Cryopreservation of in vitro-produced embryos: challenges for commercial implementation

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Abstract

In the last several years, the high demand for embryo production has resulted in the need to study new methods to make the cryopreservation of bovine embryos produced in vitro more efficient. Despite the advantages offered by in vitro embryo production (IVEP), the major challenge to its greater dissemination is to improve embryonic survival after cryopreservation. Embryos that are produced in vitro are less resistant to cryopreservation compared to those produced in vivo, which is due to the higher accumulation of lipids in their cells, among other factors. In this context, changes in the culture conditions such as the addition of lipolytic chemical substances and the adjustment of fetal calf serum in the medium have been proposed to decrease the lipid amount within the embryos. Several years ago, vitrification allowed good results for in vitro produced (IVP) embryos because of its simplicity, speed and low cost. More recently, another technique applied to simplify the embryo post-thawing rehydration step in vivo, direct transfer (DT), is a strategy that has proven to be of interest in helping to overcome limitations to the cryopreservation of in vitro produced embryos. DT has been performed by commercial laboratories, ensuring good embryo viability after thawing. However, commercial and operational limitations prevent the large-scale use of these techniques. Thus, this review aims to discuss the use of strategies to improve the post-cryopreservation survival capacity and the aspects to be overcome so that the cryopreservation of IVP embryos becomes a well-established and commercially applicable technique in addition to presenting new guidelines for embryo transfer (ET) programs from a better selection of recipients.

Keywords: bovine, commercial limitation, cryopreservation, in vitro-produced embryos, recipient cow.

Introduction

During the year 2015, almost 700,000 IVP embryos were produced, surpassing for the first time the number of bovine embryos produced in vivo. In this context, 269,353 bovine OPU IVP embryos were transferred in Brazil alone (Perry, 2016), which is considered the world’s largest producer of bovine embryos. This situation is directly related to the predominance of Bos indicus cattle. Several studies have reported that Zebu females, when submitted to ovum pick-up (OPU) guided by transvaginal ultrasonography, had a higher number of oocytes aspirated than Bos taurus females (Segerson et al., 1984; Silva-Santos et al., 2011). This feature favors large-scale in vitro embryo production (IVEP) in both dairy and beef cattle (Pontes et al., 2011).

Furthermore, IVEP has advantageous conditions for its application in Bos indicus dairy cattle, since these animals, in addition to being good donors of oocytes, are adapted to a tropical climate and can produce milk even under high-temperature conditions (Marinho et al., 2015). Another advantage is the fact that embryos are more resistant than gametes when subjected to high body temperatures due to thermal stress (Chebel et al., 2008). Thus, the pregnancy rates are better in embryo transfer (ET) than artificial insemination (AI) throughout the year (Stewart et al., 2011; Ferreira, 2013).

Additionally, in the last decade, there has been a significant increase in the production of sexed embryos, especially due to the search for genetic improvement of dairy cattle (Pontes et al., 2010). Another advantage of IVEP compared with in vivo methods is the smaller number of viable sperm required for fertilization and, therefore, more efficient results in the use of sex-sorted semen (Pontes et al., 2010; Morotti et al., 2014).

In this context, the total embryo production is sometimes higher than the number of embryos transferred, so investment in research was increased to develop an efficient protocol for the cryopreservation of the remaining embryos in a program (Sanches et al., 2016). Despite the advantages provided by IVEP, the greatest challenge of this biotechnology is the lower resistance to the cryopreservation process that these embryos present (Sudano et al., 2011).

The high sensitivity to cooling of in vitro embryos is reported to be due to the greater accumulation of lipids in their cells (Abe et al., 2002), arranged in the form of cytoplasmic lipid droplets that are constituted predominantly of triglycerides (McKeegan and Sturmy, 2012). Additionally, there are indications that this high lipid content is because of the medium in which the embryos are cultured (Abe et al., 2002; Sanches et al., 2013). Thus, some strategies for improving post-cryopreservation survival capacity have been studied and tested to produce more cryotolerant embryos (Sudano et al., 2013).

