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VETERINARY MEDICINE MASTER´S DEGREE

Equine embryo transfer: the effect of semen processing and donor mare management on recovery rates

Sónia Correia

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"Da vida não quero muito… Quero apenas saber que tentei tudo o que quis, tive tudo o que pude, amei tudo o que valia e perdi apenas o que no fundo, nunca foi meu…"

(Anonimous)

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AI - Artificial Insemination

CER - Equine Reproduction Centre (from Portuguese *Central Equina de Reprodução*)

ERR - Embryo Recovery Rate

ET - Embryo Transfer

PGF 2α – Prostaglandin F2α

P4 - Progesterone

Original Research

Equine embryo transfer: the effect of semen processing and donor mare management on recovery rates ★

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Abstract

Embryo transfer (ET) is a biotechnology that allows to get more than one foal from a single mare during a breading season and also to prevent the removal of mares from their competition careers. Nevertheless, to achieve a successful outcome, the association of many factors must be considered such as the management of donor and recipient mares, the stallions and the veterinarian´s experience.

The aims of this study were to evaluate (1) if some of characteristics related to the donor mares such as the breed and the reproductive status, may influence the embryo recovery; (2) if climate and different donor mares location may have impact in breeding programs; (3) the effect of some reproductive techniques and embryo transfer methodologies such as the day of uterine flushing, the type of semen, the stallion and the moment of inseminations and transfer as potential effectors of success.

To achieve the objectives pointed out, one hundred and fifty six uterine flushes and eighty eight embryo transfers were performed. Obtained results showed higher embryo recovery rates at day 8 (61.1%) and an overall pregnancy rate of 62.2%. An influence of the month of the year (P=0.04) and of the type of semen (fresh or frozen; P=0.03) on the number of positive embryo collections were identified. No other significant effects upon the remaining determinants under testing were observed, although stallions were responsible for inducing 11% of the variability within embryo recovery rates.

In conclusion, the main factors that had a great influence in this embryo transfer program were the type of semen and the day and month of the uterine flushing procedure. The use of fresh semen for donor mares insemination and recovery at day 8 increased the number of embryos that were collected during this period, although lower results were obtained in July-August. These procedures should be consider and implement in mare embryo transfer centres.

Keywords: Artificial insemination; Embryo transfer; Equine; Month; Pregnancy; Semen

1. Introduction

In breeding programs, assisted reproductive biotechnologies such as artificial insemination (AI) and embryo transfer (ET) have become more efficient in the last decades [1,2]. In the horse competition industry, these biotechnologies support owners and mares, to prevent the interruption of their training plans [3] allowing to obtain genetically improved animals. Brazil is one of the leading countries in equine embryo collection and transfer, presenting improved results year after year [4]. Nevertheless there are still a few difficulties in equine reproduction such as the response to hormonal treatments in particular to the superovulation protocols. In fact, these protocols are not routinely used due to the much lower ovarian response of mares compared to other species (*e.g*. bovine), but also because a reliable hormonal treatment (i.e. products, dosage regimen among others) is not commercially available. Moreover, the cryosurvival of horse embryos is lower when compared to other species and *in vitro* embryo production is quite difficult. The availability of multiple embryos from each donor mare allied to a successful cryopreservation could further expand this technology in a near future [4,5].

To improve the pregnancy rates during the breeding season, three important aspects must be considered: management and status of donor and recipient mares, semen quality of the stallions [6] and the experience of the veterinarian [2]. In fact and according to Jacob *et al*. [7] the time of the uterine flushing in relation to the day of ovulation is one of the most important factors in determining the success of obtaining an embryo from a donor mare. Nonetheless other factors such as the mare´s age, breed, reproductive category, number of ovulations as well as the location of semen deposition during AI are also essential [8]. Therefore is not surprising that the accomplishment of embryo recovery and transfer in the mare is reported as varying significantly [7,9].

The objectives of this study were to determine (1) if some of characteristics related to the donor mares such as the breed and the reproductive status, may influence the embryo recovery; (2) if climate and different donor mares location may have impact in breeding programs; (3) the influence of some reproductive techniques and embryo transfer methodologies such as the day of uterine flushing, the type of semen, the stallion and the moment of insemination and transfer as potential effectors of success.

2. Material and Methods

2.1 Embryo donor and recipient mares

This study was conducted in the Equine Reproduction Centre (CER, from Portuguese *Central Equina de Reprodução*), a commercial equine reproduction centre, accredited by the European Union located in Boituva, São Paulo, Brazil.

From July to December of 2015, eighty three mares of different breeds (Thoroughbred Arabian, Thoroughbred Lusitano and Quarter Horse) were included as donors in the embryo transfer program. Their age ranged from four to twenty years old. Most of them were housed in the CER (n=66) along

with the recipient mares (n=170). The remaining seventeen donor mares were kept in a subsidiary centre (Raphaela Haras) located in Porto Feliz, São Paulo, Brazil. Regardless of the donor mare location, in case of positive embryo recover, recipient mares of CER were used.

