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Embryo recovery following artificial insemination of mares enrolled in a small AI breeding program

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Abstract

In Portugal, there is much interest in the so-called assisted reproductive technologies as applied to horse breeding, namely at the semen cryopreservation and Artificial Insemination levels. The interest for embryo transfer in mares has also emerged despite several problems associated with this technique, such as the resistance of most breed registration authorities in accepting progeny begot by this method. The intention of this study was to evaluate the accuracy of embryo recovery techniques at a small facility and to consider the feasibility of its implementation for embryo transfer. Seven mares were alternatively inseminated in 13 successive oestrous cycles with fresh semen

from 3 stallions of varying fertility. Ovulation was left to occur spontaneously, and embryo recovery was carried out by non-surgical uterine flushing using both a closed flushing system (RS1) and an open flushing system (RS2). A total of 13 embryo collection attempts were performed on 7 possible positive pregnancy diagnoses with a rate success of 57.14% (4/7). Our preliminary results showed that the open flushing recovery system gives the best results and the high success recovery rate suggests that the technique of embryo transfer can be fully implemented even in facilities with limited resources.

Keywords: equine, embryo recovery, artificial insemination

1. Introduction

In several mammalian species embryo recovery, embryo cryopreservation and embryo transfer are routinely used in animal husbandry. In contrast in the equine these techniques are not common clinical procedures, due to the rejection of embryo transfer as a reproductive technique by the majority of horse registries. This situation has been changed and numerous studies have emerged in this field in order to obtain foals from older and problematic mares, or to increment production from genetically superior mares. Embryo recovery

as also been performed in some studies to evaluate semen quality, both in fresh and frozen-thawed samples [1].

Factors that affect the success of embryo collection include the number of ovulations, the age of the donor mare (as well as its reproductive history), the quality of stallion semen used, and, finally, embryo size and stage of embryo development at the time of recovery [2].

Embryo recovery rates vary greatly between 6 and 9 days after ovulation: Squires [1] reported a collection rate of 62% on day-6 embryos, a value slightly lower than the recovery rates obtained at days -7, -8 and -9 embryos (76%, 74% and 81%, respectively). For commercial purposes the optimal time for embryo collection is day 7 or 8 after detection of ovulation, but it results in embryos with large and variable diameters [3] It has been advanced that flushing on day 6 brings in a significant drop in embryo recovery rate because, in some mares, the embryo has not yet reached the uterus and/or that embryonic development rates differ between individual embryos [4, 5].

The procedures for embryo recovery routinely imply a daily ultrasound examination of the mare's reproductive status and the "timing" of ovulation using Human Chorionic Gonadotropin (hCG) when a growing follicle of ≥ 35 mm in diameter is observed [6]. This schedule requires an additional time commitment and personal availability that may not be feasible in all settings. Furthermore, recurring administration of hCG in mares was described as being responsible for antibody production [7]. Lastly, but not less important, most of the studies performed in this area are planned in a way that is not compatible with the reality of a Public Reproductive Centre. In fact, constraints in scheduling fresh semen inseminations are sometimes present. We don't know to what extent these issues could compromise the success of the embryo technology!

Considering the increment of equine-related activities in the past few years and the interest for equine embryo transfer that Portuguese breeders have demonstrated, it was our purpose to demonstrate that, in spite of the complexity of equine reproduction, an embryo recovery programme is viable and executively in our country.

2. Materials and methods

2.1. Stallions

Semen from three stallions (A-Lusitano, B- BWP and C- Sorraia, with histories of varied fertility with fresh semen) was used for inseminations with fresh spermatozoa during the breeding season (February to July—crescent day light period). The animals were housed at the Agricultural School, Polytechnic Institute of Coimbra, (Coimbra, Portugal). Semen was routinely collected using a phantom (Hannover model) and an artificial vagina (INRA model) and was analyzed and processed as described before. Parameters evaluated included sperm concentration mobility, morphology and vitality using the eosin-nigrosin stain [8, 9].

2.2. Mares management

The embryo donors used were 4 Lusitano (PSL) mares and 3 Cruzado Português (CP) ranging in age from 5 to 14 years old. The reproductive status of the mares was as follow: 2 barren mares with uterine problems (9 and 14 years old), 1 maiden mare (8 years old) and 4 wet mares (5, 6, 11 and 14 years old). All these females belong to the ESAC and they annually alternate between reproduction and training.

