Workshop 2: Estratégias de otimização da produção de embriões em programas de TE em equinos

Optimization strategies for embryo production in equine ET programs
Problems and Solutions in Equine Embryo Transfer Programs in Brazil

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ABSTRACT

Background: Brazil is the second country on equine embryo world production. Problems related to the application of equine embryos transfer in Brazil are discussed in this article.

Review: The main factor related to low efficacy of Equine Embryos Transfer programs consists in the difficulty to induce multiple ovulations. Among the most common difficulties and limitations faced in our country, we emphasize the utilization aspect of a higher percentage of aged females which are used as donors the interest for the continuity of their use is attached to the fact of slow genetic improvement on equine species.

Conclusion: Other important aspect is related to recipient quality.

Keywords: embryos, equine, efficacy, Brazil, embryos transfer, fertility.

I. INTRODUCTION

II. PARTICULARITIES AND LIMITATIONS RELATED TO EMBRYO TRANSFER IN HORSES

III. OTHER PARTICULARITIES THAT INTERFERE IN THE EFFICACY OF EQUINE ET PROGRAMS IN BRAZIL

IV. PROBLEMS WITH AGED MARES' IMPROPER TREATMENT

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I. INTRODUCTION

According to the last survey of Embryos Transfer International Society Statistics Committee (IETS), Brazil is the second on equine embryo world production (12,000 recovered embryos) being overtaken only by the United States of America (USA), in which approximately 14,000 embryos were recovered at the stud farm in 2008-2009. The average growth of embryos produced in Brazil has been 20% per year in the last 5 years.

Among the breeds, Mangalarga Marchador is the breed with more products born from embryos transfer registered per year, followed by Quarter Horse breed. Quarter Horse is the breed that has grown more throughout the last years in number of embryos produced yearly. Regionally, the greatest centers of embryos production are the states of Minas Gerais and São Paulo, as they are the states with major concentration of Mangalarga Marchador and Quarter Horse, respectively.

In our country, there are approximately 40 Centers of Embryos Transfer in equine, being part of them Services provider for third parties and the other part for exclusive use in animals of a sole owner. Embryos Transfers in Quarter Horses and Sport Horses are rather taken place in Centers, while in other breeds, such as Mangalarga and Campolina, Embryos Transfer are mostly taken place in structures established at the breeder's own stud. The present publication aimed to show the most important factors that interfere on the success of ET programs in Brazil.

II. PARTICULARITIES AND LIMITATIONS RELATED TO EMBRYOS TRANSFER IN EQUINE SUPEROVULATION INDUCTION LIMITATIONS

The main factor related to low efficacy of Equine Embryos Transfer programs consists in the difficulty to induce multiple ovulations. Multiple ovulations induction in mares’ main objective is to increase the chances of embryonic recovery in a cycle and, consequently, to improve the efficacy and to diminish the costs involved in programs of Embryos Transfer (TE). Today, due to the difficulty to superovulate mares, TE programs efficacy in this species is very low, being needed an average of 2 to 4 cycles to attain a gestation of an embryos donor mare, therefore, the costs for production of a young horse deriving from TE are quite expensive. The adaptation of a superovulatory protocol which consents to at least guarantee one pregnancy for each donor mare cycle is highly desirable. A great difficulty in the superovulation of mares is related to the mare refractoriness to hormonal preparations based on swine FSH commercially available for other species, such as bovines. The use of homologous FSH is being needed so that we have ovarian response to treatment. This way, Equine Pituitary Extract (EPE), purified equine FSH and most recently recombinant equine FSH are the indicated preparations to induce superovulation in female horse.

The response of cyclic mares to treatments with EPE or equine FSH allowed, according to several papers, the obtainment of a good superovulatory response, with a variation of 3 to 5 ovulations per cycle, although the embryonic recovery has been low, with an average of 30 to 40% recovery embryos per ovulation [1]. Most recent papers have demonstrated that protocols where EPE lower doses are used, allow a better embryonic recovery. However, difficulty to prepare EPE and its commercial unavailableness prevents its use in large scale. Recently, even equine FSH is no longer commercialized in USA. Recombinant hormones seem to be the most promissory perspective to the future, for it won't be necessary to extract FSH from equine pituitary anymore. Initial works using recombinant FSH in mares have shown very favorable results, although this product is still non available in the market.