Donor mares were kept in stalls with water *ad libitum* and fed with hay and concentrated dry feed. Recipient mares were provided with water *ad libitum* but were fed with forage grass. The later ones received concentrated dry feed only when taken to stocks for reproductive status evaluation.

2.2 Monitoring ovarian activity

Ovarian activity of the mares was monitored using transrectal palpation and ultrasonography (6.5 MHz rectal transducer, C40 vet™, Landwind Medical, China). The donor mares required a daily detailed evaluation, while recipient mares were submitted to the same control but only three days a week (Monday, Wednesday and Friday). When new mares with no reproductive history arrived at CER, they were examined by rectal palpation and ultrasound to check for their cycle stage. During monitoring the follicular size and firmness, uterus (edema and tone), cervical tone and eventually the possible presence of reproductive pathologies were assessed. This monitoring was important to estimate the moment of ovulation and breeding [2].

In each mare, to carry out the insemination, either with fresh or frozen semen, compliance with all the following criteria was required: follicular growth ≥ 35 mm; level 3 edema (i.e. heat edema); open and relaxed cervix; and soft uterine tone. Uterine edema was graded on a scale of 0 (no edema) to 4 (maximal) as described by William [10].

The ovulation was induced with human chorionic gonadotropin (*Vetecor*™, Hertape Calier; Brazil; 0.5 mL; IV) and deslorelin (1 mL; IM). These inducing agents helped to reduce the uterine edema with the approach of the ovulation time and to minimize the number of inseminations per cycle [6].

2.3 Stallions, semen processing and artificial insemination

Artificial inseminations were performed with fresh or frozen semen of forty eight stallions always from the same breed of the donor mare. Semen from all stallions was evaluated at the beginning of each breeding season for progressive motility, vigor, concentration and anomalies. The collection of semen from stallions that were resident at CER or at near locations was done by using an artificial vagina. During collection, the ejaculate was divided into the sperm-rich and the gel fractions, before evaluation of the color, volume, concentration and progressive motility. Semen extender (BotuSemen™; Botupharma, Brazil) was prepared before the semen collection and kept at 37ºC until dilution to obtain a final concentration of at least 500 x 10^6 viable spermatozoa but normally 800 x 10^6 to 1 x 10^9 total spermatozoa were used.

After the detection of ovulation, some mares (n=20) were inseminated with frozen/thawed semen in the uterotubal junction/uterine horn using a standard AI catheter up to six hours post-ovulation. This increased the number of spermatozoa in oviduct and thus the pregnancy rates [6]. Frozen semen straws (100 x 10^6 spermatozoa each), were thawed in water at 46ºC during twenty

seconds, dried and then spermatozoa quality was subjectively evaluated using a microscope. The number of straws per AI was dependent on the post-thaw progressive motility. On the morning after AI, if the uterus had over 15 mm of fluid it was flushed with sterile lactated Ringer´s solution (normally two liters) until the recovered flush was clear. Oxytocin (IV) twice a day during three days was also administered.

In case of fresh semen, the ovulation occurrence was confirmed by ultrasonography after the AI procedure. If the presence of uterine fluid was detected, the previously mentioned post-breeding therapies were also carried out.

2.4 Embryo recovery and transfer

Embryo recovery was performed between the 7 th and 10 th day post ovulation (day 0 = day of ovulation). In this period, donor mares were led to a stock, where the rectum was evacuated of feces, the tail was wrapped and held to one side, and the perineum was washed with povidone iodine, rinsed and accurately dried [8].

A total of 156 flushing procedures were made via a Bivona foley catheter inserted into the uterus through the cervix. The cuff was inflated with 60mL of air and a warmed sterile ringer lactate solution (3-6L per embryo recovery attempt) was infused. The fluid was recovered into a sterile filter cup and then transferred to a Petri dish [4,11]. Then the embryonic stage was identified under a stereo microscope being the majority of the recovered embryos at the blastocyst or expanded blastocyst stages. These embryos were washed 5 times with *BotuEmbryo*™ (Botupharma; Brazil) to remove cellular debris. All embryos were transferred non-surgically into synchronized recipient mares. As most of the embryos were of intermediate size, 0.5 mL straws were used to pack and transfer, along with a sterilized pipette (side opening) and cannula [12].

All embryos were transferred in fresh and placed in the tip of the uterine horns ipsi- or contra-lateral to the ovary with the corpus luteum of the recipient mare. The selection of the recipients was based on the number of the days after ovulation, uterine and cervical tone, and absence of liquid in the uterine horn [1]. Once the procedure for embryo transfer was completed, a dose of 6 mL (IM) of progesterone (P4) was administrated to each recipient mare.

Immediately after embryo recovery, 2 mL (IM) of prostaglandin (PGF2α) (Pfizer, USA) were administered to each donor mare. This administration of PGF2α could be delayed (*e.g.* to the end of the afternoon or to the next morning) if gastrointestinal disorders (*e.g.* colic) occurred to avoid the PGF2α side effects including transient decrease of body temperature and sweating. Less often increased respiratory and heart rates, ataxia, abdominal pain and lying down occurred [13].