Among cryopreservation techniques, vitrification...
has been used worldwide (Dode et al., 2013) because of its simplicity, speed, and low cost. However, this technique requires a high concentration of cryoprotectants in addition to a trained person to perform a morphological evaluation of embryo quality before the loading process (Vajta et al., 1998).

In contrast, Sanches et al. (2016) demonstrated that a technique used since the 1990s to simplify the post-thawing rehydration step of in vivo embryos — direct transfer (DT) — can also be used for frozen in vitro embryos. The DT strategy has been demonstrated to be helpful for overcoming limitations to in vitro embryo cryopreservation, since it has been recently performed by commercial laboratories, providing good embryo viability after thawing.

The choice of embryo recipients is another important step in the implementation of IVEP programs (Peixoto et al., 2004). Age, sanitary and nutritional conditions, such as synchrony between the recipients and embryonic stage, are important attributes to take into account in the choice of embryo recipients (Hasler et al., 1987; Seenan and Diskin, 1987; Callesen et al., 1996; Jones and Lamb, 2008). Moreover, recent strategies for the synchronization of estrus/ovulation and the selection of recipients by fertility have been achieved (Marinho et al., 2012).

In contrast, there are still many commercial and operational limitations of bovine IVEP embryos and cryopreservation processes, which prevent its use on a large scale. Examples include the need for a qualified person to perform all stages of IVEP and the cryopreservation process, logistics between laboratory and recipients, as well as a trained technician in the field due to the particularities of warming those cryopreserved embryos before transfer (Hasler, 2010; Saragusty and Arav, 2011).

Therefore, considering the importance of implementing an efficient IVEP and cryopreservation program, this review aims to discuss i) the use of strategies to improve embryo post-cryopreservation capacity; ii) the choice of recipients with good sanitary/nutritional conditions and reproductive characteristics to maintain a healthy pregnancy; and finally, iii) a team able to perform all stages of IVEP with rigorous quality control and the logistics necessary for making ET feasible in the field.

**Cryopreservation of bovine embryos**

Methods and differences of cryotolerance in embryos produced in vivo and in vitro

The process of embryo cryopreservation is the most challenging aspect of embryo biotechnology, and despite advances in recent years, the results are still inconsistent (Sudano et al., 2013). During embryo freezing, the cryopreservation method aims to avoid the formation of intracellular ice crystals and to decrease the toxic effects generated by the cryoprotectant agent, minimizing the osmotic stress to the cells (Pryor et al., 2009).

Cryopreservation protocols are based on two variables: type and concentration of cryoprotectant and cooling rates (Vajta and Kuwayama, 2006). Currently, slow freezing (classic) and vitrification (ultra-rapid) are the two main methods used commercially for IVEP embryo cryopreservation (Saragusty and Arav, 2011).

Vitrification is the predominant technique used for IVEP (Dode et al., 2013) due to being a simple, fast and low-cost method (Sanches et al., 2016). In this method, a high-osmolarity solution is used so that the embryonic intracellular water exits rapidly, dehydrating the embryonic cells and making them permeable to the cryoprotectant. Thus, the embryo is able to withstand direct immersion in liquid nitrogen (-196°C) without the formation of ice crystals (Vajta et al., 1998).

On the other hand, high cryoprotectant concentrations have been described as promoting high cellular toxicity, even if exposed for a short period and a minimum volume of this solution (Vajta et al., 1998). Thus, different strategies have been developed for embryos to have rapid contact with liquid nitrogen and to reduce the volume of the cryoprotectant agent, such as the open pulled straw (OPS; Vajta et al., 1998), cryoloop (Lane et al., 1999), microdroplets (Papis et al., 2000) and cryotop techniques (Kuwayama et al., 2005).