On a paper published at the SBTE convention annals, Nagao et al. [7] mention that the use of deslorelin acetate in a special carrier has allowed induction of double ovulation in most treated mares. On this protocol, deslorelin low doses applied twice a day, animals monitored daily through ultrasound and at the moment they have presented a follicle between 23-25mm and at least a second follicle e≥ 20mm (diameter), the treatment with deslorelin acetate was initiated. It is worth to emphasize that the mares didn’t always present this follicular dominance and co-dominance standard and consequently they were not treated. 85% of treated mares presented double ovulation during their embryonic recovery per cycle almost duplicated (0,9 x 0,55 embryos per wash). This protocol using GnRH has two advantages: 1- it’s synthetic, and 2- it has lower cost.

The age factor

Another particularity of TE in equine concerns the use of aged donor mares. In no other production animal species there is a percentage so high of aged procreators in reproductive activity as in equines. The interest for the
continuity of their use is attached to the fact of slow genetic improvement observed in equines for their small quantity of descendents produced yearly, either by limitations imposed by breeders associations as well as by breeding and market profile. Stallions considered as their breeds’ representative hardly produce more than 200 products per year. Similarly, mares are conserved in reproductive activity for a long time. Even mares in TE programs produce few young horses per year, an average of 2 to 4, and take a few years to show potential improvement. The age factor, in its own, is a low fertility determinant.

It is known that mares older than 18 years old present follicular maturation problems, and consequently oocyte maturation, which lead to future problems in their embryonic development capacity, increasing the precocious embryonic loss rate which is almost 60% from the fertilization to 30 days of pregnancy [3].

Difficulties with the use of frozen semen

Difficulties faced for the application of frozen semen in large scale of embryos is another factor which restrains a faster improvement of the species. The application problem of frozen semen in large scale is related to mares long estrus period (5 to 7 days) associated to a large period of time until the ovulation happens during the estrus (last 48 hours), associated to limited time of survival in spermatozoon genital handling after frozenness and defrosting (12 hours), that obliges the accomplishment of artificial insemination at a moment very close to ovulation, which is only possible with control of intensive follicular development. On that basis, trying to trespass frozen semen limitations, the refrigerated semen is routinely used as it has survival time longer than the frozen semen (24 to 30 hours) which facilitates its use in large scale. It is thought that 80% of produced embryos are derived from insemination with refrigerated and transported semen. A bigger limitation to the application of refrigerated semen concerns the short storage period, being ideal not to exceed 24 hours. Some stallions have better fertility results with frozen semen than with refrigerated one. In situations where it’s difficult to get to the mare which will be inseminated, the use of frozen semen is an alternative very appropriated.

In vitro embryo production limitations

Systems employed in other species to mature oocyte, to capacitate spermatozoon and to cultivate “in vitro” embryos do not adapt to equine species. In other species, such as bovines, the IVF application result in the birth of thousands of products yearly, being applied in large scale at a commercial level in Brazil where the number of bovine embryos derived from IVF is higher than the ones produced in vivo, that is, recovered straight from uterus, on the other hand, up to this moment rare products are created through IVF in equines [2].

III. OTHER PARTICULARITIES THAT INTERFERE IN THE EFFICACY OF EQUINE TE PROGRAMS IN BRAZIL RECEIVERS’ SCARCENESS

The increase of interest for use of TE in equines led not only to a significant increase of receptors’ market price as well as reduced the offer. The average prices adopted in the state of São Paulo have increased an average of 30 to 40% per year. In other words, a receptor that was worth 500 Brazilian Reais 3 years ago, at the last stud farm (2009-2010) was worth approximately 1200 Brazilian Reais. That led to two situations which certainly interfere negatively in the program: purchase of lower quality animals and reuse of receptors which would be dismissed for its bad fertility history. According to Central Bimbryo observation, located at São João da Boa Vista – SP, the chances of a receptor which gets pregnant after the loss of an embryo with more than 15 days old is lower than 25%.