2.5 Pregnancy diagnosis

After embryo transfer was performed, the recipient mare returned to the group of the remaining mares to minimize any situation of stress. Five days later, pregnancy was confirmed by detecting an embryonic vesicle using rectal palpation and ultrasonography. Cases of dubious diagnosis included

uterine tone examined by rectal palpation compatible with a positive pregnancy, with failure to identify embryonic vesicle through ultrasonography. In such cases the ultrasonography examination was repeated a couple of days later to confirm the pregnancy or possible occurrence of an early embryonic death.

Prior to pregnancy confirmation through a positive heart beat (fourteen days after), the mares stayed with the rest of the group and afterwards they were placed in the pregnant lot. Progesterone was administered weekly until accomplishment of one hundred and twenty days of gestation.

2.6 Statistical analysis

The breeding records of the mares were analyzed from July up to December of 2015. The available information was statistically analyzed with the objective of evaluating the effects of environmental conditions; the location and breed of donor mare's; stallion´s breed; type of semen (fresh vs. frozen); the month of embryo collection; interval between AI and ovulation; day of uterine flushing; day of embryo transfer; reproductive stage of recipient mare and day of pregnancy diagnosis. Initially the frequency of the studied factors was estimated by Microsoft Excel 2010 (Microsoft Office 2010™; Microsoft Corporation, USA) and the PROC FREQ of programme Statistical Analysis System Institute (SAS International™, Heidelberg, Germany). The results of the pregnancy diagnosis and uterine flushing were analyzed by logistic regression using PROC LOGISTIC with a model that included the effects that were mentioned before. A P-value ≤ 0.05 was considered significant.

3. Results

One hundred and fifty six uterine flushing procedures were performed from July to December of 2015 which enabled the recovery of 88 embryos (overall embryo recovery rate of 56.7%). No differences (P>0.05) were observed between the results of the headquarters (CER) and the subsidiary center (Haras Raphaela). The effect of the day in which the uterine flush was performed on embryo recovery rate (ERR) is depicted in table 1.

Table 1- Embryo recovery rates according to the day of uterine flushing (day 0 = day of ovulation).

Day of uterine flush	Positive embryo recoveries n (%)	**Total n (%)**
7	1 (100 %)	**1 (0.67 %)**
8	77 (61.1[a] %)	**126 (84.0 %)**
9	7 (31.8[b] %)	**22 (14.7 %)**
10	0 (0 %)	**1 (0.67 %)**
Total	**85 (56.7 %)**	**150 (100 %)**

The majority of the embryos (97.7%) was recovered at the day 8 or 9 post ovulation. Moreover, increased embryo recovery rates were obtained at day 8 (P=0.008). Significant differences between the months in which embryo recovery was carried out were identified (P=0.04, figure 1). During July-August period only 41.3% of uterine flushes were positive, which corresponded to the worst results in this experimental period. From September until December the total number of positive flush increased significantly, although showing similar ERR rates (September: 72.4%, October: 63.6% and November/December: 58.2%).

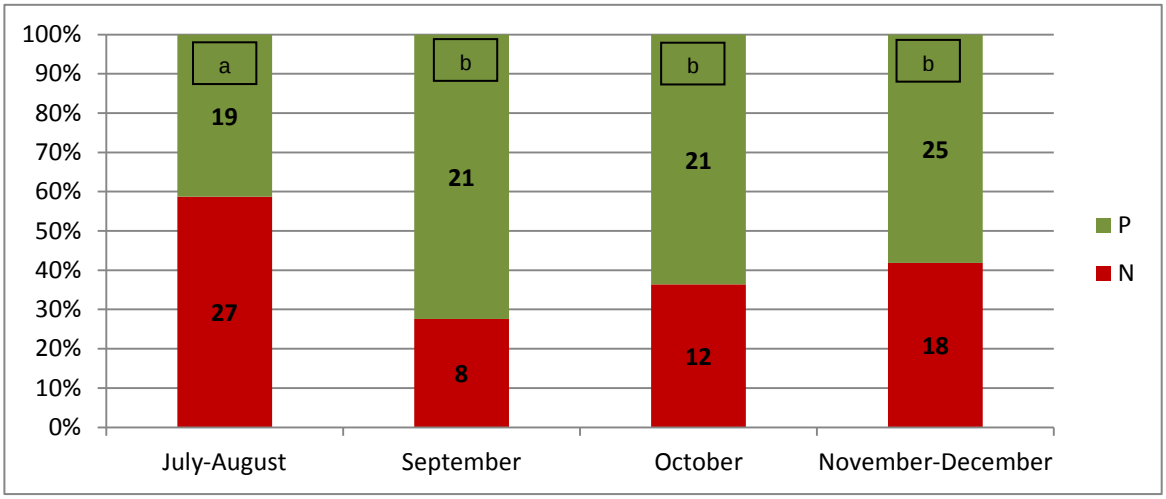

Fig. 1- Influence of the month of the year on the number of positive embryo collections with detection of an embryo (P, positive; N, negative, months with different letters are statistically different P<0.05).

