General techniques and organization of large commercial embryo transfer programs
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Introduction
Equine embryo transfer (ET) started in Argentina in 1990 primarily in polo ponies. This presentation is a review of the practical experience gained over the last 20 years of commercial ET in Argentina. Although the author has been involved in several ET programs since 1990, the data analyzed in this work were obtained only at Centro de Reproducción Equina Doña Pilar, located in Lincoln (B), Argentina and involves the production of 7,939 pregnancies obtained from 13,942 uterine lavages for embryo collection (flushings) between 1997 and 2010. A summary of this work is detailed in Table 1.

The ET industry grew tremendously during the last 10 years in Argentina. Many of the best playing mares were sold overseas at the peak of their athletic careers so their bloodlines were being lost. Embryo transfer became the solution to this problem allowing us to produce offspring before the mares were sold abroad. The technique rapidly showed its advantages and became widely accepted by polo players and breeders. This resulted in a need to rapidly adjust to the market demand. We introduced changes in many procedures to make them faster and simpler and also changes in general and reproductive management. Despite these changes the fast increase in labor caused several “crises” that affected the overall efficiency of our program. The analysis of these data helped us to identify some of the factors that affect the efficiency of an embryo transfer program.

Keywords: Embryo transfer, estrus synchronization, recipient management

Procedures in an ET program
Selection and management of donors
Most mares brought to our center are polo ponies. Many of these mares are still at the peak of their athletic careers. They are enrolled in the program for three to four months after the polo tournaments and during this time undergo intensive reproductive management to produce several pregnancies. Other donors are old retired polo pony mares that come to the program to maximize the number of pregnancies produced per season. In some instances the mares have failed to produce offspring naturally due to fertility problems.

Table 1: Number of flushings performed at Doña Pilar 1997-2009 (n=13942 flushings)

