovulation detection was recorded. To detect additional ovulations, follow-up of follicles 30 mm or larger was continued for 2 days after the first ovulation.

A uterine swab was taken before the first insemination using a guarded swab and an aseptic technique *(Blanchard et al.* 1991).The tip of the swab was streaked onto a blood agar and the side of the swab was used to prepare a smear. The smears were stained with May-Grünewald

Giemsa. Numbers of polymorphonuclear neutrophils (PMN) per 10 fields at a magnification of 400 were scored as follows: no PMN (0), 1 to 10 PMN (+), >10 but mostly isolated PMN (++), and large clumps of PMN (+++). Bacterial colonies were counted and identified after incubation of 48 h at 37°C. Identification was based on colony morphology, haemolysis, catalase test and Gram's stain. Eosin methylene blue agar was used when necessary. Bacterial growth was considered significant when more than 10 colonies were recovered per plate.

Embryos were recovered nonsurgically 7 days after detection of ovulation (in case of asynchronous double ovulation, from the second ovulation) using a two-way equine embryo flushing catheter (Ch 2; Ludwig Bertram Gmbh, Hannover, Germany). The actual time of embryo recovery was recorded. The uterus was flushed 3 to 4 times with 1 litre of phosphate buffered saline (PBS) supplemented with 2 g of bovine serum albumin (BSA) and 25 mg kanamycin. Flushing medium recovered from the mares was passed through an embryo filter (Emcon – Immuno Systems, Spring Valley, WI), and the fluid in the filter cup was examined for the presence of embryos and degenerative ova using a stereomicroscope. Once an embryo was located, it was washed with PBS supplemented with 5% foetal calf serum (FCS)(Gibco BRL, Life Technologies, Paisley, Scotland), photographed, classified according to *McKinnon & Squires* (1988) and measured with an eyepiece graticule. The age of the embryo was estimated to be the time from ovulation detection to the time of embryo recovery plus 6 h (the ovulation was estimated to have occurred midway during the 12 h interval between 2 consecutive examinations).

To assess viability, the embryos were stained with fluorescein diacetate (FDA) *(Pruitt et al.* 1988). FDA passes easily into the embryos where esterases turn it into fluorescein. If the cell membranes are intact, fluorescein accumulates intracellularly. The embryos were placed in PBS solution containing 5% FCS and 2.5 μg/ml FDA. After 1.5 min of incubation at room temperature, the green colour of intracellular fluorescein was visualised using an inverted microscope (Olympus, IMT-2) with dichroic mirror unit (IMT2-DMU). After staining, the embryos were washed with PBS supplemented with 5% FCS and bisected with a microblade attached to a Narishige micromanipulator (Model MO-204). After bisection, the embryos were stained with Hoechst 33342 (Sigma Chemical Co., St. Louis, MO) to count the number of cell nuclei. The staining was done in a four-well dish. The first well contained 10 μl of 2% Hoechst-stock solution, 100 μl of ethanol and 400 μl of PBS + 5% FCS. The other wells contained 0.5 ml of PBS + 5% FCS. Each demi-embryo was placed in the first well for 10 min, then washed in the 3 other wells. After washing, each demi-embryo was transferred onto a slide and gently pressed with a coverslip before being exposed to UV light. The UV irradiation of FDA- and Hoechst-stained embryos were recorded on a videotape.

A uterine biopsy was taken after embryo recovery using an instrument with alligator-type jaws. The biopsy specimens were fixed in Bouin's solution and transferred to 70% ethanol after 24 h. The fixed tissues were embedded in paraffin, sectioned at a thickness of 6 μm, and stained with haematoxylin and eosin. The biopsies were evaluated and the non-lactating mares classified according to the principles outlined by *Kenney & Doig* (1986). From the endometrial biopsies of p.p. mares, the degree of involution, degenerative changes, and inflammation (acute and chronic) were individually evaluated from each sample.

The differences between the 2 groups of mares (foal heat vs control) regarding embryo recov-

ery rates, and the differences between foal heat mares that had positive or negative uterine swabs were analysed using Chi-square analysis. The differences between the 2 groups of mares in diameters of embryos and cell numbers were analysed using linear model methods *(SAS Institute Inc.* 1988). The effect of uterine biopsy parameters on embryo recovery results in the foal heat group was analysed using the T-test.

Results

The mares in the p.p. group were swabbed and inseminated for the first time an average of 7.6 ± 1.8 (SD) days after foaling (range 5 to 15 days). One mare had retained foetal membranes and was treated with oxytocin and antibiotics. The mares ovulated an average of 10.8 ± 1.9 days post partum (range 8 to 15 days). Double ovulations were detected in 3 mares (1 p.p. mare, 2 control mares).