No differences were identified for the effect of the interval between ovulation and AI on ERR (P>0.05) as the majority of the mares ovulated at the same day or within an interval of twenty-four hours.

In this study, donor mares were more often inseminated with fresh semen (87.2%; [136/156]) compared to frozen semen (12.8%; [20/156]). The type of semen clearly influenced the ERR (fresh: 61.6% vs frozen: 30.0%; P=0.03) as shown in figure 2.

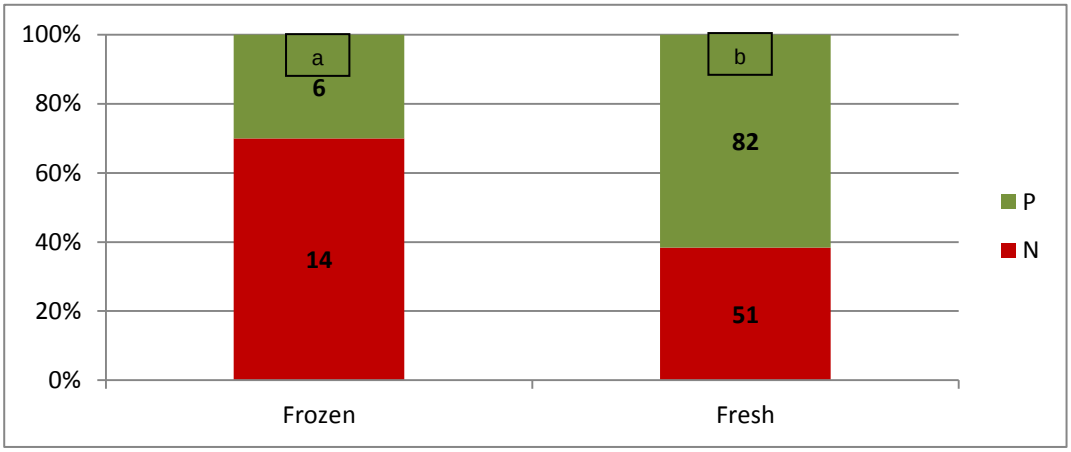

Fig. 2- Embryo recovery rates according to the type of semen used in artificial insemination. (P, positive; N, negative, columns with different letters are statistically different P<0.05).

The distribution of positive and negative embryo recoveries according to the breed of the donor mares is represented in figure 3. The Quarter horse, breed presented better results (ERR=70.7%, P=0.05) but also had the highest percentage of fresh semen AI (98.4%) while Thoroughbred Arabian and Lusitano presented only 46.7% and 52,6% positive recoveries, respectively. So although differences were identified in ERR among breeds, those differences could be related to the type of semen used.

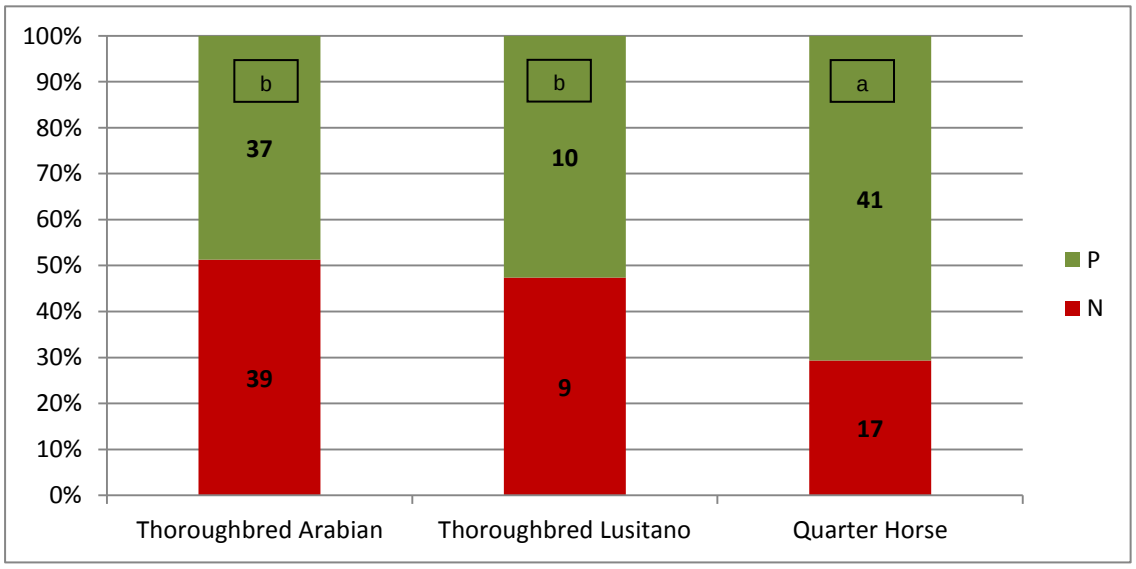

Fig. 3- Embryo recovery rate according to the donor mare breed. (P = positive; N = negative, columns with different letters are statistically different P<0.05)

In addition, the stallion had no significant effect on the EER despite inducing great variability (11% of the variability can be attributed to the stallion). Some of stallions were used more than once (Fig. 4).