In the classical slow-freezing protocol, the cooling rate is controlled to maintain a constant curve until the straws with embryos are immersed in the liquid nitrogen. The use of low concentrations of cryoprotectants is the main advantage of this technique since high concentrations are toxic to embryos. In addition, the process of thawing and the DT of embryos to cows make the slow freezing protocol more efficient for commercial use.

However, ice crystals can form and damage the structure of the embryo’s membranes and organelles (Dode et al., 2013). In this way, the success of slow freezing and direct transfer of in vitro produced embryos invariably depends on the equilibrium between the rate of dehydration of the cell and the rate at which water is transformed into ice crystals (Visintin et al., 2002).

Despite the advances in cryopreservation methods, freezing and thawing processes impair the viability of the embryo. This impairment occurs due to the physical and chemical damages induced during the cryopreservation process (Overstrom, 1996; Baguisi et al., 2000). Sudano et al. (2012a) reported the effects of this damage by comparing the apoptosis rate caused by the stress of cryopreservation between fresh and vitrified blastocysts. In this study, there was a 2.4-fold increase ($P < 0.0001$) in the apoptosis rate of vitrified (49.4 ± 1.9) in relation to fresh embryos (20.8 ± 1.1). Similar apoptosis profiles were observed in other studies, which demonstrated increases of 3.7-fold (Park et al., 2006) and 1.7-fold (Márquez-Alvarado et al., 2004) in the apoptosis rate of cryopreserved embryos compared with fresh embryos.

Moreover, it has been definitively demonstrated that in vitro embryos are more sensitive to cryopreservation than in vivo embryos (Pollard and Leibo, 1994). This lower cryotolerance has been associated with the high lipid content present in the
cytoplasm of these embryos (Abe et al., 2002; Mucci et al., 2006) and the decrease in the density of mature mitochondria compared to embryos produced in vivo (Crosier et al., 2001; Farin et al., 2004). Additionally, the most abundant lipids in the plasma membranes of cells (phosphatidylcholine and sphingomyelin) also have different profiles (Sudano et al., 2012b).

Researchers suggest that lipid accumulation may be due to the uptake of the culture medium itself or to the inefficient and unregulated metabolism of the embryonic mitochondria (Farin et al., 2004; Barceló-Fimbres and Seidel, 2007a; Moore et al., 2007). Further, in vitro embryos have fewer transcripts levels for genes related to lipid metabolism compared to in vivo-produced embryos (Gad et al., 2012). Therefore, the addition of substances to the culture medium has been proposed in addition to adjusting the cryopreservation method to make the embryos more cryotolerant (Dode et al., 2013).

**Strategies to increase the cryotolerance of in vitro embryos**

Despite many advances in the last decades, the cryopreservation process of IVEP remains a major challenge in livestock, and the results are still inconsistent (Sudano et al., 2013). For example, the low cryotolerance of in vitro embryos is the main obstacle to the use of cryopreservation protocols (Sudano et al., 2011). The role of embryonic lipids in this regard is well described in the literature (Abe et al., 2002). Furthermore, strategies such as the use of serum-free culture media, the addition of chemical substances to promote changes in lipid metabolism, and the modulation of the membrane lipid composition can help improve the survival of in vitro embryos after cryopreservation (Sudano et al., 2013).

The cause of cytoplasmatic lipid deposition in in vitro embryos is not well established, but it has been suggested that the presence of serum in the culture medium may be directly involved in this process (Sanches et al., 2013). Studies have shown that the fetal calf serum (FCS) concentration affects the number of cryoplastic lipid droplets of embryos (Leroy et al., 2005; Sudano et al., 2012a). Moreover, in vitro embryos cultured in a serum-free medium had decreased lipids and higher cryotolerance (Pereira and Marques, 2008).