<table>
<thead>
<tr>
<th>Season</th>
<th>Donor mares</th>
<th>Stallions</th>
<th>Flushings</th>
<th>Embryos</th>
<th>Preg 14-21 days</th>
<th>Preg 60 days</th>
<th>Embryo rec. (%)</th>
<th>Pr 1 (%)</th>
<th>EED (%)</th>
<th>Efficiency (%)</th>
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<tr>
<td>97-98</td>
<td>56</td>
<td>12</td>
<td>213</td>
<td>141</td>
<td>69</td>
<td>66.2%</td>
<td>83.1%</td>
<td>69.3%</td>
<td>21.05%</td>
<td>57.58%</td>
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<tr>
<td>98-99</td>
<td>50</td>
<td>11</td>
<td>231</td>
<td>192</td>
<td>133</td>
<td>105</td>
<td>91.8%</td>
<td>55.2%</td>
<td>10.74%</td>
<td>50.68%</td>
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<tr>
<td>99-00</td>
<td>58</td>
<td>14</td>
<td>294</td>
<td>270</td>
<td>149</td>
<td>133</td>
<td>86.5%</td>
<td>67.0%</td>
<td>13.23%</td>
<td>57.98%</td>
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<tr>
<td>00-01</td>
<td>76</td>
<td>20</td>
<td>326</td>
<td>282</td>
<td>189</td>
<td>164</td>
<td>75.5%</td>
<td>11.11%</td>
<td>68.07%</td>
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<td>01-02</td>
<td>96</td>
<td>16</td>
<td>357</td>
<td>322</td>
<td>243</td>
<td>216</td>
<td>90.2%</td>
<td>75.5%</td>
<td>11.11%</td>
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<td>02-03</td>
<td>128</td>
<td>23</td>
<td>487</td>
<td>426</td>
<td>325</td>
<td>271</td>
<td>87.5%</td>
<td>76.3%</td>
<td>14.62%</td>
<td>66.74%</td>
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<td>03-04</td>
<td>188</td>
<td>31</td>
<td>964</td>
<td>870</td>
<td>558</td>
<td>484</td>
<td>90.2%</td>
<td>64.1%</td>
<td>13.26%</td>
<td>57.88%</td>
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<tr>
<td>04-05</td>
<td>275</td>
<td>35</td>
<td>962</td>
<td>1043</td>
<td>671</td>
<td>574</td>
<td>108.4%</td>
<td>64.3%</td>
<td>14.46%</td>
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<td>05-06</td>
<td>352</td>
<td>48</td>
<td>1744</td>
<td>1413</td>
<td>861</td>
<td>750</td>
<td>81.0%</td>
<td>69.9%</td>
<td>12.89%</td>
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<tr>
<td>06-07</td>
<td>431</td>
<td>44</td>
<td>2287</td>
<td>1845</td>
<td>1036</td>
<td>889</td>
<td>80.7%</td>
<td>56.2%</td>
<td>14.19%</td>
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<td>07-08</td>
<td>448</td>
<td>55</td>
<td>2331</td>
<td>1823</td>
<td>1286</td>
<td>1162</td>
<td>78.2%</td>
<td>70.5%</td>
<td>9.64%</td>
<td>55.17%</td>
</tr>
<tr>
<td>08-09</td>
<td>500</td>
<td>53</td>
<td>2288</td>
<td>1951</td>
<td>1454</td>
<td>1278</td>
<td>85.3%</td>
<td>74.5%</td>
<td>12.1%</td>
<td>63.55%</td>
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<tr>
<td>09-10</td>
<td>359</td>
<td>44</td>
<td>1671</td>
<td>1313</td>
<td>1034</td>
<td>949</td>
<td>78.6%</td>
<td>78.8%</td>
<td>8.2%</td>
<td>61.88%</td>
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Breeding soundness evaluation. Before admission to the center, every mare is tested for equine infectious anemia. Once the mare arrives, a general physical examination is performed. All mares are dewormed and vaccinations are updated. Records and fertility history, including previous ET records, are carefully reviewed. Each donor is accompanied by the following information upon arrival: number of pregnancies to achieve, sires to be used on each cycle, and type of housing and feeding to which the mare is accustomed. The mares are carefully identified with tags, and samples are submitted for DNA typing. Every mare undergoes a breeding soundness evaluation after arrival at the ET center. In some old mares with defective perineal conformation, surgical correction is performed before starting the program. If a fertility problem is detected, appropriate therapy is instituted.

Management of embryo donors. At Doña Pilar, embryo donors are housed in groups of 10 to 25 to optimize management and decrease stress and chances of injury. Once a mare is introduced and adapted to a social group, she will stay with that group for the rest of the season. Donor groups are assigned to one of two examination facilities directed by two different veterinarians on each team. An average of 150 donor mares are examined, inseminated, and flushed by each team.

Selection and management of recipients

One of the most critical aspects of an ET program is the selection, management, and quality of the recipient mares. Good recipient mares should meet all of the following requirements: (1) good health and body condition, (2) easy to handle and halter broken, (3) body size similar to that of the embryo donor, (4) 4–10 years of age, (5) sound breeding condition with a uterine biopsy grade I or IIA according to the criteria of Kenney, and (6) regular estrous cycles. We prefer mares that have foaled normally at least once and that have shown good ability to nurse the foal. Although primiparous mares can be used, it is important to advise the owner of the embryo that the mare may need more attention at the time of foaling and that foals can be of smaller size at birth. Our recipient herd consists of crossbred mares weighing between 400 and 600 kg. Health requirements for recipient mares are the same as those for donor mares. In addition, all recipient mares are freeze branded. Careful records include identification information, age, markings, vaccination status, deworming status, and reproductive history, if available. The breeding soundness examination for the recipient mare is similar to that performed on the donor mare. Special emphasis is given to the size and tone of the uterus and cervix. We prefer to use recipient mares with documented, well-known reproductive histories.