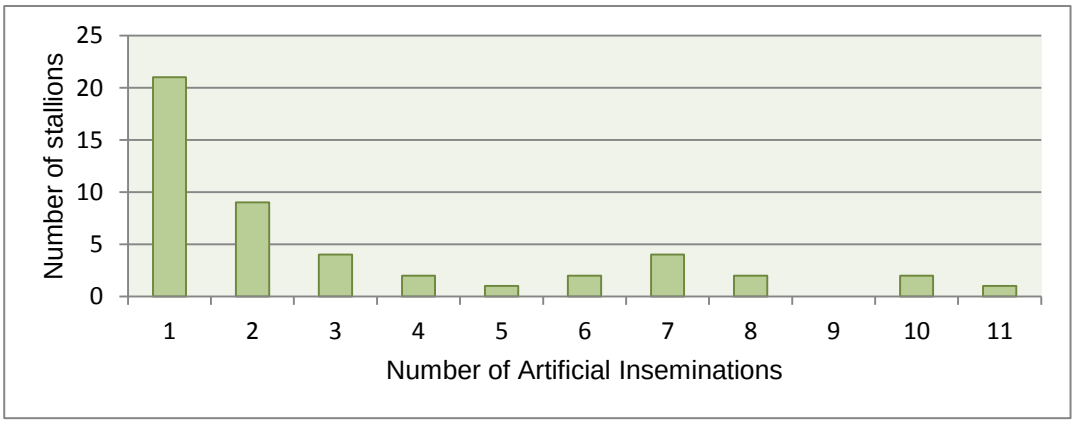

Fig. 4- Number of artificial insemination performed by stallion.

All the remaining studied factors (stallion breed, moment of AI, number of flushing/mare, oxytocin treatment) did not have a significant influence in the embryo recovery programme (P>0.05).

The majority of recipient mares was classified as “good” or “good plus” corresponding to 76.8% and 21.4%, respectively. No effect of the post ovulation day of the recipient mares transfer on the pregnancy rates was identified (P>0.05). The degree of synchrony of ovulation between the donor and recipient mares ranged from - 4 to 0 and did not influence the pregnancy rate (P>0.05).

An ultrasonography-confirmed positive pregnancy of 64.4% and 68.7% was obtained five or six days after ET respectively by the presence of an embryonic vesicle, that was confirmed by rectal palpation in 92% of the cases. Only four cases were considered doubtful. The overall pregnancy rate was 62.2% (both fresh and frozen semen). This value includes the total pregnancies that were confirmed by rectal palpation and at the same time by ultrasonography. The type of semen had no effect on the pregnancy rate. No differences were identified for donor mare location although CER had thirty one recipient mares pregnant as compared to fifteen of Haras Raphaela, thus displaying the higher number of embryos that were collected at CER (Fig. 5).

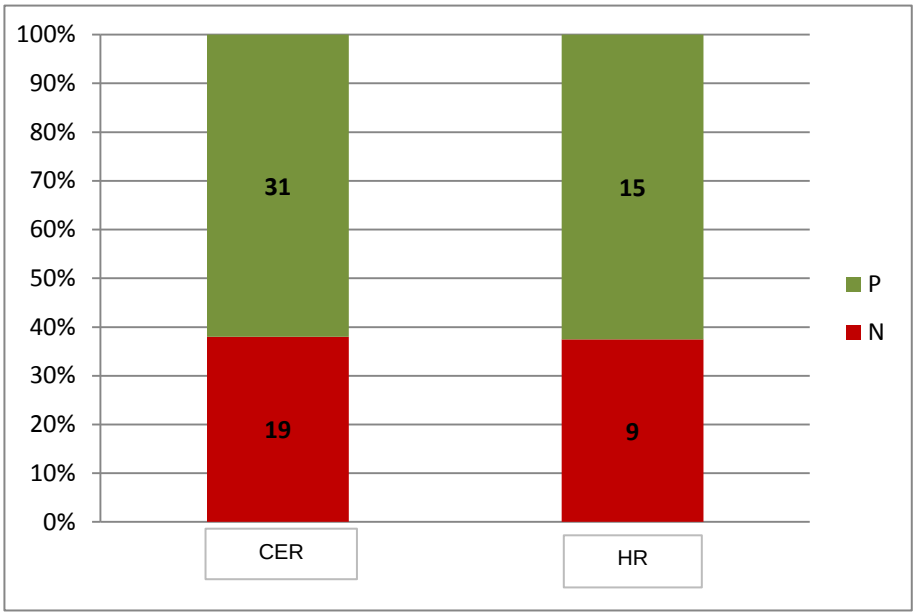

Fig. 5. Pregnancy diagnosis by location (CER= Equine Reproduction Centre; HR= Haras Raphaela).

