Anim. Reprod., v.9, n.2, p.86-92, Apr./Jun. 2012

# Developmental rates of *in vivo* and *in vitro* produced bovine embryos cryopreserved in ethylene glycol based solutions by slow freezing or solid surface vitrification

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## Abstract

The aim of this study was to compare *in vitro* survival rates of *in vivo* and *in vitro*-produced bovine embryos by slow freezing or solid surface vitrification. *In vivo*-produced blastocysts (n = 210) and *in vitro*-produced blastocysts (n = 445) were randomly allocated in two cryopreservation groups. Group 1 - embryos were exposed to 1.5 M ethylene glycol (EG) for 5 min, loaded in 0.5 ml straws, frozen at -6.5ºC and seeded. After 10 min of equilibration, straws were cooled at -0.6ºC/min until -35ºC, and then plunged into liquid nitrogen (-196ºC). Group 2 - embryos were exposed to a 15% EG + 0.25 M trehalose solution for 1 min and then a 30% EG + 1 M trehalose solution for 30 sec to be vitrified using the Cryologic Vitrification Method (CVM®). After at least one week of storage, embryos in the slow freezing group were thawed in a water bath at 30°C for 12 sec and then placed in holding medium for 5 min and transferred into SOF culture media. Vitrified embryos were placed directly into a 0.25 M sucrose solution for 5 min then cultured in SOF medium. Re-expansion and hatching rates were evaluated at 24 and 72 h, respectively. *In vivo*-produced embryos had higher (P < 0.01) re-expansion (179/210, 81% *vs*. 244/445, 54%) and hatching rates (159/210, 72% *vs*. 177/445, 39%) than *in vitro*-produced embryos, regardless of the cryopreservation method. However, re-expansion and hatching rates were higher (P < 0.01) for *in vitro*-produced vitrified embryos (155/223, 69% and 132/223, 59%) than *in vitro*-produced embryos cryopreserved by slow freezing (89/222, 40% and 45/222, 20%). Although similar re-expansion rates were obtained with *in vivo*-produced embryos cryopreserved by the two systems, hatching rates tended to be lower (P = 0.09) with *in vivo*-produced embryos that were vitrified as compared to slow freezing. In conclusion, solid surface vitrification improved the cryosurvival rates of *in vitro*-produced embryos compared to the conventional slow freezing procedure.

**Keywords:** blastocyst, cryopreservation, expansion, hatching.

## Introduction

Embryo cryopreservation has been a useful tool for the different assisted reproductive technologies (i.e., embryo transfer, cloning, nuclear transfer, *in vitro* embryo production, etc.), allowing for the wide spreading of genetic resources (Pereira and Marques, 2008). A successful embryo cryopreservation procedure must avoid intracellular ice crystal formation with minimal toxic and osmotic stress to cells (Campos-Chillon *et al*., 2006). The two principal techniques that currently rule bovine embryo cryopreservation are slow freezing (Whittingham *et al*., 1972; Wilmut, 1972) and vitrification (Rall and Fahy, 1985). The slow programmable freezing with ethylene glycol (EG) as a cryoprotectant is the most commonly used method for cryopreservation of *in vivo* produced bovine embryos (Vajta, 2000), allowing direct transfer of frozen-thawed embryos into the uterus (Voelkel and Hu, 1992) and consequently, multiple births around the world (Palasz and Mapletoft, 1996).

Although commercial production of *in vitro* bovine embryos has increased significantly in the last 10 yr (Stroud, 2010), most embryos are still transferred fresh, and require large numbers of recipients available at any given time. Therefore, it is essential to develop a method of cryopreservation that allows for the dilution of the cryoprotectants within the straw after thawing and direct transfer into the uterus. However, *in vitro*-produced embryos are less cryotolerant than *in vivo* embryos due to the commercial methods currently used to cryopreserve *in vivo* produced embryos (Kaidi *et al*., 2001; Assumpção *et al*., 2008; Dinnyes and Nedambale, 2009; Nicacio *et al*., 2011). This decreased cryotolerance is probably due to differences in the biochemical composition of *in vitro* produced embryos, especially the amount of lipids within the cytoplasm, and an increased susceptibility to low temperatures or an increase in cellular damage occurring during the procedure (Kaidi *et al*., 2001; Seidel, 2006; Barcelo-Fimbres and Seidel, 2011). For this reason, vitrification became an alternative for *in vitro* embryo cryopreservation because it avoids exposure of the embryos to chilling temperatures for a prolonged period of time and the formation of intracellular ice crystals, and reduces cell damage (Vajta *et al*., 1998; Vajta and Nagy, 2006; Pereira and Marques, 2008; Saragusty and Arav, 2011). For this reason, many devices and methods have been introduced for the commercial vitrification of bovine

