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Review Article

Cryopreservation of Equine Embryos

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ABSTRACT

Equine embryo transfers have increased dramatically in the past decade, but in spite of the advantages of cryopreservation of equine embryos, this technology has not increased proportionally. Lack of a superovulation protocol for mares and the inability to freeze embryos >300 μm have been the limiting factors impeding equine embryo cryopreservation. Data from both controlled laboratory settings and commercial embryo transfer facilities have shown that small embryos can be slow cooled or vitrified and, after thawing and transfer, provide pregnancy rates of 50%–70% similar to that obtained with bovine embryos. In contrast, studies have shown that embryos >300 are damaged more during slow cooling or vitrification than those <300 μm and result in low pregnancy rates after transfer. The presence of the acellular capsule in the equine blastocyst and the large volume of blastocoele fluid were thought to be the major reason for poor survival of cryopreserved large equine embryos. However, deflating the embryo before freezing has been shown to improve the survival of cryopreserved large equine embryos dramatically. Unfortunately, this must be done using very expensive equipment. Developments that could improve the success of equine embryo cryopreservation are as follows: having hormones available for superovulation, a means of hastening the embryo into the mare's uterus to consistently collect <300 μm embryos, or the development of a simple means of collapsing embryos >300 μm.

1. Introduction

Equine embryo transfer is a technique that has been used in the equine industry since the late 1970s and early 1980s. Most embryos collected during those decades were transferred as fresh embryos, and the recipients and donors were on the same farm. With the development of techniques for cooled storage of embryos at 5°C (Carnevale et al [1]) for 12 to 24 hours, shipment of embryos to recipient stations became a reality. This stimulated the embryo transfer business and resulted in the establishment of several large embryo transfer programs that receive shipped embryos. In contrast, the concept of cooled, transported embryos has not been embraced by the cattle industry. Generally, when a donor cow is flushed, the embryos are transferred into the available recipients and any extra embryos are frozen.

Equine embryo transfer numbers have increased dramatically in the last couple of decades [2], generally driven by the change in breed regulations by the major breeds in the United States as well as the polo industry in Argentina and the sport horse industries in Brazil. Many breeds now allow unlimited registration of embryo transfer foals. However, the number of embryos frozen has not increased proportionately. A significant difference between embryo transfer in cattle and horses is that a very predictable superovulation regime is available for cattle and typically six transferable embryos are available from each flush. In contrast, superovulation is not currently available in horses, and consequently, embryo recovery is based on only one ovulation and generally ranges from 50% to 70% embryo recovery per cycle. As a consequence, implementation of

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cryopreservation procedures in clinical practice has been limited by the number of embryos available and, to a lesser extent, relatively low demand by the equine breeding. Despite the relative limited use of cryopreservation in the horse, there are some distinct advantages:

1. Minimize the number of recipients and thus decrease the cost of embryo transfer;
2. Ability to bank embryos, especially from young mares while their performance and the genetic value is being determined;
3. Exportation or importation of embryos;
4. Transport of frozen embryos may have less health risks then importing live animals;
5. Collection and cryopreservation of embryos in the off season so they can be transferred early the following breeding season;
6. Cryopreservation of an embryo while genetic testing or sexing is being conducted;
7. In vitro produced (IVP) embryos can be taken out of culture as a morulae or early blastocyst and cryopreserved successfully.

However, there also are some disadvantages of frozen embryos over fresh or cooled embryos. Although pregnancy rates with small (<300 μm) embryos can be 50%–65%, these rates are still lower than those for fresh or cooled embryos [3]. In addition, because the embryo develops and increases in diameter rapidly once it reaches the uterus, it is difficult to recover small embryos unless the time of ovulation is accurately determined by frequent examination of the mare. Embryos >300 μm have very poor survival after cryopreservation, providing a 20%–30% pregnancy rate [4]. As mentioned previously, without the hormones available for superovulating the mare, there are very few extra embryos for freezing. In addition, just recently, the American Quarter Horse Association changed their rule stating that frozen embryos can only be used for transfer for 2 years after the death of the mare. This more than likely will discourage the idea of embryo banking.

2. Embryo Size

Size of the embryo is the major factor affecting survival after cryopreservation. When the embryo enters the uterus from the oviduct, it is 150 to 220 μm in size and has the morphology of a morula or early blastocyst. Within 0.5 to 1.0 days, the embryo will increase in diameter to >300 μm and become a blastocyst [5]. Morula and early blastocyst are surrounded by a thick zona pellucida. However, by day 7 postovulation, the zona pellucida has thinned out and the underlying acellular glycoprotein capsule has formed [6]. There are several reasons why embryos >300 μm do not survive freezing and thawing:

1. The capsule impedes the penetration of the cryoprotectant;
2. Thickness of the capsule has been shown to be correlated to freezability of the embryo [7];
3. Small surface-area-volume ratio;
4. The large amount of blastocoele fluid.

A discussion will follow later as to how researchers have tried to modify the large embryo to allow better survival after cryopreservation.

Needless to say, most of the equine embryos that are currently frozen are small, morulae, or early blastocysts obtained by performing embryo recovery 6.0–6.5 days after ovulation. This requires that the mare be examined every 4–6 hours after hCG or GnRH agonist administration until ovulation is detected or rely on a timed-ovulation protocol and flush 8 days after injection of hCG as demonstrated by Eldridge-Panuska et al [8].

3. Slow Cooling

The first equine embryos were frozen by a slow cool method. A pregnancy was produced but subsequently lost [9]. Yanamoto et al [10] reported the birth of the first frozen/thawed embryo foal. Slade et al [11] in our laboratory in 1985 used essentially a bovine protocol to slow cool equine embryos. Glycerol was used as the cryoprotectant and embryos packaged in either ampules or 0.5-mL plastic straws. Embryos were cooled at 4°C/min from room temperature to −6°C, seeded at −6°C, and held for 15 minutes then cooled at 0.3°C/min to −30°C and 0.1°C/min to −33°C then plunged into nitrogen. Six of 23 embryos were unsuitable for transfer after thawing, and the remaining 17 were transferred. Eight of 10, with a mean diameter of 173 μm, resulted in a pregnancy, whereas only one of seven classified as blastocysts resulted in a pregnancy.

Lascombes and Pashen [12] as part of a commercial program used a similar slow cooling procedure with glycerol added in two steps. A 56% pregnancy rate was reported after transferring 43, day 6 to 6.5 embryos (<220 μm). Combined these studies demonstrate that pregnancy rates following transfer of small equine embryos using a slow cooling method provide an pregnancy rate similar to what is obtained with frozen bovine embryos. However, the problem with slow cooling small equine embryos is that the appropriate size is difficult to acquire, slow cooling method takes 1–2 hours, and an expensive programmable freezer is needed. This more than likely prompted the search for alternative methods such as vitrification which is quicker and requires no expensive equipment.

4. Vitrification

The alternative to slow cooling of embryos that has gained in popularity is a process called vitrification. This is an ultrarapid cooling method that prevents ice crystals by cooling so fast that the liquid changes to a solid, glass-like phase without ice formation [2]. This has the advantage of being fast and easy but does expose the embryo to high concentrations of cryoprotectants. Thus, the type of cryoprotectants and the timing of exposure to these agents become very important. The effect of size of the equine embryo on survival may be even more critical with vitrification than with slow cool.

Hochi et al [13] reported on the viability following vitrification of equine embryos of various sizes: <200, 200–300, and >300 μm. Reexpansion of the small embryos after freezing and thawing was quite good for <200 (7/8) and