Likewise no differences were identified among breeds or month of embryo transfer on pregnancy rates (P>0.05). Quarter horse breed had twenty four positive pregnancies (63.2%) followed by Thoroughbred Arabian (18 – 60.0%) and Thoroughbred Lusitano (4 – 66.7%) (Figure 6).

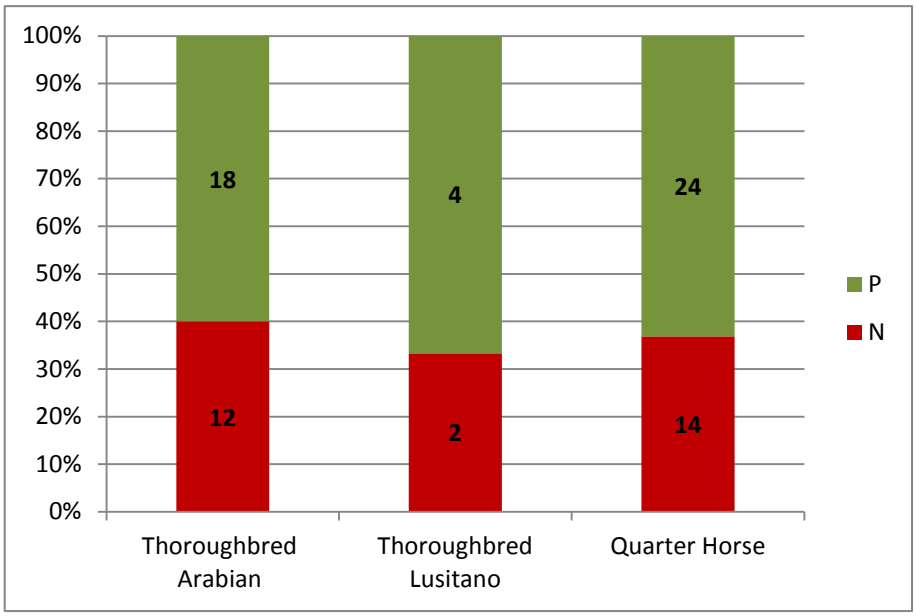

Fig. 6. Pregnancy results per breed (P=positive pregnancy diagnosis; N=negative pregnancy diagnosis).

4. Discussion

In this business, uterine flushes in mares are generally performed between the 7th and 8th day post ovulation [14], although a broad range of days may be successfully practiced. Our results were already foreseeable as the procedure was deliberately performed mostly at day 8. Lower results of embryo recoveries performed at day 9 were obtained. Indeed the range of 8-9 days of age, generally adopted in the present program, can be justified by the fact that at that age, the embryos are easily visualized in the filter system, which speeds up the process of collection, identification, evaluation and transfer [15]. Thus it minimizes the risk for the people involved, the stress and the risk for the donor by reducing the time and intensity of transrectal manipulation of the uterus as well as the cost of material and time of exposure of the embryo to unfavorable conditions [1]. Although no flushings for embryo recovery were performed at day 6, a previous study [7] reported a lower EER compared to days 7, 8, 9, and 10. Similar results were reported by Lopes *et al*. [1] during six breeding seasons, however the achieved ERR (72.8%) was higher compared to our results. Their ERR was also higher compared to the 64% reported by Aurich *et al*. [9] and the 64.2% obtained by Youngquist & Threlfael [12] that are near the range achieved herein. On the other hand, Carnevale *et al*. [16] mentioned that day 7 embryos produced higher pregnancy rates compared to 6, 8 or 9 days old. Similar results were reported by Lopes *et al*. [7]. These authors further demonstrated that embryo age should be related to the post-ovulation day of the recipient mare being the pregnancy rates after TE of embryos with a degree of synchrony until – 4 or -5 days very similar as also showed herein. Camargo *et al.* [17] also obtained improved results for a synchronization of 0 to 4 days. The above mentioned data demonstrated that the degree of synchrony between donor and recipient mares does not need to be as restricted as previously reported.

An increased age of donors which can affect the embryonic development and recovery or even the use of a large number of stallions with different fertilities which is a very common practice in large scale commercial programs have been reported to explain the above differences [18,19]. Thus, the use of stallions and donors with good fertility in horse breeding farms and the conduction of programs by the same experienced operators most likely contribute to the similarity among embryo recovery

results [1]. Moreover, Squires *et al*. [20] argued that the embryonic development and its transport in the oviduct of an old mare may be delayed. Therefore embryonic recovery on days 8 and 9 post-ovulation may be more appropriate for older mares. Their worse results may also be related to the reduction in the quality of oocytes and reproductive parameters that occurs after 12-13 years of age [21].