This group of mares was subject to the reproductive mare management implemented in the Animal Reproduction Laboratory, at ESAC. Briefly, this involves daily teasing to monitor reproductive behaviour. Three main

behaviour signs are associated with receptivity towards the stallion: 1) standing for mating, 2) tail raising and 3) winking of the vulva lips. Negative (-) and positive (+) values were assigned to each of these categories, if they were absent or present in a given mare, respectively. Thus, combinations of these values were used as an intensity index for estrus behaviour. Gynaecological examination (vaginal redness and fluid, cervical tone and aperture, transrectal uterine and ovarian palpation) complemented with ultrasonography (FALCO - 6 MHz transducer) were performed to monitor ovarian follicular activity, uterine relaxation and endometrial oedema during the estrous cycle. When in heat, mares were examined each 48 hours to monitor follicular development, which allows optimal timing to inseminate with fresh semen. Apart from the palpable feature of follicular consistency, the time of an impending ovulation was estimated by shape and size of the follicles. We confirmed that mares used were all cycling normally using this routine. In these exams the follicular and uterus status were also evaluated to monitor any pathology such as the presence of cysts, endometritis or mucometra.

2.3. Artificial Insemination (AI)

Semen dilution was performed at a final concentration of 20×10^6 viable sperm cells/ml in a milk extender medium [half skim ultra high temperature (UHT) pasteurized, milk supplemented with Gentamicin (50 µg/ml) and Penicillin (50 UI/ml)] maintained at 35°C prior to semen collection. Standard doses (15 ml) of diluted semen were used in AI within the first 30 min following recovery.

When a dominant follicle exceeding 35 mm in diameter was observed, mares were inseminated every 48 h with 300×10^6 motile sperm until the mares refused the stallion (non-estrus behaviour). The time of ovulation was then estimated by the observance of the echogenicity of the corpus luteum at the site where the predominant follicle had been detected.

2.4. Embryo recovery

An examination (6 MHz transducer) of the uterus was performed 6 days after the mare refused the stallion (designated as Day 0). Although it is a challenge to correctly identify a developing embryo at this stage, any sign of a possible pregnancy was considered, and embryo collection was then performed by non-surgical, trans-cervical uterine lavage using a Saline Solution (0.9% NaCl). The flushing fluid (pre-warmed at 37°C) was infused into the uterus by gravity flow via a balloon-tipped embryo collection catheter (1400mm length, 60ml balloon, 8 mm inside diameter, sterile). Four litters of flushing medium were used and after the first flush the uterus was massaged via the rectum during subsequent flushes. Each litre of medium used to flush the uterus was analysed separately by passing it through a 0.75 μ m embryo grid filter cup (IMV- Technologies). The remaining fluid was then examined in search of the embryo.

Each embryo was analysed in terms of diameter, shape, size, colour and stage of development using a Nikon SMZ-10 Binocular Stereoscope at approximately 14x magnification.

2.5. Experimental design

Seven mares and a total of 13 estrous cycles were used: 3 for stallion A, 6 for stallion B and 4 for stallion C. No inducer of estrus was used, and mares were allowed to ovulate spontaneously.

A recovery systems composed by a Foley catheter coupled to a disposable Y-junction tubing with clamps was used to infuse the flushing medium into the uterus. For embryo recovery two techniques were applied: a closed flushing system (RS1) and an open flushing system (RS2). In the RS1 (n=6), the uterine fluid was drained out via Y-junction tubing and in the RS2 (n=7) it was allowed to flow back out through the Foley catheter.

2.6. Statistical analysis

The statistical analysis was performed using the SPSS version 14.0 software (SPSS Inc., Chicago, IL, USA).

Fertility rates were calculated according to the rules of the French National Studies, as previously describes [8].

3. Results

3.1. Clinical Semen parameters and Fertility results

All semen samples were obtained during the breeding season (February to July) and the ejaculates used to inseminate the mares as described before [8]. Stallions A (PSL) and B (BWP) presented good fertility results at the end of the breeding season (87.82% and 88.72%, respectively). Stallion C, a Sorraia horse, presented bad semen characteristics (Table 1) and high levels of inbreeding (50% in the previous breeding season and 28.57% in the breeding season related to this study).