An alternative to improving embryo freezeability is the use of lipolytic chemical agents, such as phenazine ethosulfate (PES), which reduces lipid accumulation and regulates energetic metabolism by NADPH to NADP oxidation (De La Torre-Sanchez et al., 2006; Sudano et al., 2011). Interestingly, it has been reported that PES, when used in the post-compaction period, promoted an increase in post-cryopreservation survival (Barceló-Fimbres and Seidel, 2007b).

In this sense, a study involving supplementation with FCS and PES showed an improvement in the blastocoele re-expansion rate after the embryo vitrification process when the serum concentration was reduced to 2.5% concomitant to the addition of PES to the culture medium on day 4 (Table 1; Sudano et al., 2011).

**Table 1. Effects of fetal calf serum (FCS) and phenazine ethosulfate (PES) on blastocoele re-expansion (means ± SEM).**

<table>
<thead>
<tr>
<th>Responses</th>
<th>Vitrified embryos (n)</th>
<th>Cryotolerance</th>
<th>Re-expansion rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>233</td>
<td></td>
<td>90.5 ± 2.7*A</td>
</tr>
<tr>
<td>2.5%</td>
<td>346</td>
<td></td>
<td>81.6 ± 2.5*B</td>
</tr>
<tr>
<td>5%</td>
<td>332</td>
<td></td>
<td>78.0 ± 2.8*Bc</td>
</tr>
<tr>
<td>10%</td>
<td>405</td>
<td></td>
<td>67.3 ± 3.5*C</td>
</tr>
<tr>
<td><strong>In vivo control</strong></td>
<td>15</td>
<td></td>
<td>93.3 ± 6.7*A</td>
</tr>
<tr>
<td>PES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>474</td>
<td></td>
<td>72.0 ± 3.0*B</td>
</tr>
<tr>
<td>PES day 2.5</td>
<td>362</td>
<td></td>
<td>79.9 ± 2.8*C</td>
</tr>
<tr>
<td>PES day 4.0</td>
<td>480</td>
<td></td>
<td>86.2 ± 2.4A*C</td>
</tr>
</tbody>
</table>

*a-b* Within a column, means without a common superscript differ (P < 0.05). *a-b* Within a column, means without a common superscript differ (P < 0.05). Adapted from Sudano et al. (2011).

It is important to note that the addition of medium with 2.5% FCS did not decrease the embryonic cryotolerance (represented by the blastocoele re-expansion rate) compared to the group without FCS. However, independent of FCS concentration in the medium and the use of PES, the embryos in the in vivo group (control) had the highest survival after vitrification (Sudano et al., 2011).

Forskolin is another lipolytic chemical agent used to reduce the lipid content of in vitro embryos. This agent acts directly by activation of the adenylate cyclase, thus increasing the levels of cAMP and stimulating lipolysis to activate lipases (Men et al., 2006). Recently, Paschoal et al. (2017) demonstrated that forskolin was an effective lipolytic agent even at low concentrations, resulting in the formation of blastocysts with a larger number of cells than the untreated group. Additionally, this substance decreased embryo apoptosis caused by the cryopreservation method.

Therefore, it has been previously reported that treatment with forskolin before vitrification with the...
cryotop method (a polypropylene rod in which the embryos are allocated next to minimum volumes of cryoprotectant solution) improved the cryotolerance and pregnancy rates of Bos indicus in vitro embryos after transfer to recipients (Sanches et al., 2013). The results are shown in Table 2.

Table 2. Pregnancy rates of Bos indicus in vitro embryos treated with or without the lipolytic agent forskolin for 48 hours in culture before the vitrification process.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Transferred embryos (n)</th>
<th>Pregnancy rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>65</td>
<td>18.5 b</td>
</tr>
<tr>
<td>Forskolin</td>
<td>80</td>
<td>48.8 a</td>
</tr>
</tbody>
</table>

**Within a column, rates without a common superscript differed (P < 0.05). Adapted from Sanches et al. (2013).**

According to these results, the use of forskolin and vitrification with the cryotop system as a strategic cryopreservation system could be a great alternative to facilitate the transport and export of embryos over long distances (Sanches et al., 2013).