The AI with fresh semen resulted in a significantly higher embryo recovery per ovulation compared to frozen semen (61.6% vs 30.0%, respectively). Although a meticulous management of the mare was performed to guarantee good results, in general pregnancy rates decreased when using frozen semen due to the inferior viability of the spermatozoa [22]. This could also be related to the "capacitation-like" changes inflicted on the spermatozoa during the process of freezing and thawing responsible for the shorter longevity of cryopreserved sperm [6]. Recently, Govaere *et al*. [23] proved the superiority of deep horn insemination over uterine body insemination that was reflected by the better pregnancy rates obtained after the former insemination using the same low doses of frozen thawed semen (30.6%). Therefore, the insemination technique is one of the factors that must be considered as crucial to achieve a successful pregnancy or embryo collection results. Nevertheless, although a deep horn insemination was performed in the present study, this technique could not overcome the negative effect of using frozen semen. Therefore it is no surprising that, in the current study, the majority of the mares were inseminated with fresh semen. Previously Squires *et al*. [4] also referred that mares inseminated with fresh semen are more likely to produce an embryo compared to those inseminated with either cooled or frozen-thawed semen. Later on, Panzani *et al*. [8] reported similar results for AI performed with fresh, cooled or even the association of cooled plus frozen semen resulting in significantly more positive flushes compared to the AI with frozen semen. Stallions fertility, insemination doses, insemination method and mares management are some of the factors that must be considered to explain the variability in the reported results. Govaere *et al*. [23] showed that the dose of frozen/thawed semen and AI protocol have a great impact on pregnancy rates and outcomes. So to achieve good results, if only a low-dose of semen is available, a deep uterine horn insemination can significantly improve the pregnancy results. However no significant differences were observed in mares that were inseminated with high doses of frozen/thawed semen in the uterine body. Moreover, an inadequate semen manipulation (especially when using low doses of frozen semen) will lead to adverse effects and limitations on semen quality and thus to suboptimal pregnancy rates per cycle. Conversely higher doses due to lower semen quality may be related to the presence of free uterine fluid.

In the mare, a non-pathological response to spermatozoa is normally associated to the AI procedure, which can lead to persistent endometritis, unless it disappears within 24-36 hours [24]. Uterine flushing and oxytocin administration are typically used to prevent persistent endometritis. Recently new therapies emerged to modulate the inflammatory response. Ryan *et al*. [25] mentioned the use of mesenchymal stem cells and autologous conditioned serum. Both strategies reduced the presence of uterine inflammation and the number of neutrophils at six hours post-breeding by over 50%. The authors reported that mesenchymal stem cells treatment had a better ability to modulate the

immune response. However, it was already mentioned [6] that common therapies should only be implemented in mares really showing signs of uterine inflammation or fluid accumulation.

Nowadays, there is still controversy on whether mares should be bred just prior to or after ovulation with frozen semen. Nevertheless, in either case it is evident that the timing of ovulation is highly desirable in order to reduce the interval between breeding and ovulation [6]. Recently, Avanzi *et al*. [26] compare two protocols for equine frozen semen AI using either post-ovulation (the same as in the present study) or fixed-time insemination, and evaluated both pregnancy rates and intrauterine fluid accumulation. The pregnancy results were not influenced by the technique of insemination (41.4% vs 51.7%) but the presence of intrauterine fluid accumulation was higher in the post-ovulation protocol (58.6% vs 34.0%). Although the number of mares inseminated was higher in the study of Loomis & Squires [27], similar rates between mares inseminated twice or once (48.1% vs 47.3%) were observed. They also reported uterine fluid presence in 23% of the mares but still lower than mentioned before. In the present study, seventeen mares had this problem (20.5%), only six were submitted more than once at this procedure and four (23.5%) were inseminated with frozen semen. These differences may be related to the fact that an uniform definition of the “free fluid” terminology does not exist.

The impact of breed on ERR was not often reported in the literature. In this work, apparently the Quarter Horse breed had the best results compared to the other two breeds (Lusitano and Arabian). However the higher number of Quarter Horse mares included in this ET program that were bred with fresh semen could have contributed to these differences. Panzani *et al*. [8] recognized that the Standard breed and the Quarter Horse mares had significantly higher positive flushes and embryo recovery per cycle and per ovulation rates compared to Show Jumping mares. Nevertheless, such reported differences were also related to the different management of the semen market in Italy. Likewise, it is very common in these ET programs, that mares can be used as embryo donors without neglecting their competitive careers. Recently Pessoa *et al*. [28] compared embryo recovery and pregnancy rates between athletic and non- athletic breeding Quarter Horse mares in a tropical warm climate (similarly weather conditions as in our research). They mentioned that embryo production from donor mares under well-conditioned, appropriate training, were similar to non-athletic as no differences were observed in EER (76% vs 71%) as well as in the pregnancy rates on days 15 (78% vs 79%) and 40 (69% vs 70%).

