Evaluation of embryo collection and transfer days on pregnancy rate of Mangalarga Marchador mares during the breeding season

Evaluación de los días de recolección y transferencia de embriones sobre las tasas de preñez en yeguas Mangalarga Marchador durante la estación de monta

Avaliação do dia da colheita e da transferência de embriões equino da raça Mangalarga Marchador durante a estação de monta

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Abstract

Background: Proper timing for embryo collection and transfer in horses -which is critical for the success of this biotechnology- is still debated. Additionally, there is little information on this technology under tropical conditions. **Objective:** To determine the best day for collection and transfer of embryos in Mangalarga Marchador mares under Brazilian northeast's conditions. **Methods:** Donors (n= 30) and recipients (n= 76) in diestrus phase were selected based on both clinical and gynecology examinations. Estrus was induced on both donor and recipient mares by intramuscular injection of 5 mg Dinoprost, aiming to obtain an ovulation interval of -1 to +3 between recipient and donor. Ovulation was induced with buserelin acetate when the largest follicle reached at least 35 mm in diameter. At this time, mares were subjected to artificial insemination at 48-hour intervals until ovulation. The embryos were collected on days 7, 8, and 9 after ovulation. **Results:** The embryo collection on day 8 was more efficient ($p<0.05$) than on day 7, but it was not more effective ($p>0.05$) than day 9, which presented the same efficiency ($p>0.05$) as day 7. From a total of 76 embryos transferred to the recipients, that were between days 4 and 9 after ovulation, there was no influence ($p>0.05$) of the day of transfer on pregnancy rate. **Conclusions:** The embryo collection must be performed on day 8 after ovulation, and transfer can be performed on any day of that interval (4-9) without affecting the pregnancy rate.

Keywords: *assisted reproduction; embryo; embryo collection; embryo recipients; embryo transfer; Equus caballus; estrous synchronization; Mangalarga Marchador; mare.*

Resumen

Antecedentes: El momento mas apropiado para la recolección y transferencia de embriones en equinos -que es fundamental para el éxito de ésta biotecnología- continua siendo sujeto de estudio . Además, es escasa la información sobre ésta tecnología en condiciones tropicales. **Objetivo:** Determinar el momento mas adecuado para la recolecta y transferencia de embriones en yeguas Mangalarga Marchador, en las condiciones del nordeste Brasileño. **Métodos:** Donadoras (n= 30) y receptoras (n= 76) en la fase de diestro se seleccionaron con base en los exámenes clínicos y ginecológicos. El estro de las yeguas donadoras y receptoras fue inducido con 5 mg de Dinoprost, vía intramuscular, intentando obtener un intervalo de ovulación de -1 a +3 entre la receptora y la donadora. La ovulación fue inducida con acetato de buserelina cuando el folículo mayor alcanzó 35 mm de diámetro. En ese momento, las yeguas fueron sometidas a inseminación artificial en intervalos de 48 horas hasta la ovulación. Los embriones fueron recolectados en los días 7, 8 y 9 después de la ovulación. **Resultados:** La recolecta de embriones en el día 8 fue más eficiente ($p<0,05$) que en el día 7, pero no fue más efectivo ($p>0,05$) que en el día 9, el cuál presentó la misma eficiencia ($p>0,05$) que en el día 7. De un total de 76 embriones transferidos a las receptoras, que se encontraban entre el día 4 y 9 después de la ovulación, no se registró influencia ($p>0,05$) del día de la transferencia en la tasa de preñez. **Conclusiones:** La recolecta embrionaria debe ser realizada el día 8 después de la ovulación, y la transferencia puede ser realizada en cualquier día de este intervalo (4 a 9) sin que se afecte la tasa de preñez.

Palabras clave: *colección de embriones; embrión; Equus caballus; Mangalarga Marchador; receptora de embriones; reproducción asistida; sincronización del estro; transferencia de embriones; yegua.*