The receptor is known to be the TE program success key point [6]. In two moments accentuated losses in pregnancy rate and increase of precocious embryonic loss rate are observed in TE programs: the first during stud farm when new receptors are added to breeding stock [8]; and at the stud farm end when we start to reuse receptors which lost pregnancies throughout the stud farm [9]. In the first moment, when there is a need to replace receptors in the middle of stud farm, it is important to try to isolate those animals, because during the adaptation period they routinely develop strangles. Pessoa et al. [8] mentioned decrease from 70% to 40% on pregnancy rates in a TE commercial program in equines when new receptors are added. However, Riera et al. [10] mentioned decrease from 70% to 40% on pregnancy rates after TE when they compared the last two stud farm months (March and April) against the period from October to January.
The receptor's selection should be based in the reproductive system's age (<12 years) and health. To minimize the receptors' scarceness problem at stud farm beginning and end, the use of progesterone in anoestrus mares has become a routine strategy. This way, we can increase the number of susceptible mares to be used, because either at stud farm beginning or at its end many receptors are not cycling different from donors which initiate the cycle early and finish it later at stud farm. So, 4 to 6 donors' cycles are lost throughout the year. An average of 2 to 3 more embryos are produced per year with the progesterone use. However, progestogens use may not be effective if appropriated drugs and protocols were not use. Another problem concerns the application responsibility. An application error may be an embryonic loss determinant factor as they are for weekly or biweekly use. We advise the establishment of a progesterone application day at the stud or central which should be applied, preferential, by the Veterinarian.

Most recently, protocols using cycle receptors treated with P4 in diestrus have also allowed a better improvement from receptors. In this protocol, published as recapitulation of this SBTE Meeting [4], progesterone treatment was initiated in diestrus after one only dose of estradiol benzoate application. Pregnancy rates were close to 50% when using this protocol.

The mistakes most often observed in receptors' selection are related to the purchase moment, as they are usually purchased during the winter when mares are in anestrus. At the anestrus phase, it is known, that problems such as improper vulvar conformation and even uterine infections may not be clinically detected. A way out would be to apply estradiol benzoate exogenously on the day before when the receptors should be evaluated for purchase.

Another routine mistake concerns the exclusive use of ultrasound as uterine inflammatory processes diagnosis method, where mares with uterine fluid are rejected. The equivocation lies on the fact that, in some situations, mares with uterine infection don't have uterine fluid as well as in the estrus the presence of small quantities of fluid may be normal. The ideal would be to associated cytology exam to the ultrasound.

IV. PROBLEMS WITH AGED MARES IMPROPER TREATMENTS

Every time we face an aged mare donor with fertility problems we should consider the situation in a different way than the one used for younger mares. It is important to have in mind that aged mares present, because of their advanced age, the following characteristics which may lead to fertility loss:

- Low LH serum levels at periovulatory period [5]
- They show high percentage of atypical ovulations
- Have increased interovulatory interval because the follicular phase is longer
- In spite of a good fertilization rate, they show higher percentage of embryonic losses between 2 to 5 days after the fertilization
- They show embryonic development slower in the fallopian tube making, in several situations, the embryos to reach morula phase and migrate to the uterine environment only 8 days after the ovulation.

What can we do to improve embryonic recovery rate of aged mares?

- To supplement with LH equine low doses during the follicular phase
- To induce ovulations associating it to HCG and Deslorelin
- To collect embryos between D 9 and D 10 after ovulation
- To use stallions of high fertility

It is always important to have in mind that aged mars need more care related to well being, such as differentiated feeding, to avoid environmental caloric stress and social stress as well as they should remain in pickets close to reproductive treatment center to avoid long courses.

Finally, other problems such as: non accomplishment of a proper andrologic evaluation of stallions to determinate the fertility capacity, improper treatment of semen to be refrigerated and transported, use of material improperly sterilized (catheters and others), manipulation inaccuracy during the uterine washes for embryos collection which after years of collections injures the cervix and excess of uterine treatments are usually observed in TE programs in Brazil and should be avoided.
REFERENCES


Particularities and difficulties related to equine embryo transfer in Northeast of Brazil

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ABSTRACT

Background: The use of Embryo Transfer (ET) presents significant advantages in its application, however some particularities impose certain limitations on its use.