Recipient mares are kept in mixed pastures of grass and alfalfa. Pregnant and transferred recipients receive the best pastures, especially from the day of transfer up to 40 days of gestation. Non-pregnant mares are kept in groups of approximately 50 to 100. These groups are examined periodically depending upon the synchronization requirements to determine follicular activity. Time of ovulation is determined within a range or “window of synchrony” as described below.

Recipient management. Recipient management to avoid stress is a critical factor that affects pregnancy rates in a large-scale ET program. It is very common for recipient mares added to the program in the last trimester of the breeding season not to become pregnant and go into anestrus earlier than the rest of the group. The ability to overcome this problem is one of the major challenges in a large commercial program.

Synchronization

Synchronization between the estrous cycles of donor and recipient mares is the most time-consuming activity in an ET center. Mares are routinely examined by transrectal palpation and ultrasonography of the ovaries and internal genitalia. Donor mares in estrus should be examined periodically once a dominant follicle has been detected. This is essential for deciding the time for artificial insemination and for determining the day of ovulation (day 0). At Doña Pilar we use recipients that ovulate from the same day as the donor (synchrony 0) up to four days after the donor (sync +4). Synchronization between donors and recipients is better understood if the days of progesterone influence of the recipient uterus at the time the embryo transfer are considered. We
prefer recipients to have been under progesterone influence for at least four and not more than seven days at the time of transfer. The progesterone influence can be from endogenous progesterone if the recipient has effectively ovulated or from exogenous progesterone in those cases when the recipient is not cycling. An artificial estrous cycle in the recipient can be produced by means of injections of estrogens and progesterone and be effectively used as it is described later in this work.

The method used for synchronization depends upon the number of donors and recipients involved in the program. If there are a large number of recipients available, synchronization may be performed by administration of a luteolytic dose of prostaglandin F$_2$α (PGF) or an analog given to one or two recipients one or two days after administration to the donor.$^{15,16}$ In large programs recipient availability can sometimes be a limitation. The use of ovulation-inducing agents such as human chorionic gonadotropin (hCG) and deslorelin acetate is common in ET programs.$^{17,18}$ Injection of 1500 IU of hCG intravenously when there is a 35 mm follicle induces ovulation 36–48 hours after injection. Deslorelin acetate (1.5 mg sc) is also commonly used at Doña Pilar to tighten the synchrony between donors and recipients.

The use of intact non-cycling mares supplemented with progesterone to mimic a regular estrous cycle has been a useful alternative when recipients stop cycling at the end of breeding season.$^{19,22}$ As previously mentioned, it is common that new mares added to the program at the end of the season enter anestrus. In these cases we administer estradiol benzoate (2 mg/day for two to three days) and then 300 mg of progesterone daily for four to five days before using the mare as a recipient.$^{23}$ On the day of ET the anovulatory mares are treated with progesterone in oil (300 mg im) plus biorelease progesterone (1.8 gm im). The biorelease progesterone (P4LA) treatment is repeated on a weekly basis thereafter, until day 110 of gestation. Progesterone administration could be discontinued once secondary corpora lutea are detected. Some ET programs use altrenogest instead of progesterone. Altrenogest does not cross react with progesterone so endogenous progesterone produced by secondary corpora lutea can be determined which indicates if altrenogest administration can be safely discontinued.

In a study conducted at our clinic, a total of 469 transfers were performed between February 15 and April 30, 2008 and pregnancy rates achieved in normal cycling mares vs. intact non-cycling mares supplemented with progesterone were compared. The results in both groups were analyzed in 15-day periods. Overall, pregnancy rates in anovulatory, progesterone-treated recipients were significantly lower than those for ovulatory recipients (164/192 [56.1%] vs. 197/277 [71.1%]). This finding was probably related to the season in which this study was conducted (early fall); mares that become anovulatory early in the fall are typically those with lower body condition scores. Later in the anovulatory season, many mares, regardless of body condition score, will be in transition or anestrous and the pregnancy rates obtained in both groups are similar. However, the use of noncycling progesterone-treated mares is still a viable alternative to extend the ET season by one or two months.