Resumo

Antecedentes: A importância do momentoda colheita e da transferência do embrião equino para o sucesso dessa biotécnica em equino continua sem ser completamente entendida. Adicionalmente, existe pouca informação sobre essa tecnologia em condições tropicais. **Objetivo**: Determinar o melhor dia para colheita e para transferência de embriões em eguas manga larga marchador nas condições do nordeste brasileiro. **Métodos**: Doadoras (n = 30) e receptoras (n = 76) na fase de diestro foram selecionadas com base nos exames clínico e ginecológicos. O estro das éguas doadoras e receptoras foi induzido com 5 mg de Dinoprost administrado por via intramuscular, buscando obter um intervalo de ovulação de -1 a +3 entre a receptora e a doadora. A ovulação foi induzida com acetato de buserelina quando o foliculo maior alcançou o tamanho de 35 mm de diâmetro. Nesse momento, as éguas foram submetidas a inseminação artificial em intervalos de 48 horas até a ovulação. Os embriões foram colhidos nos dias 7, 8 e 9 depois da ovulação. **Resultados**: A colheita de embriões no dia 8 foi mais eficiente ($p<0,05$) do que no dia 7, porem não foi mais efetivo ($p>0,05$) do que o dia 9, o qual apresentou a mesma eficiência ($p>0,05$) que o dia 7. De um total de 76 embriões transferidos para as receptoras que se encontravam entre os dias 4 e 9 depois da ovulação, não se registrou influência ($p>0,05$) do dia da transferência sobre a taxa de prenhez. **Conclusões**: A colheita embrionária deve ser realizada no

dia 8 depois da ovulação, e a transferência pode ser realizada em qualquer dia desse intervalo (4-9) sem que a taxa de prenhez seja afetada.

Palavras-chave: *colheita de embriões; egua; embriões; Equus caballus; Mangalarga Marchador; receptoras de embriões; reprodução assistida; sincronização do estro; transferência de embriões.*

Introduction

Embryo transfer (ET) biotechnology in horses is a useful tool for allowing rapid genetic dissemination of valuable animals (Arruda *et al*., 2001; Azevedo *et al*., 2015). The ET makes it possible to obtain foals from aged mares, mares with problems to maintain gestation, and mares under training or competition. This biotechnology also allows obtaining foals of young mares (1 to 2 years) without impairing its development and shortens the generation interval of mares with a promising pedigree (Sieme *et al*., 2018).

The ET technology is less complex than other reproductive biotechnologies, such as *in vitro* embryo production (Lira *et al*., 2009; Claes *et al*., 2016; Sanchez *et al*., 2016; Rua *et al*., 2016). Its efficiency depends on proper selection and management of mares (Squires *et al*., 1999; Vanderwall, 2008), and estrus and ovulation synchrony (Iuliano *et al*., 1985; Taveiros *et al*., 2003/2008; Rabelo *et al*., 2009; Hinrichs, 2012; Azevedo *et al*., 2014).

Equine embryos are transported from the oviduct to the uterus from the compact morulae to the blastocyst stages, between the 5th and 6th days after ovulation (Oguri and Tsutsumi, 1972). Embryos obtained by post-ovulation artificial insemination (AI) reach the uterus later compared to their counterparts obtained by pre-ovulation AI, possibly due to delayed fertilization (Bertani *et al*., 1997). Therefore, embryo collections should be performed around 12 hours after day 7 (Day 7.5) and should not exceed day 8 (Cuervo-Arango *et al*., 2009).

The most appropriate day for embryo collection remains controversial. On one hand, for collections between days 6 and 9 after fertilization, collections made between days 7 and 8 retrieved greater embryo numbers (Cuervo-Arango *et al*., 2009; Sieme *et al*., 2018). On the other hand, similar embryo recovery rates could be achieved from days 5 to 8 post-ovulation (Vogelsang *et al.,* 1985). Also, according to Squires and Seidel (1995), day 6 is the most advantageous for embryo cryopreservation, and day 9 -although less used for ET- shows lower results in comparison to days 7 and 8.

The ET into previously synchronized mares can be performed by either surgical or transcervical methods (Fleury *et al.,* 2001). Despite ongoing controversy, the most frequently used period for ET has been from days 5 to 9 after donor ovulation (Iuliano *et al.,* 1985; Vogelsang *et al.,* 1985; Fleury *et al.,* 2001; Hajčić *et al.,* 2008; Sieme *et al*., 2018).

Given that day for collection and ET in horses plays a crucial role in its overall success, a limited number of reports on the subject under the Brazilian northeast condition are available. The northeast of Brazil is a semi-arid region that in spite of stable luminosity throughout the year, faces substantial variation in forage supply due to low and uneven distribution of rainfall. The breeding season spans from September to March, which is during the dry season. Therefore, the objective of this study was to define the most appropriate days for collection and transfer of embryos in Mangalarga Marchador mares under Brazilian northeast's conditions.

Material and Methods

Ethical considerations

The study was approved by the Ethics Committee for Animal Research at Universidade Federal Rural de Pernambuco (License: 011/2013).