Review: The use of ET has expanded in recent years with a focus on Brazil, United States and Argentina. Virtually all Equine Associations support ET, confirming its benefits. With the main objective to maximize the success of equine ET programs, it becomes essential the understanding of main factors affecting reproductive efficiency with differences between regions. Among those factors include reproductive seasonality, donor history, quality of semen or Stallion individuality, selection and management of recipients, treatments for a better sincrony between donor and recipients. It is the responsibility of the technician to clarify Breeders in which situations is best to use this technique.

Conclusion: The goal of this work is to discuss particularities and difficulties related to equine ET in the Northeast region of Brazil.

Keywords: equine, embryo transfer, donors, recipients.

I. INTRODUCTION

II. SEASONALITY

III. DONOR

IV. SEMEN V. RECIPIENT

VI. HORMONAL THERAPY

VII. PROGESTERONE CONCENTRATION VS. EMBYO STAGE

VIII. USE OF ACYCLIC RECIPIENTS SUPPLEMENTED WITH PROGESTERONE

IX. CONCLUSIONS

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I. INTRODUCTION

The use of equine ET has grown rapidly, however some biologic peculiarities as well as technical issues has limited its extensive use when compared with bovine species [17]. The ET is the procedure where the donor mare donates an embryo to a recipient that will take this gestation to term. Most Breeding Associations already accept in its regulation the use of this technique confirming its benefit. In Brazil several Breeding Associations benefit of this technique such as Brazilian Quarter Horse Association (ABQM), Brazilian Warmblood Association (ABCCBH), Brazilian Mangalarga Marchador Association (ABCCMM), Brazilian Paint Horse Association (ABCPaint), Brazilian Campolina Association (ABCCC), and Brazilian Arabian Association (ABCCA). These standards follow legislation drawn up by each Technical Association and approved by Brazilian Department of Agriculture. Brazil is the country that most employs this biotechnology in the world being estimated at an average of 6000 pregnancies/year [11].

II. SEASONALITY

Mares are classified as seasonal poliestric due to the breeding season being characterized during Spring and Summer. There are 3 basic factors which explain the estrous cycle characteristic in tropic mares: nutrition, temperature and photoperiod [1]. Mares retained under long days (16h light/day) show cyclic ovarian activity, and mares kept in short days (8.5h) show no ciclicity (anestrous). This variable occurs according to short days associated with the decrease in pituitary secretion leading to a reduction in gonadotrofins and subsequent ovarian activity [12]. However, when we consider the conditions of northeastern Brazil where the photoperiod variation is minimal during the course of the year, mares estrous cycle is closely related to other factors such as temperature, air humidity and nutrition of the animals. In Recife (8º03´14´), capital of Pernambuco State, in 2009 were observed a minimum light exposition (Table 1).

| Table 1. Time of sunrise and sunset at Winter and Summer solstice and number of hours on sunshine exposition and dark in Recife-PE (8º03´14´). |
|-----------------|-----------------|
| **June 21st**   | **December 21st** |
| **Winter Solstice** | **Summer Solstice** |
| Sunrise: 05h:17min | Sunrise: 05h:02min |
| Sunset: 17h:25min | Sunset: 17h:40min |
| 12h08 light: 11h08 dark | 12h38 light: 11h38 dark |

Cintra Filho et al., [6] evaluated the ovarian activity and pregnancy rate in 268 Westfallen Breed mares during 14 months in a stable located in Recife-PE. As a result, mares cycled all year and there was no difference in pregnancy rate or early embryonic loss, indicating that in this region, breeding season can be prolonged in order to maximize reproductive efficiency (Table 2).
Table 2. Pregnancy and Early Embryonic Loss rate during 14 months in a Westfallen Heard located at Recife – Pernambuco, Brazil.