In a retrospective analysis of our records over two consecutive breeding seasons we studied the effect of number of days of progesterone supplementation on pregnancy rates achieved at day 14-21 days after donor ovulation (Table 2). In addition, we studied the effect on early embryonic death (EED) rates. A total of 551 non-cycling hormonally treated mares were used as embryo recipients after being supplemented with 300 mg of progesterone in oil (P4) daily for five to eight days before transfer. The pregnancy rates achieved appeared to be different in mares that were treated for five, six, seven or eight days (70.45; 57.71; 56.11 and 44.44%, respectively). Early embryonic death appeared to be different among groups (9.68; 12.93; 27.42; 5.00%) for mares that received P4 for five, six, seven and eight days, respectively. Mares that received P4 for seven days experienced a much higher incidence of EED.
Artificial insemination

Mares are artificially inseminated with fresh, extended semen collected at the center. Mares that ovulate within 24–48 hours after breeding are not rebred if the semen is of good quality and sperm longevity is adequate. Mares that are susceptible to endometritis are inseminated using minimal contamination breeding procedures. Due to the large number of donors enrolled in our program we intended to decrease the number of examinations per mare to allow us to better organize the work. We analyzed the effect on the efficiency of our program if ovulation was detected in a range of 48 hrs instead of 24. Donors received either hCG (1600 IU iv) or deslorelin acetate (1.5 mg sc) when a 35mm follicle was detected with other signs of estrus (uterine edema). Donors were artificially inseminated on the same day or the day after administration of the inducing agent, depending upon semen availability and then were re-examined 48 hrs later to detect ovulation. Flushings were performed eight days after detection of ovulation. The embryo recovery rate (679/1079 [63%] vs 127/181 [70%]) was not different between these two groups. This has been a major change in our donor management because it has helped us to better organize the work and to decrease workloads on Sundays. The fact that the embryo recovery rate was higher in the group of mares examined every 48 hrs reflects the fact that difficult mares susceptible to endometritis or mares bred to stallions of short sperm longevity were all examined in a daily basis to be managed using minimal contamination techniques, whereas “normal” donors with a good reproductive history bred to stallions with good semen quality were examined every 48 hrs.

Embryo collection and processing

The embryo enters the uterus from 5 days 10 hours to 5 days 22 hours after ovulation. The possible reasons for this variability could be the delay between ovulation and fertilization, embryonic factors related to timing of prostaglandin E2 secretion, sex of the embryo, or other individual factors. Flushings are usually performed between days six and eight after ovulation. Recovery rate is lower when performed at day six. This can be due to one or more of the following reasons: (1) failure of the embryo to descend into the uterus by day six, (2) failure of the technician to recover the embryo from the uterus because of a higher gravity weight of the embryo, (3) failure of the technician to find the embryo because of its smaller size, and (4) loss of the embryo at some point during the process. We prefer to attempt embryo recovery on day eight after ovulation. At this stage, embryos are large enough to be easily found even with the naked eye, so chances of missing or losing the embryo are decreased.

Flushing technique. The uterine lavage, or uterine flush, is a simple procedure performed with the mare restrained in stocks. Before the flushing, the mare’s rectum is evacuated of feces, and the size and tone of the uterus and cervix are evaluated. In addition, follicular status of the ovaries is established to determine whether the mare will receive PGF immediately after the uterine flush. When the mare has a follicle >35 mm, PGF treatment is delayed or a half dose is given to prevent premature ovulation. This allows the uterus to recover from the uterine flushing and increases the chances of a normal subsequent heat before re-insemination. Tranquilization is usually not necessary, but some mares may require light sedation with 50–100 mg of xylazine intravenously. Acepromazine maleate can also be used for this purpose, but we prefer not to use it because it induces relaxation of the uterus, making it more difficult to recover the fluid in some instances. Once the mare has been