Table 1. Clinical semen parameters^a in tree stallions of varying fertility and reproductive parameters obtained with the 7 donor's mares

	Stallion (Breed)		
	A (PSL)	B (BWP)	C (Sorraia)
Sperm Concentration ($\times 10^6$)	179.57 ± 67.74 (28)	131.92 ± 39.79 (26)	246.97 ± 92.29 (39)
Sperm Vitality ^b (%)	39.29 ± 16.84 (24)	58.98 ± 22.71 (24)	37.83 ± 15.48 (39)
Sperm Motility ^c (% progressive)	39.29 ± 16.84 (24)	37.31 ± 12.10 (26)	19.67 ± 7.15 (39)
Abnormal Morphological Sperm ^d (%)	48.38 ± 16.63 (30)	28.85 ± 9.24 (26)	53.50 ± 10.15 (36)
Semen Volume (gel-free) (ml)	66.81 ± 41.72 (26)	35.38 ± 16.57 (21)	19.38 ± 9.18 (31)
pH	7.38 ± 0.27 (31)	7.41 ± 0.74 (26)	7.53 ± 0.28 (39)
Fertility (%)	66.7	83.3	28.57
Total heat cycles	3	6	4
Nº AI/ cycles	2.33 ± 0.58	1.50 ± 0.55	2.00 ± 0.00
FC (%)	66.7	83.3	0.0
Heat cycles/pregnancy	1.5	1.2	0

Values in parentheses indicate number of ejaculates observed for each stallion.

^a Values are means $\pm$ S.D. of measurements.

^b Values are mean ($\pm$ S.D.) percentages of the viable (not eosin stained) cells. For each ejaculate, counts were performed on 200 cells.

^c Progressive motility observed after collection.

^d Values are mean ($\pm$ S.D.) percentages of the cells that had abnormal morphology. For each ejaculate, counts were performed on 200 cells.

FC: fertility per heat cycle

3.2. Embryo recovery

Embryo recovery took place at day 6 (n=7), 7 (n=2), day 8 (n=2), day 9 (n=1) and day 10 (n=1) after the day that the mare refused the stallion (Day 0) which corresponded to 6 and 7, 9, 10, 11 and 12 days after last AI, respectively (Table 2).

Table 2. Embryo recovery attempts

Mares/ Breed	Age	Gestations	Reproductive State	Uterine Cysts	Estrous Cycle	Stallion	Embryo Recovery				
							Embryo Recovery System	Days after Day 0	Days after CL detection	Days after last AI	Pregnant Diagnosis/ Embryo Recovery
1 PSL	14	7	Barren	Yes	1 ^o	A	RS1	6	7	9	-/-
					2 ^o	C	RS2	6	6	8	-/-
					3 ^o	B	RS2	8	9	10	+/+
2 PSL	9	1	Barren	No	1 ^o	A	RS1	7	8	9	+/-
					2 ^o	B	RS2	10	10	12	+/+
					3 ^o	C	RS2	7	7	9	-/-
3 CP	8	0	Maiden	No	1 ^o	C	RS1	6	7	9	-/-
					2 ^o	B	RS2	8	9	10	-/-
4 PSL	5	1	Wet	No	1 ^o	B	RS1	6	7	7	+/-
5 PSL	6	1	Wet	No	1 ^o	B	RS1	6	7	7	+/+
					2 ^o	C	RS2	6	7	7	-/-
6 CP	11	4	Wet	No	1 ^o	B	RS1	9	10	11	+/-
7 CP	14	9	Wet	Yes	1 ^o	A	RS2	6	7	8	+/+

In general, embryo recovery was performed at 7 ± 1.35 days after the mare refused the stallion, 7.77 ± 1.30 days after corpus luteum detection and 8.92 ± 1.55 days after the last AI. A mean of 1.85 ± 0.56 AIs were performed

per cycle. For RS1, embryo recovery was performed at 6.67 ± 1.67 days after the mare refused the stallion, 7.67 ± 1.51 days after corpus luteum detection and 8.67 ± 1.97 days after the last AI. For RS2, embryo recovery was performed at 7.29 ± 1.50 days after the mare refused the stallion, 7.86 ± 1.46 days after corpus luteum detection and 9.14 ± 1.68 days after the last AI.

A total of 13 embryo collections attempts were performed. In 7 cases the collections were performed on presumed positive pregnancies (i.e. situations in which an embryo was thought to have been visualized). In these cases, the success rate for embryo recovery was 57.14% (4/7). In each case we used four litters of flushing medium in each recovery attempt but the four embryos recovered were obtained in the first flush. In contrast, the remaining 6 embryo collections were done on presumed negative pregnancies (i.e. situations in which no sign of embryo presence was detected). In these attempts no embryos were recovered, suggesting that our detection methods for the presence of an early equine embryo in uterus were accurate.