<table>
<thead>
<tr>
<th>Months</th>
<th>Mares (n)</th>
<th>Pregnancies (n)</th>
<th>Early Embryonic Loss (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>March</td>
<td>19</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>April</td>
<td>18</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>May</td>
<td>19</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>June</td>
<td>27</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>July</td>
<td>18</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>August</td>
<td>9</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>September</td>
<td>17</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>October</td>
<td>27</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>November</td>
<td>16</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>December</td>
<td>18</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>January</td>
<td>26</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>February</td>
<td>6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>March</td>
<td>33</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>April</td>
<td>15</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>268</td>
<td>188 (70.14%)</td>
<td>19 (7.08%)</td>
</tr>
</tbody>
</table>

Camargo [3] evaluated 695 harvested embryos in the Breeding Season (September to February) and out of Breeding Season (March to August), there was no difference (p > 0.05) between embryo recovery or pregnancy rates between the two periods. These data are suggestive that you can work all year. In both cases there was an adequate nutritional supply of these animals. It is important to stress that within the same region there are differences in estrous behavior of animals.

The State of Pernambuco (Figure 1) despite being one of the smallest of the Federation is divided into 3 distinct areas: (a) Zona da Mata - closest coastal region with low altitude, with predominantly pseudotropical climate with heavy rain in autumn and winter, high air humidity and medial temperature; b) Agreste – with an average elevation of 400 meters, with a diverse climatic conditions showing some humid and dry areas prevailing a semi-arid climate; c) Sertão – where there is a shortage of rainfall due to dry air currents from South of Africa, semi-arid climate and high temperatures [16]. The 3 regions have similar average sunshine during the year and distinct estrous cycle behavior influenced by other factors such as management, nutrition, soil and weather conditions. This is one of the critical points in equine ET in the northeastern region, where the technician must consider on a case by case basis and set the ET schedule according to ciclicity of each herd worked. Regarding the influence of seasonality in early embryonic loss, there are controversies. Scherbarth [15] demonstrated greater incidence (7.7%) of early embryonic loss in Hanoverian mares at the beginning of Breeding Season compared to mares in the end of breeding season (2.7%). On the other hand, [18] assessed the average rate of pregnancy among mares kept pregnant towards those who have embryonic loss and did not show any difference.
III. DONOR

One of the biggest problem that the technician face in equine ET programs concerns the age of donors. Generally most donors are older mares which compromises the final results of the program because it is known that older mares have lower reproductive efficiency compared to younger mares [4]. In this study, older mares showed more incidence of endometrial inflammation, lower pregnancy rate and increased incidence of embryonic death. In another study, Carnevale and Ginther [5] performing gamete intrafalopian transfer (GIFT) transferred oocytes of older mares to oviduct of younger mares and vice versa. After artificial insemination, 30.7% (8/26) of oocytes from older mares transferred to young mares resulted in embryonic vesicle formation, while the oocytes of young ones generated 91.6% (11/12) of gestation suggesting that reduction of fertility in older mares is associated with the production of defective oocytes.

IV. SEMEN

The quality of semen to be used in an ET program is crucial for the results. A complete breeding soundness evaluation in Stallions to be used in programs of ET or if to have an accurate diagnosis of seminal quality becomes crucial for increase the efficiency and improve the results in the program. We know also that there is variability in fertility between stallions. Fresh semen provides better embryo recovery results than frozen semen [17]. Most ET programs in Northeast of Brazil are performed using cooled semen. There are problems inherent to the semen transport due to long distances in Brazil.

V. RECIPIENT

The appropriate selection and management of recipients is one of the most important factors affecting the results in equine ET programs [17]. Recipients are hard to find due to the great demand and is becoming very expensive. Selection criteria include size, age, docility and good maternal ability (mostly difficult to evaluate). The recipient needs to cycle normally and be absent of reproductive problems [17]. The recipient evaluation the receiver
must include a full gynecological examination by ultrasound and an endometrial biopsy. The color Doppler ultrasonography enables a more accurate assessment of blood flow and vascularity of pre-ovulatory follicles, corpus luteum and endometrium and becomes a powerful tool for a better selection of recipients [9].