<table>
<thead>
<tr>
<th>Days of P4</th>
<th>Total embryos</th>
<th>Pr1 (%)</th>
<th>Pr2 (%)</th>
<th>EED (%)</th>
<th>EED (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>44</td>
<td>31</td>
<td>70.45</td>
<td>28</td>
<td>63.64</td>
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<tr>
<td>6</td>
<td>201</td>
<td>116</td>
<td>57.71</td>
<td>101</td>
<td>50.25</td>
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<tr>
<td>7</td>
<td>221</td>
<td>124</td>
<td>56.11</td>
<td>90</td>
<td>40.72</td>
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<td>8</td>
<td>45</td>
<td>20</td>
<td>44.44</td>
<td>19</td>
<td>42.22</td>
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</tbody>
</table>

Table 2. P4 Supplementation (2007/2008 and 2008/2009 combined) n=551
examined, her tail is wrapped and hung in a vertical position. The perineal area is carefully washed with soap, rinsed with tap water, and dried with a clean paper towel. A small piece of wet cotton is used to clean the vestibule. Flushings are performed with lactated Ringer’s solution with the addition of 0.5% fetal calf serum. There are several brands of flushing medium on the market. We prefer to use lactated Ringer’s solution because of its lower cost and easy availability. Fetal calf serum prevents the embryo from sticking to the tubing and filter although the use of fetal calf serum is controversial. We believe that fetal calf serum facilitates embryo handling but probably does not make any difference on embryo survival or recovery rate.

The technician, wearing a sterile sleeve with a small amount of lubricating jelly on the dorsal part of the hand, introduces the arm into the vagina to identify the external os of the cervix. With the index finger, the technician dilates the external os to a size large enough to pass the tip of a 24-gauge Foley catheter into the body of the uterus. The cuff is inflated with 30 ml of air, and the catheter is pulled back gently, forming a tight seal at the internal cervical os. A total of 2–3 L of flushing medium is infused by gravity flow in aliquots of 500–1000 ml, depending on the size of the uterus. The uterus should not be overfilled which will produce discomfort for the mare and also can result in fluid lost through the cervix. The flushing catheter is connected with a Y junction to the delivery tubing on one end and to a large-volume filter on the other end. The system should be completely purged with flushing medium to eliminate the air before the procedure is started. This prevents the formation of foam and bubbles. Several brands of catheters can be used. Integrity of the air cuff should be checked before the catheters are introduced into the mare. The fluid is passed through the filter connected in line with the catheter by means of the Y junction and silicone tubing. The amount of fluid recovered is measured in a graduated receptacle and should be more than 95% of the volume infused into the uterus. In some cases, especially in old mares with a large pendulous uterus, fluid recovery can be difficult. In such cases, the use of 20 IU of oxytocin iv during the flushing procedure can aid in the recovery of the flushing medium. The use of ultrasonography to locate pockets of fluid can be helpful during the subsequent manipulation of the catheter toward these areas. Gentle massage of the uterus is performed to ensure that the medium has reached the entire uterus. This also produces a slight turbulence to get the embryo in suspension, thereby increasing the chances of recovery.

Handling and evaluation of the embryo. After the uterine flush is completed, the filter is drained so that approximately 20 ml of fluid are left in the filter. This content is swirled gently to prevent the embryo from sticking to the filter walls, and then it is poured into a sterile Petri dish. The filter is then rinsed with flushing medium to ensure that the embryo is not lost in the filter. Many of the expanded blastocysts recovered at day seven and almost all embryos recovered at day eight can be found with the naked eye or with a small magnifying glass if the effluent is clear. Consequently, the embryo can be easily found and transferred within a short period of time. If we fail to find the embryo with the naked eye, we search with a dissecting microscope first at a lower magnification and then with a higher magnification to grade the embryo quality. Embryo searching is facilitated when bubbles or foam are not present in the Petri dish. Once the embryo is found, it is rinsed at least three or four times and then transferred to a small Falcon dish containing holding medium (ViGro or SYNGRO™, Bioniche Animal Health, Athens, GA) by means of a 10 to 20 µl Unopette adapted to a 1 ml syringe. Large embryos will be handled with a 0.25-ml sterile straw since they are too large to be loaded in a Unopette. Depending upon the diameter embryos should be transferred using other devices. Embryos can be kept at room temperature in holding medium for two or three hours before transfer. If transfer is delayed, we usually cool them to 18°C.