Some authors [1,9] mentioned that in mare embryo collections, no differences were verified within breeding seasons but they did not exclude the fact that if a higher number of embryos collections could be analyzed these difference could become significant. Although the studied period did not cover the entire breeding season, in the present study it was observed that on July-August a lower number of embryos were obtained when compared to the rest of the months. This difference might be explained, at least in part, by the environmental circumstances such as the temperature and rainfall conditions along with the number of mares in the program. Mares are seasonal polyestrous breeders. So they have multiple estrous cycles during the breeding season being their ovulatory activity related to the long days [29]. In the south hemisphere, on July-August the temperature and the

length of days begin to increase and in consequence the mares gradually start to cycle while emerging from the anestrus period. From September onwards better results were achieved as their cycles become more regular and thus the chances of obtaining a greater number of embryos per cycle was higher.

In our research, all donor mares were submitted to a PGF2α intramuscular administration after embryo recovery so that four to five days later an estrous cycle could again be monitored. In fact, as mentioned by Goretti *et al*. [30], giving PGF2α to the donor mares 48h after embryo collection helps to reduce the average interovulatory interval by approximately 2.5 days, thus increasing the number of embryos that could be collected during the breeding season. This administration had no deleterious effects on the embryo quality, the interval from PGF to ovulation or in the pregnancy rate of recipient mares. On the other hand, the study of Cuervo-Arango *et al*.[31] concluded that the interval from PGF – induced luteolysis to ovulation had a significant effect on the posterior pregnancy and EER in the mare. Fertility was reduced as this interval became shorter. Differences in sample size, breed or management of the mares could explain these discrepancies.

5. Conclusions

In conclusion, the day of embryo collection, the type of semen and month of the year were the most important factors affecting embryo recovery rates and thus the embryo transfer program Moreover, the specific conditions of this study, working simultaneously on different breeds, with different types of semen and donors management in two different breeding stations, allowed several technical approaches that may be relevant to consider and implement in mare embryo transfer centres.

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Supplementary material

Table 2- History of mares, location, number of embryo collection and embryos recovered during the experimental period.

Breeding Farm	Mares	Breed	Embryo collections attempts (n)	Embryo recovered (n)
CER	Brentina	TL	1	0
	Champgne		1	1
	Wilrona		1	0
	Hervilha		4	4
	Wilrona		2	2
	Fama		1	0
	Xerez		1	1
	Cartagena		4	1
	Qualidade		3	1
	Violeta		1	0
	Afirestar	TA	6	3
	Beauty		3	2
	Hamona		6	2
	Morana		1	0
	Hestoriah JM		1	0
	Jaritza		1	1
	La Rosa		1	0
	Rene Al Jamaal		1	0
	Shannaya		1	1
	Mayka		1	0
	Samara Valentina		2	2
	Tshakira Di Pscore JM		2	0
	Ursulla Di Pscore		1	1
	Monicha		1	1
	Stig Jhustine		2	1
	Tzarinna		1	0
	Uhu Lady Juliant		1	0
	Dasha FF		1	0

Margot HVP		1	0
Monallisa		1	0
Michelle Carol		1	0
Mystic Lady HVP		1	1
Magda HVP		1	1
Shaklinna El Jahd		2	2
Emocione		1	0
Nalina Power		1	0
Diamond		1	0
Nice Dream		1	0
She Star P´score		1	0
Usherrie		1	1
Jessica		1	1
Yasmin		2	2
Ballerina		1	1
Rose		2	1
Valkirie		1	0
Shavanna Al Madan		4	3
Star		1	1
Sonora		2	2
Venitia		1	1
Vitoria		1	1
Honey Moon		1	1
Shining		3	0
Jade		1	1
Psynsation		1	0
Spartana		2	1
Greta By Ashiraf		2	2
Sovering Almond		1	1
Stig Jhustine		1	1
Marcessa		1	0
Java Reyna		1	0
Mirabella Moon		1	1
Hopeful Eye	QH	1	1
Baby Rojo		5	1

	Nick Failas		3	3
	Pamplona		4	4
	Flame Baby		1	0
	Star		2	2
	Eternaly Relic		4	4
	Indiana Fames		1	0
	Sarambu		1	1
	ST Creek Fly		4	3
	Roseana Seis		6	3
	Linda Ta Fame		1	1
	That Blazin		4	3
	Go Like		1	1
	Serata		3	1
	Very Tempting		5	2
HR	Gana Apollo		1	1
	Little Zita		4	4
	Primetime		1	1
	VPJ Cherry Merry		1	1
	Star Bryan		1	1
	Vivid Apollo		3	0
	Beapeaceful		1	1
	Dash Dee Fame		1	0
	Famous Pawn Star		1	1

(CER, Equine Reproduction Centre; HR, Haras Raphaela; QH, Quarter Horse; TL, Thoughbreed Lusitano; TA, Thoughbreed Arabian)

Annex 1

(retrieved from the “Guide for authors” of the website of the *Equine Veterinary Science* journal, available at: http://www.j-evs.com/content/authorinfo)

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