VI. HORMONAL THERAPY

The use of hormone treatment and/or supplementation in recipients is a topic of researchers. Fleury & Arruda [7] observed the effects of different hormonal treatments in recipients at the time of transfer on pregnancy rates and Progesterone (P4) plasma concentrations. In this study 67 recipients were used divided into 4 groups: a control (5 ml of saline IM), GnRH (40 µg Buserelin IM), hCG (3,000 IU IM), and Norgestomet (3 mg subcutaneous). Plasma blood samples were taken for P4 analysis starting the day of transfer (D5-D8) until the first pregnancy diagnosis (D13). The P4 levels were greater in hCG Group hCG (average of 11.7 ng/mL), compared with the other groups (p<0,001). No differences were seen in pregnancy rates, but there was a trend in both D13 and D16 with the hCG group demonstrating a luteotropic factor. Treatments that increased P4 concentration also increased pregnancy rate showing a relationship between P4 plasma concentration and pregnancy rate. This study demonstrates an increase in pregnancy rate of pregnancy with the use of hCG on the day of transfer (Table 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>P4 average Concentration from Day of transfer to D13</th>
<th>Pregnancy D13</th>
<th>Pregnancy D16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>7,7 ng/ml</td>
<td>66,7%</td>
<td>56,0%</td>
</tr>
<tr>
<td>GnRH</td>
<td>17</td>
<td>9,3 ng/ml</td>
<td>70,6%</td>
<td>70,6%</td>
</tr>
<tr>
<td>Hcg</td>
<td>16</td>
<td>11,7 ng/ml</td>
<td>87,5%</td>
<td>81,3%</td>
</tr>
<tr>
<td>Norgestomet</td>
<td>16</td>
<td>9,9 ng/ml</td>
<td>75,0%</td>
<td>75,0%</td>
</tr>
</tbody>
</table>

VII. PROGESTERONE CONCENTRATION VS. EMBryo STAGE

Foss & Crane [8] evaluated the P4 concentration in a commercial ET program with the following objectives: (1) assess P4 concentrations at ET trying to identify which animal would need a hormone supplementation; 2) to assess changes in serum P4 levels post ET; 3) correlate these data with embryonic stage. In this study 297 embryos were transferred. The donor has been collected on D7-D8 post-ovulation and were classified as morulas, blastocysts, expanded blastocysts and highly expanded blastocysts (> 700 µ). Only embryos classified as Grade I or II based on IETS were used in the study. Pregnancy diagnosis was performed 5-7 days post ET and blood sample were taken at the time of transfer and measured by RIA. Another sample were collected in pregnant mares in D12-D14. From those, the mares which had P4 serum concentration less than 4 ng/ml or showed signs of endometrial folds at ultrasound examination would start P4 supplementation with 0.044 mg/kg Altrenogest oral on a daily basis. The total pregnancy rate was 83.2% (247/297). Morula embryos presented a pregnancy rate significantly lower than blastocysts (p<0.001 - vide Table 4).
Table 4. Embryo stage effect in pregnancy rates

<table>
<thead>
<tr>
<th>Embryo Stage</th>
<th>Pregnancy/ET</th>
<th>% Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mórula (M)</td>
<td>16/28</td>
<td>57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Initial Blastocyst (Bi)</td>
<td>32/39</td>
<td>82.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Expanded Blastocyst (Bex)</td>
<td>151/177</td>
<td>85.3</td>
</tr>
<tr>
<td>Highly Expanded Blastocyst (BaEx)</td>
<td>48/53</td>
<td>90.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The pregnancy rate in recipients with initial serum P4 concentration < 4 ng/ml was 80.5% (29/36), 9 recipients had P4 concentration > 4 ng/ml in the second sample collected at the moment of pregnancy diagnosis. A week after ET, 31% (77/247) of pregnant recipients had P4 concentration < 4 ng/ml. Recipients who received mórulas showed a decrease in P4 serum concentration compared with the recipients who received blastocysts (Table 5).

Table 5. Effect of embryonic stage at the time of transfer in the progesterone concentration post transfer.