Embryos were recovered an average of 17.8 ± 2.0 days post partum (range 15 to 22 days). The recovery rate of fluid was identical in p.p. and control mares (96% and 94%, respectively). Eleven embryos were found from 23 p.p. mares (48%), and 10 embryos from 14 control mares (71%), but although lower for the p.p. mares, this difference was not statistically significant ($p = 0.16$). No twin embryos were recovered. Four control mares yielded 6 degenerative ova. Three of these mares also gave an embryo.

The mean age of the embryos was 7.2 days ± 0.25 days, but they were clearly divided into 2 age groups depending upon the time of ovulation (morning vs evening). One control mare that had double ovulations was flushed 7 days after the second ovulation, and an embryo 980 μm in diameter was recovered. This supposedly day 8 embryo was removed from statistical analyses.

Diameters and numbers of cells for embryos are shown in Table 1. Embryos were smaller ($p<0.05$) in p.p. mares (279 μm) than in control mares (465 μm) in the age group of 7.3 to 7.6 days (± 0.25 days). The diameter of younger embryos (6.8 to 7.0 ± 0.25 days) was about equal in p.p. and control groups (248 μm vs 260 μm, respectively). The numbers of cells did not differ between embryos from p.p. mares and control mares ($p>0.05$). Three embryos were excluded from the table either because one of the halves was lost during the Hoechst-staining procedure or because the number of the cells was impossible to count.

According to morphological evaluation all embryos were considered normal and of excellent or good quality. A few extruded blastomeres, irregular shape or trophoblastic separation from the zona pellucida were the only imperfections seen. All embryos were able to hydrolyse FDA and produce intracellular fluorescein, and were therefore considered to have esterase activity, intact cell membranes, and to be viable. Unfertilised ova, stained with FDA, did not accumulate fluorescein.

Fourteen out of 19 uterine swabs from p.p. mares yielded bacteria and/or neutrophils (Table 2). Five mares had neither bacteria nor neutrophils in their swabs, and were therefore excluded from Table 2. Four p.p. mares were not swabbed. The amount of neutrophils and/or bacteria in the uterine swab had no statistically significant effect on embryo recovery rate ($p>0.10$). In control mares all but one of the swabs ($n = 13$) were negative for bacteria and neutrophils. From the positive control mare which had 2 colonies of bacteria and very few neutrophils in the uterine swab, a normal appearing embryo was recovered.

According to histological analysis of the uterine biopsies, most of the mares in the control group were classified as normal (4 mares in category I and 9 mares in category IIA); only one mare belonged to category IIB. In the p.p. group of mares, no statistically significant differences re-

Table 1. Diameters and cell numbers of embryos from post-partum and control mares.

	Post-partum		Controls	
Embryo age ± 0.25 days	Embryo diameter, μm (n) mean (range)	Cell number (n) mean (range)	Embryo diameter, μm (n) mean (range)	Cell number (n) mean (range)
6.8-7.0 days	(4) 248 (170-420)	(3) 1260 (306-2569)	(5) 260 (220-330)	(5) 1107 (635-1678)
7.3-7.6 days	(7) 279[a] (160-420)	(7) 1015[c] (137-2331)	(4) 465[b] (360-580)	(2) 2027[d] (1783-2270)

(n) : Number of embryos.
Statistically significant differences between a and b : $p<0.05$;
no statistically significant difference between c and d : $p>0.05$.

Table 2. Bacteria and neutrophils in positive uterine swabs taken before the first insemination from post-partum mares.

Mare	Bacteria colonies	Bacteria identification	Neutrophils	Embryo
Ensjus V.	> 100	β-haemol.str.	+++	–
Matrosa	> 100	β-haemol.str.	+	–
Good L.	> 100	gram + rod	+	–
Ungena	25	mixed growth	+	+
Lerkka	17	mixed growth	+++	–
Neili	12	mixed growth	+	–
Seirene	2	β-haemol.str.	+++	+
Pink C.	2	mixed growth	++	+
Armbro U.	2	?	+	–
Miss C.A.	5	E. coli	–	–
Cindy	1	?	–	+
Ulrica	0		+	+
Hiluntähti	0		+	–
Nietro	0		+	–

garding the stage of involution, degenerative or inflammatory changes were found between mares that gave an embryo vs those that did not.

Discussion

The average interval from parturition to ovulation in our foaling mares (10.8 days) is in agreement with the result (10.2 days) of 456 thoroughbred mares in Kentucky *(Loy* 1980). The average interval from parturition to ovulation in other Finnish studies concerning untreated mares has been somewhat longer (12.2 days – *Katila et al.* 1988, 13.0 days – *Koskinen* 1991). The shorter interval from parturition to ovulation in our study was probably caused by artificially prolonged daylight, which has been reported to shorten the interval from parturition to first ovulation in mares foaling in spring *(Koskinen et al.* 1991).