Location and animals

The experiment was conducted in Limoeiro (Pernambuco state, Brazil). The farm is located at the following geographic coordinates, 7º 52' 29'' S latitude and 35º 27' 01'' W longitude. Mean annual temperature is 24.6 ºC and mean total rainfall is 1,007 mm^3.

Mangalarga Machador donor and recipient mares were pluriparous, non-lactating, aged between 5 and 15 years, and body weight ranged from 450 to 550 kg. All mares were raised under semi-extensive conditions, mostly grazing on cultivated pastures (*Digitaria decumbes*) with access to water and mineralized salt (Suprafós 73, Supranor®, Recife, PE, Brazil) *ad libitum*. Mares were also supplemented with hay (*Cynodon spp*), alfalfa (*Medicago sativa*), and 4 kg/day of a pelletized commercial horse ration containing 16% crude protein, 12.0% fiber, 3,080 kcal/kg fat, 1.3% calcium, 0.75% phosphorus, and 6.0% ether extract (Corcel Reprodução, Presence, São Lourenço da Mata, PE, Brazil).

The body condition score of mares was between 5 and 7, in a 1 to 9 scale, as described by Henneke *et al.* (1983). Other factors considered for selection were: reproductive performance, clinical condition, and gynecological examination by ultrasonography for identification of uterine fluid and endometrial alterations that could compromise fertility, as suggested by Taveiros *et al.* (2008). For this examination, an Aquila Pro, Esaote, equipped with a linear multi-frequency transducer (6 and 8 MHz) was used.

Estrus was induced in donors (n= 30) and recipients (n= 76) with IM injection of 5 mg Dinoprost (Lutalyse®, Pfizer, São Paulo, SP, Brazil), and a single estrous cycle was used per mare. This strategy was carried out to obtain an -1 to +3 ovulation synchronies between the recipient and donor ovulations, as proposed by Squires (1993). Ovulation was induced with buserelin acetate (Conceptal®, MSD Saúde Animal, São Paulo, SP, Brazil) after the largest follicle had reached at least 35 mm diameter. At this time, mares were inseminated with fresh semen from a single stallion of proven fertility, using 500 x 10^6 viable sperm concentration in 48-hour intervals until ovulation occurred, as recommended by Rabelo *et al.* (2009).

Embryo collections were performed on days 7 (D7), 8 (D8), and 9 (D9) post- ovulation (single embryo collection per mare). The procedure was carried out by the open method, which requires the introduction of a catheter (Bivona, St. Paul, Minnesota, USA) into the uterus body to fixate the balloon to the uterine walls. A total of 3 L of Ringer-Lactate solution was used, divided into three fractions (Fleury and Alvarenga, 1999). After the last uterine flushing, the balloon was emptied, and the catheter was removed. The filtered content was deposited in Petri dishes for embryo recovery using a stereomicroscope (80X), as suggested by Taveiros *et al.* (2008). Immediately after collection, donor mares received an additional Dinoprost injection to avoid an unwanted pregnancy.

After recovery, embryos were evaluated for the stage of development and graded based on their morphology, as described by McKinnon *et al.* (1993). Only grades I and II embryos (n= 76) were transferred immediately to recipients at various post-ovulation stages (D4, D5, D6, D7, D8, and D9). Pregnancy diagnosis was performed by ultrasonography on day 25 after ET. All ultrasonographic exams were carried out by the same technician.

Statistical analysis

The statistical analysis of binomial data was performed using a chi-square test with Yates correction (Preacher, 2001). The significance level of 5% was used.

Results

Table 1 shows results relative to embryo collection at different days post-ovulation. Day 8 was more efficient than day 7 for embryo recovery ($p < 0.05$), but it did not differ with day 9 ($p > 0.05$). Moreover, day 9 was not more efficient than day 7 ($p > 0.05$), although showing a trend towards such a difference ($p = 0.061$). Regarding transferable embryos, there was no difference among the days of collection ($p < 0.05$).

Table 1. Influence of the day of the collection on embryo recovery (Positive) and morphological evaluation (Transferable) in Manga Larga Marchador donor mares.

Collection (Day)	Uterine flushing		Transferable embryos n (%)
	Total (n)	Positive n (%)	
D7	21	9 (42.8)^{A}	6 (66.6)^{A}
D8	70	51 (72.8)^{B}	39 (76.4)^{A}
D9	59	39 (66.1)^{AB}	31 (79.4)^{A}
Total	**157**	**97 (61.7)**	76 (78.3)

Different superscript letters ($^{A, B}$) within the same column indicate significant difference ($p < 0.05$).