<table>
<thead>
<tr>
<th>Embryonic Stage</th>
<th>M</th>
<th>Bi</th>
<th>Bex</th>
<th>BaEx</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>16</td>
<td>32</td>
<td>151</td>
<td>48</td>
</tr>
<tr>
<td>Initial P4 (ng/ml)</td>
<td>7.8</td>
<td>6.3</td>
<td>6.8</td>
<td>6.6</td>
</tr>
<tr>
<td>P4 2nd sample (ng/ml)</td>
<td>4.0</td>
<td>4.81</td>
<td>4.92</td>
<td>5.04</td>
</tr>
<tr>
<td>P4 decrease (ng/ml)</td>
<td>3.8 ± 2.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.49 ± 1.78&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.88 ± 2.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.53 ± 2.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% P4 decrease</td>
<td>46.3%</td>
<td>22.6%</td>
<td>22.8%</td>
<td>16%</td>
</tr>
<tr>
<td>P4 Difference 1st and 2nd samples</td>
<td>3.35 ng/ml</td>
<td>2.05 ng/ml</td>
<td>1.65 ng/ml</td>
<td>0.95 ng/ml</td>
</tr>
</tbody>
</table>

The results of this study showed that a low P4 concentration can occur in pregnant recipients possibly because receiver of manipulation during the procedure. In transfers of blastocysts compared to morula there was a decrease in P4 concentration. The morula ET showed a lower pregnancy rate, and P4 serum concentrations were significantly lower. The hypothesis raised was whether the morula has ability to stimulate maternal recognition of pregnancy. Argument would be if the mórula obtained in this study on D7-D8 would not be delayed on development or stagnated and/or do not have the ability to interact in the endometrium.

VIII. USE OF ACYCLIC RECIPIENTS SUPPLEMENTED WITH PROGESTERONE

This procedure would facilitate reproductive management in commercial ET programs particularly in the starting of breeding season where most recipients are still at transition stage. The use of ovariectomized mares supplemented with progestagens has already been reported [10]. In this study the researchers tested 4 treatments: 22 mg altrenogest oral during 5 days; 66 mg altrenogest oral 6 days; 300 mg injectable P4 for 5 days; control group with cyclic mares. The result was 1/6, 2/6, 2/5 and 13/9 respectively. Pessoa et al. [13] tested two treatments with injectable short duration P4 (200 and 400 mg IM every other day and two treatments of long-term P4 (1500 mg IM
every 6 or 7 days). In this study, 264 embryos were evaluated during 3 ET programs in 2001, 2002 and 2003. Subsequently, Rocha Filho et al. [14] assessed in more detail this data. All treatments of progesterone presented in this study were effective for synchronize recipients. Early embryonic loss rates were within the standards considered normal [2] with the exception of long P4 duration treatment (7 days). No difference were seen between the treatment and control group (Table 6). It was concluded that anestrous or transition stage mares, supplemented with P4 by 5 to 8 days before ET can be used as recipient facilitating management particularly in the most critical periods of the year where the availability of cyclical mares decreases.

Table 6. Pregnancy rate and Early Embryonic Loss (EEL) at D12-25 e D25-50 post ET for cycling mares (control) and non cycling mare receiving different Progesterone Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Pregnancy D12</th>
<th>EEL D12-25</th>
<th>EEL 25-50</th>
<th>EEL TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>152</td>
<td>75%</td>
<td>9,6%</td>
<td>7,9%</td>
<td>17,5%</td>
</tr>
<tr>
<td>P4 200 mg/Day</td>
<td>54</td>
<td>75,9%</td>
<td>9,7%</td>
<td>9,8%</td>
<td>19,5%</td>
</tr>
<tr>
<td>P4 400 mg/Day</td>
<td>13</td>
<td>76,9%</td>
<td>10,0%</td>
<td>10,0%</td>
<td>20,0%</td>
</tr>
<tr>
<td>P4 LD 1500 mg/7 Days</td>
<td>30</td>
<td>76,6%</td>
<td>8,6%</td>
<td>21,8%</td>
<td>30,4%</td>
</tr>
<tr>
<td>P4 LD 1500 mg/6 Days</td>
<td>15</td>
<td>73,3%</td>
<td>0</td>
<td>18,2%</td>
<td>18,2%</td>
</tr>
</tbody>
</table>

IX. CONCLUSIONS

The equine ET technique is absolutely dominated and of common usage in all regions of the world particularly in Brazil. The major obstacle is the selection, handling and precise control of recipients. The variability of seasonality at different regions should be taken into consideration and assessed individually by region, management, nutrition, etc. The use of acyclic mares through P4 application is a reality that facilitates the management of recipients and is a viable alternative to increase the recipient options. More studies will be necessary in superovulation and cryopreservation of embryos to transfer them at more suitable time.