Embryo biopsy for gender diagnosis. Embryo biopsy samples can be obtained from day seven or eight embryos for PCR determination of genetic diseases as well as gender diagnosis. At Doña Pilar embryos are micromanipulated and a few cells from the trophoblast are obtained and subsequently processed for PCR analysis. Embryos are only transferred if the gender corresponds to the one requested by the owner. This service has been recently started at Doña Pilar. In a limited trial we performed biopsies on 36 embryos that were immediately transferred. Thirty-one mares were detected pregnant (86.11%) indicating that this is a promising technique that will be applied commercially in a larger scale in future ET seasons.
Selection of the transfer device. There are several disposable instruments on the market developed to transfer equine embryos. It is essential to choose the appropriate instrument depending upon the size of the embryo to be transferred. Embryos collected on day eight or later may be too large to be transferred in a 0.5-ml straw in those instruments in which the opening is smaller than the 0.5-ml straw diameter. In some instances, the embryo can fit into the straw but it is larger in diameter than the opening of the transfer gun, which means that the embryo is destroyed when it is transferred. If the embryo is too large to be transferred in a conventional transfer gun, we use an AI pipette or even the outer part of a 0.25-ml disposable transfer gun.

Preparation for transfer. In preparation for ET we follow these steps:

1. Select the recipient to be used from among the ones available. The records of the recipients available should be carefully reviewed. It is important to note if the mare has received an embryo in the same season and did not get pregnant, or if the mare was pregnant and suffered EED. It is important to note if there were any signs of uterine abnormality such as presence of fluid or any evidence of endometritis. The recipients are evaluated before transfer and notes are taken about tone of the uterus and cervix and the presence of a corpus luteum.

2. Prepare the recipient carefully; the mare is restrained in stocks and prepared as for any other intrauterine procedure. The mare should be properly restrained at the time of transfer. If sedation is required we like to use 50–100 mg xylazine iv. In rare cases we may use detomidine at a dose of 1 mg iv given before transfer.

3. The embryo is aseptically aspirated into a 0.5-ml straw. The embryo is aspirated between two columns of medium separated by air as has been described. An assistant separates the mare’s vulvar lips and the operator introduces his/her sterile gloved hand into the vagina. The external os of the cervix is located with the index finger. Care should be taken not to dilate the cervix in this procedure, which in our experience will lower pregnancy rates. The index finger should be used just to find the external os but not to dilate it. A sanitary protector is used to pass through the vagina and halfway into the cervix. The anterior end of the transfer gun is introduced gently halfway into the cervix where the sanitary sleeve is punctured by the gun. The operator removes his/her hand from the mare’s vagina and introduces it into the rectum. Gentle manipulation of the body and right horn of the uterus through the rectal wall is performed to position the transfer gun deep into the uterus. It is essential to avoid scratching the endometrium, which would induce prostaglandin release and could cause pregnancy failure. The embryo is transferred by gentle pressure to the transfer gun plunger. At our clinic most clinicians use their left hand for all gynecological procedures making it easier to transfer the embryo into the right horn. I do not think it makes a difference if the embryo is transferred deep in the body of the uterus or in the uterine horn, but in any case it is essential to avoid scratching the endometrium at the time of transfer.

Recipient mares are given a dose of long acting progesterone at the time of transfer. Although it has not been yet proven that it makes a difference, we do this in a regular basis. The author personally believes that progesterone supplementation is very important at the end of the breeding season to reduce the incidence of EED.

References