Table 2 contains the results of pregnancy and conceptus loss about the recipients' post-ovulatory stage (in days) at the time of ET. There was no effect of recipient's post-ovulation day at ET on pregnancy rates and conceptus loss ($p > 0.05$).

Table 2. Influence of the recipients' post-ovulatory stage (Day) on pregnancy rates in Manga Larga Marchador mares submitted to embryo transfer.

Embryo Transfer			
Day (post-ovulation)	**Embryos (n)**	**Pregnancy n (%)**	**Conceptus loss n (%)**
D4	6	2 (33.3)	4 (66.7)
D5	20	18 (90.0)	2 (10.0)
D6	17	13 (76.4)	4 (23.6)
D7	15	9 (60.0)	6 (40.0)
D8	11	7 (63.6)	4 (36.4)
D9	7	3 (42.8)	4 (57.2)
Total	**76**	**52 (68.4)**	**24 (31.6)**

Discussion

The equine embryo reaches the uterus between the fifth and sixth day after ovulation (Oguri and Tsutsumi, 1972; Webber *et al.,* 1991). That is why the day of collection is described as an essential factor on quantity (Montechiesi, 2015) and quality of retrieved embryos (Betteridge *et al.,* 2000; Allen, 2001). The studies mentioned above tested different days for embryo collection. While day 7 was the least preferable, these reports are in contrast to Vogelsang *et al.* (1985), Iuliano *et al.* (1985) and Fleury *et al*. (2001), who suggested day 7 as the most promising day for embryo collection. The most plausible explanation for this discrepancy is that embryos from Mangalarga Marchador mares at day 7 post-ovulation are smaller in size than other breeds, thus making their recovery more challenging during uterine flushing. Despite the embryo potential for adapting to unsynchronized uterine environments, it is probable that under such conditions, it may have initially faced slower development at gene, cellular, and tissue levels (Gibson *et al.,* 2017).

The results described herein may further support that collection can be preferentially performed at day 8, but can also be carried out at day 9 without diminishing embryo recovery. In a previous report, Iuliano *et al.* (1985) assumed that collections could be performed at day 8, while Fleury *et al.* (2001) supported that day 8 is the most promising day for embryo collection. Albeit the possibility that embryos at day 7 post-ovulation can be smaller than their day 9 counterparts, these later embryos are free from the zona pellucida and are more likely to succumb to mechanical damage during uterine flushing (Stout *et al.,* 2005). Nevertheless, their morphological quality was similar, since the percentage of transferable embryos did not differ.

The ET in horses can be performed by surgical or transcervical methods into previously synchronized recipients (Fleury *et al.,* 2001). Despite the controversy among reports on the best day for ET, it has been mostly described during days 5 to 9 after donor ovulation (Iuliano *et al.,* 1985; Vogelsang *et al.,* 1985; Fleury *et al.,* 2001; Stout, 2006; Hajčić *et al.,* 2008; Azevedo *et al.,* 2015).

As described above, donor and recipient mare management, selection, ovulation synchrony, and -particularly- embryo quality for ET could have positively affected the results. This reasoning is based on the fact that pregnancy rates from day four to nine could be similar is ruled out. Thus, the remaining data would be in agreement with those published by several researchers (Wilson *et al.,* 1987; McKinnon and Squires, 1988; Fleury *et al.,* 2001; Taveiros *et al.,* 2008).

Although the absence of statistical difference among pregnancy results, those obtained on days five and six can be outlined from the remaining. In spite of lack of scientific support for these days as the best time-points for ET, it is possible to account for this pregnancy data to the more extended period that embryos have to develop for maternal to fetal crosstalk in the uterus. This crosstalk, among other possibilities, can be in consequence of conceptus migration within uterine horns, thus inhibiting PGF2α production and release (Conley, 2016).

Therefore, embryos transferred more precociously may not have acquired the necessary competency to promote the signaling above, since it is the exact

moment of embryonic activation (Stout, 2006). It is possible that this problem overlaps with fluctuating P4 concentration during this time, and P4 available may not be sufficient for establishing uterine conditions to support embryonic development (Irvine *et al.*, 2000).

In conclusion, equine embryos can be collected eight days post-ovulation, and their transfer can be performed in a relatively broad period without any detrimental effect on pregnancy rates.

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Conflict of Interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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