REFERENCES


Cryopreservation of equine embryos: ice or glass?

L.F. Campos-Chillon¹ & J.L. Altermatt¹

ABSTRACT

Background: Gamete cryopreservation has permitted widespread preservation of valuable genetics. However, cryopreservation of equine embryos is still not a routine procedure in the industry due to biological reasons [16] and inconsistent results in the field.

Review: The basic principle of cryopreservation is to avoid ice crystal formation. Small (< 250 mm) equine embryos can be successfully frozen by slow-cooling techniques to allow slow dehydration of the embryo, thus avoiding ice crystal formation and subsequent lysis of plasma membranes, cytoskeleton disruption, and organelle dysfunction [10,14-15]. Embryos are dehydrated with glycerol, loaded in 0.25 ml straws and frozen by a programmable freezer with liquid nitrogen. Embryos are thawed in a water bath followed by serial dilutions in glycerol and sucrose [14]. Recently, a variation of the slow freezing technique [1] in field conditions produced 16 day pregnancy rates (70%), not different from controls. Vitrification is an alternative to slow-freezing and is defined as a physical state similar to glass [13]. There is no ice crystal formation or concentration of solutes. The methodology requires a high concentration of cryoprotectants, small volumes, high viscosity and rapid cooling rates. Numerous studies with different formulas and containers have resulted in successful vitrification of small equine embryos [6,8,10-11]. An economical and simple vitrification technique was developed [6] that permits in-straw dilution of embryos for direct transfer in the field. Pregnancy rates on day 16 were 65% and not different from controls. In a subsequent study [9], embryos (n=40) < 250 mm in diameter were obtained from eFSH treated mares, half cooled for 16-20 h and vitrified and the other half vitrified only. The pregnancy rate on day 16 after direct transfer under field conditions was 65 and 70% respectively. However, personal communications from practitioners around the world have found poor pregnancy rates (<30%) with vitrification. Furthermore, a recent study [1] found a significant difference in pregnancy rates of 27 and 70% for vitrification and slow-freezing respectively. Generally, large embryos (>250 mm) have poor survival rate after cryopreservation. One explanation is that the equine embryonic capsule composed of mucin like glycoproteins [12] that replaces the zona pellucida and surrounds the embryo until embryonic attachment (16- 21 d) may impede the proper penetration of cryoprotectants, leading to intracellular ice crystal formation. Another possibility is that the small surface area to volume ratio of embryos with large amounts of fluid in the blastocoele cavity may slow addition and removal of cryoprotectants [2]. A vitrification technique was developed with a modified three-step vitrification procedure for large embryos [3]. However, the pregnancy rate on day 16 (37 %) was unacceptable for commercial use. In a recent and promising study [5] after piercing the embryonic capsule with micromanipulation tools, pregnancy rates were 70% (5/7) at day 16. In vitro produced embryos have been cryopreserved successfully. Pregnancies (~50%) after slow freezing and thawing of equine blastocysts produced after ICSI have been consistently obtained [7]. In addition, pregnancies (65%, 5/8) have been obtained after vitrification in super open pulled straws, warming and transfer of 2- to 8-cell embryos produced by ICSI [4]. However, more studies are needed in this area.

Conclusions: Even though vitrification offers theoretical advantages to minimize cell damage, the learning curve and practicality of the methods need to be standardized for commercial use. Nonetheless, depending on embryological stage, both methods (ice or glass) of cryopreservation can be used successfully to produce pregnancies.

Keywords: cryopreservation, vitrification, equine embryos.

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