In addition, the stage of development of blastocysts at the time they undergo cryopreservation is another factor that needs to be considered as a strategy to improve cryotolerance. For example, Kocyigit and Cevik (2016), showed a correlation between the diameter of embryos and their cryosurvival, in which early and expanded blastocysts were more sensitive to the damage promoted by vitrification and posterior warming compared to the blastocyst stage. In our experience, the ideal developmental stages are blastocyst and expanded blastocyst for both vitrification and direct transfer methods.

The slow freezing of embryos for later DT, despite having higher costs, eliminates the evaluation before transfer, which makes it more practical than vitrification. Moreover, smaller concentrations of cryoprotectants may also be used, thereby reducing toxicity to the embryos (Voelkel and Hu, 1992).

Briefly, in the DT method, the in vitro embryos are cryopreserved by the slow freezing method previously described for in vivo embryos (Vajta et al., 1998). The in vitro embryos are next exposed to a freezing solution consisting of 1.5 M ethylene glycol (EG), and at the end of the freezing curve, they are directly immersed in liquid nitrogen and stored until being transferred into the recipients.

Surprisingly, this strategy has been demonstrated to help overcome obstacles to in vitro embryo cryopreservation. On Table 3 pregnancy rates for fresh, vitrified, and frozen (direct transfer) in vitro embryos from dairy cows are presented.

Table 3. Pregnancy rates at 30 days after the transfer of fresh, vitrified or frozen (direct transfer) in vitro-produced embryos after ovum pick-up of Girolando cows.

<table>
<thead>
<tr>
<th>Group</th>
<th>Transferred embryos (n)</th>
<th>Pregnancy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>259</td>
<td>43.24 ± 1.23 a</td>
</tr>
<tr>
<td>Vitrified</td>
<td>234</td>
<td>31.19 ± 4.01 b</td>
</tr>
<tr>
<td>Frozen</td>
<td>311</td>
<td>34.72 ± 4.15 b</td>
</tr>
</tbody>
</table>

**Different letters in the same column indicate a significant difference (P < 0.05). Adapted from Sanches et al. (2016).**

The results of this study revealed the possibility of using frozen embryos because the direct transfer optimized the logistics and can become a more practical approach for the transfer of cryopreserved in vitro embryos in the field (Sanches et al., 2016).

The direct transfer protocol has been used in large-scale operations, especially in the US and Brazil. In the near future, once other companies incorporate the direct transfer protocol in their operation, the majority of commercial IVEP embryos will probably be frozen, as currently occurs in the semen industry.

Despite the advances in cryopreservation methods, few players are using this technique, and some challenges remain in relation to the greater efficiency of the technique.

**Importance of recipient cow selection**

The choice of recipients is an important part of the success of bovine ET programs, since many of problems with this biotechnology application are related to the female conditions that will allow embryo implantation and maintenance of gestation until the fetus is born (Andrade et al., 2012).

Among the factors that directly interfere with the performance of fresh or cryopreserved transferred embryos, major highlighted aspects are the recipient’s age, the sanitary and nutritional conditions of the recipients, and the degree of synchrony between the embryo stage and its recipient (Sreenan and Diskin, 1987; Hasler et al., 1987; Callesen et al., 1996; Peixoto et al., 2004, Jones and Lamb, 2008).

An interesting study evaluated the effects of synchrony between embryo stage and recipient on conceptus elongation and pregnancy rate. In this study, the authors showed that conceptus length was greater following transfer to an advanced uterus and that supplementation with progesterone resulted in short cycles in approximately 50% of recipients. Transfer of day 7 embryos to a synchronous uterus (day 7) resulted in a pregnancy rate of 47.3%. Transfer to an asynchronous uterus of day 5 (40.8%) or day 8
embryos (41.3%) moderately impacted the pregnancy rate (P < 0.01), but transfer to the uterus 2 days in advance (day 9, 24.4%) or 3 days behind (day 4, 27.0%) reduced (P < 0.001) the pregnancy rate compared with synchronous transfer (Randi et al., 2015). Interestingly, this study emphasized the importance of greater possible synchrony between the embryonic stage and the cycle day of the recipient.