The embryo recovery rate of 71% of the non-lactating control mares was comparable to embryo recovery rates obtained by other researchers (*Squires et al.* 1982, *Woods et al.* 1986). Also the recovery of degenerative ova from the uteri of cycling mares is in agreement with previous results (*Wilson et al.* 1991). To our knowledge, no reports have been published on embryo recovery from mares bred at foal heat, although these mares are being flushed in commercial embryo transfer programs. In the present study, the embryo recovery rate of 48% for p.p. mares agrees with foal heat pregnancy rates reported previously (*Caslick* 1937, *Sullivan et al.* 1975, *Lieux* 1980, *Badi et al.* 1981, *Loy* 1988, *Katila et al.* 1988).

In our study, embryos were smaller in p.p. mares than in control mares when the estimated age of the embryos was between 7.3 and 7.6 days. However, the average time between ovulation detection and flushing happened to be 2.4 h shorter in p.p. mares than in the controls. A few hours can be very important at this stage because the size of the embryo increases rapidly (*McKinnon & Squires* 1988). Due to this difference in age, it is difficult to draw any conclusions about the effect of uterine environment after the first p.p. on embryonic growth. *Griffin & Ginther* (1991) have reported that the size of embryonic vesicles on days 14 to 16 was about the same in p.p. mares and nonparturient mares. Hence, the embryos from mares bred at foal heat would reach the same size at the time of uterine fixation.

Fluorescein diacetate has been successfully used to evaluate the viability of embryos (*Schilling et al.* 1979, *Mohr & Trounson* 1980, *Pruitt et al.* 1988 & 1991). We found very little variation in the intensity of fluorescence between embryos, only unfertilised ova were clearly nonfluorescent. Scoring embryos by percentage of fluorescent cells or fluorescent intensity was therefore impossible. The reasons for not detecting any difference between embryos might have been due partly to the good quality of embryos, partly to the automatic video-camera, which corrected the visualised intensity of the staining.

The number of p.p. mares that had significant bacterial growth in their uterine swab is in agreement with previous studies (*Cygax et al.* 1979, *Saltiel et al.* 1987, *Katila et al.* 1988, *Purswell et al.* 1989). Cytological smears and bacteriological cultures have been shown to have no correlation with fertility in foal heat, although mares with large numbers of bacteria just before ovulation had low pregnancy rates (*Katila et al.* 1988). The same applies to this study, where the amount of neutrophils and/or bacteria in the uterine swab at the time of first insemination had no statistically significant effect on embryo recovery rate ($p>0.05$).

The results of the present study suggest that lowered fertility of mares bred at foal heat is probably caused by other reasons than early embryonic death in the uterus prior to the first pregnancy detection. The uterine environment might be hostile at the time of insemination and may therefore prevent spermatozoa from reaching the site of fertilisation (*Fischer & Beier* 1986, *LeBlanc et al.* 1988, *Squires et al.* 1989). Oviductal secretions, motility and the effect on gamete interaction during foal heat remains still an unknown area which needs further investigation.

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Sammanfattning

Frekvensen funna embryon och deras kvalitet från ston inseminerade i den första post-partum brunsten.

Dräktighetsprocenten är lägre hos ston inseminerade i den första post-partum brunsten. Embryourspolning användes för att utreda om defekter i embryon var en av orsakerna till lägre dräktighetsprocent. Embryon från 23 post-partum och 14 icke-lakterande kontrolllston urspolades 7 dagar efter en uppskattad ovulation. Frekvensen funna embryon var 11/23 (48%) och 10/14 (71%) hos post-partum respektive kontrollston. Skillnaden var inte statistiskt signifikant (p = 0,16). Embryona färgades med fluorescein diacetat för att bedömma deras vitalitet, och med Hoechst 33342 för att räkna antalet cellkärnor. Embryona i de båda grupperna uppvisade samma vitalitet och deras cellantal var inte signifikant olika. Storleken hos 7,3 till 7,6 dagar (± 0,25 dagar) gamla embryon var mindre i post-partum gruppen än i kontroll gruppen. Orsaken till att fölbrunst embryona var mindre, kan vara en 2,4 timmars skillnad i tidsintervall från ovulationen till embryourspolningen. Frekvensen funna embryon motsvarade tidigare rapporterad dräktighetsprocenten i den första post-partum brunsten. Därför är en tidig embryodöd i livmodern inte den mest troliga orsaken till den lägre dräktighetsprocenten hos ston inseminerade i den första post-partum brunsten. Spermatransporten och äggledarens kondition under den första post-partum brunsten kräver mera forskning i framtiden.

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