Each embryo collected was measured and graded, and its developmental stage determined according to McKinnon and Squires [1] – Table 3 and Figures 1-3.

Table 3 – Classification of the equine embryos recovered

Mares	Days after Day 0	Embryo diameter (mm)	Capsule	Developmental stage	Visible (naked eye)	Grade quality^a
1	8	4.5	Yes	ExpB	Yes	1
2	10	8.6	Yes	ExpB	Yes	1
5	6	1.8	No	LM	No	1
7	6	4.3	Yes	EB	Yes	1

a – according with McKinnon and Squires [1]

Figure 1 - After entering the uterus, some blastocysts were free within a zona pellucida (A). In others, still less than 200 mm in overall diameter, the trophoblastic cells had become closely apposed to the inside of the zona pellucida (B).

Figure 2 – The embryo capsule was not quite discernible on the freshly collected spherical embryos greater than 400 mm in diameter (A) but became very obvious when the embryo shrank during examination (B). 7177

Figure 3 – An 8 day embryo where the zona was shed. The capsule is clearly visible where it is separated from the trophoblast by a fluid-filled space

When considering the many variables in this study no statistically significant differences were found between mare, stallion, number of estrous cycles, number of AIs or time at embryo collection (Day 0, Day after CL detection and Day after last AI). However individual stallions significantly ($P < 0.05$) determined the pregnancy rates, confirming varying fertility among stallions. Indeed, the worse semen quality of the male horse C was confirmed by embryo recovery results as this stallion presented the worst results, while the best per Cycle Fertility was observed for stallion B (Table 1).

The only factor that seemed to affect embryo collection success was the recovery system ($P < 0.1$); Using the RS1 system, 66.7% of the cycles explored ($n=6$) resulted in a presumed positive pregnancy but just 25.0% of the embryos were successfully collected ($n=1$, 6 days old). These results contrast with those obtained using the RS2 system, where 100% of the cycles with a presumed positive pregnancy ($n=3$) resulted in the recovery of a viable embryo. One embryo had 6 days by Day 0, one 8 days and the other 10 days.

4. Discussion

In most studies the day of ovulation is determined by daily or twice daily scanning of the donor's ovaries, and embryo age is determined within 24h or 12h from ovulation. That is, if embryo collection is attempted 7 days after the detection of ovulation, the embryo could be 7 days $\pm$ 24h or 7 days $\pm$ 12h of age. These schedules require a time commitment that is not feasible in most clinical settings, and also imply the use of compounds to induce ovulation. In this study, ovulation was allowed to occur spontaneously.

Ovulation in mares is considered to occur 48-24h before the end of estrus. Accordingly, in this study its occurrence was determined by virtue of: 1) the

observance of the anestrus behaviour in the mare; 2) analyzing the day at which a preovulatory follicle was observed and 3) by observing an intense ultrasonic echogenicity at the site where the preovulatory follicle had been detected. McKinnon and al. [10] consider that a bright echogenic border and an irregular shape are predictive of imminent ovulation. In our observations all preovulatory follicles presented a hyperechogenic border and a pear form, with a long axis of ≥ 4.5 cm.

At the time of insemination the appearance and fluidity of vaginal secretions were observed and we noticed that when ovulation occurred the vaginal secretions were very fluid and more translucent than before. These characteristic, associated with those stated before, seem to be good indicators for the time of ovulation.

By cross analyzing the data we conclude that the RS2 system is better to collect embryos, and that between 5.79 and 8.78 days after the mare refused the stallion is the appropriate timeframe to successfully retrieve a viable embryo.

Mares 1 and 7 presented uterine cysts that were all mapped before the beginning of this study, and it was possible to collect an embryo from each one of these mares. Thus, the presence of uterine cysts seems not to be a problem for fertilization, although it could constitute an obstacle towards the normal establishment of pregnancies. The use of ovulation inducers seems not be needed for the success of an embryo recovery routine.

In conclusion, these results show that, with the breeding management routinely used at the ESAC Equine Reproduction Laboratory, it is possible to implement an embryo recovery practice, with a good recovery rate, that could be used in an embryo transfer programme, even one with limited resources in terms of staff or animals.

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