Additionally, new technologies have been developed with the aim of helping the selection of recipients be more accurate by searching for genetic markers related to desirable characteristics. The sequencing of the bovine genome allowed genome-wide association studies (GWAS) to be conducted, which examine specific sites, such as single-nucleotide polymorphisms (SNPs), and associate them with certain phenotypes (Dairy Herd Management, 2017).

Recently, it has been possible to use commercial programs aimed at the identification of genomic loci associated with fertility in heifers and dairy cows. Some specific genes associated with fetal abnormalities that lead to abortion, embryonic death, or lower fertility, as well as genes associated with better reproductive efficiency, have been identified.

Therefore, this information associated with other methods and criteria for choosing the recipient can help the optimization and practical success of ET and consequently can improve the efficiency of IVEP in the field.

Commercial and operational limitations to cryopreservation of in vitro-produced embryos

Cryopreservation of bovine embryos is a biotechnology that allows the storage of surplus embryos produced in vitro or through superovulation/embryo transfer programs, making feasible commercialization between countries and the transfer of embryos at a more convenient time (Sudano et al., 2012b).

However, the number of embryos cryopreserved in the past several years represented only 3 to 7% of the total embryo production in Brazil (Stroud, 2011, 2012; Viana, 2012). These data reflect the great challenges to consider is the atmosphere (lower or higher oxygen tension) in which the embryos are grown, which has been widely used to minimize oxidative stress; low oxygen tension improves metabolism and decreases the production of free radicals (Dode et al., 2013).

Finally, the qualification of the field veterinarian/technical responsible for performing the embryo transfer into the recipient uterus is another factor limiting the use of cryopreserved embryos in ET programs. In general, the professional must perform the process in a careful, rapid and accurate manner.

In our experience, this job position (embryo transfer) will be the next limitation to using IVEP cryopreservation on a large scale and globally. Once the technology is proven and well accepted, there will not be a sufficient number of field technicians able to perform embryo transfers.

Therefore, these factors, when considered together, will directly reflect pregnancy rates and may have positive impacts on the large-scale application of IVEP and embryo cryopreservation in cattle.

Final comments

In the last decade, several technical advances have increased the efficiency of IVEP, making this reproductive strategy to have a greater impact on selection and genetic dissemination in cattle. On the other hand, the need for an efficient method to cryopreserve the surplus volume from embryo programs was generated. For in vitro embryos, vitrification has become the most frequently used technique for cryopreservation worldwide, which has contributed widely to the storage of embryos, as well as making IVEP programs more efficient.

Therefore, the implementation of a commercial program for IVEP and cryopreservation needs to overcome many challenges when using a vitrification protocol. There is no question that we must develop and improve the efficiency of direct transfer techniques to make the IVEP technology accessible to everyone everywhere. Along with the selection of the recipient according to good sanitary and nutritional status, adequate synchrony between embryo stage and recipient cycle, high maternal ability, and the choice of females with characteristics linked to fertility are aspects fundamental to the success of this biotechnology. Finally, the entire in vitro process for production or cryopreservation requires a highly qualified and trained team to perform each step of this journey.

References


Baguïs A, Lonergan P, Overstrom E, Boland M, Santos RM. 2013. In vitro embryo production in Brazil (Stroud, 2011, 2012; Viana, 2012). These data reflect the great challenges to consider is the atmosphere (lower or higher oxygen tension) in which the embryos are grown, which has been widely used to minimize oxidative stress; low oxygen tension improves metabolism and decreases the production of free radicals (Dode et al., 2013).