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Received: April 2, 2012
Accepted: June 26, 2012

embryos in recent years. Most of the devices reduced the volume of samples in order to increase heat transfer and thus promote higher cooling rates. Some of these systems are Minimum drop size (MDS; Arav, 1992), Electron microscope grids (EM; Martino *et al*., 1996), Open-pulled straw (OPS; Vajta *et al*., 1998), Cryoloop (Lane *et al*., 1999), Hemi-straw (Vanderzwalden *et al.,* 2000), Gel-loading tips (Tominaga and Hamada, 2001), Closed-pulled straw (CPS; Chen *et al*., 2001), Nylon mesh (Matsumoto *et al*., 2001), Flexipet denuding pipette (FDP; Liebermann *et al*., 2002), Superfinely open-pulled straw (SOPS; Isachenko *et al*., 2003), Cryoleaf (Chian *et al*., 2005), Cryotip (Kuwayama *et al.,* 2005), Cryotop (Kuwayama *et al*., 2005), sealed pulled straw (Yavin *et al*., 2009), plastic blade (Sugiyama *et al*., 2010) and cryopette (Portmann *et al*., 2010). Solid surface vitrification is another vitrification technique that has been successfully applied to preserve bovine oocytes (Dinnyes *et al*., 2000; Sripunya *et al*., 2009) and embryos (Lindemans *et al*., 2004; Fry *et al*., 2005; Peachey *et al*., 2005). The Cryologic Vitrification Method (CVM®, Cryologic, Australia) uses this technique to vitrify on a metal surface which is precooled to -196°C by partial immersion into liquid nitrogen ($LN_2$), avoiding the generation of the gas phase of $LN_2$ and serving as a template to cool microdrops of vitrification solution containing embryos or oocytes (Fry *et al*., 2005). Furthermore, it is one of the vitrification procedures that not only prevents direct contact with $LN_2$, but also avoids risk of contamination during storage because it uses a closed container system (Lindemans *et al*., 2004; Beebe *et al*., 2011). The aim of this study was to compare *in vitro* survival rates of *in vivo* and *in vitro*-produced bovine embryos by two commercially available methods of cryopreservation: slow freezing and the solid surface vitrification system.

## Materials and Methods

### *Chemicals*

Unless specified, all chemical reagents used for this experiment were purchased from Sigma Chemical Company (St. Louis, MO, USA), except for fetal calf serum (FCS), which was obtained from Natocor (Carlos Paz, Argentina).

### *In vitro embryo production*

Bovine ovaries were obtained from a slaughterhouse and kept in PBS at 37°C during transport to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2-8 mm in diameter. COCs were washed in Tissue Culture Medium 199 (TCM-199) and transferred to 4-well plates containing 500 µl of maturation medium per well (30-50 COC per well) and matured for approximately 24 h at 38.8°C in an atmosphere of saturated humidity and 5% $CO_2$. The maturation medium consisted of TCM-199 supplemented with 10% (v/v) fetal bovine serum, 0.2 mM sodium pyruvate, 0.05 µg/ml porcine FSH (Folltropin-V; Bioniche Animal Health Inc., Pullman, WA, USA) and antibiotics. After *in vitro* maturation, COCs were washed and transferred into Fert-TALP fertilization medium (500 µl) in a 4-well dish. The fertilization medium consisted of modified Fert-TALP with 10 µg/ml heparin, 0.5 µg/ml hypotaurine and 0.5 µg/ml epinephrine. Frozen semen was used from one Angus bull. The semen was thawed at 37ºC for 60 sec and selected through a 90-45% Percoll (P-1644) gradient system in a 15-ml conical tube and centrifuged at 700 g for 15 min. The pellet was resuspended in the same amount of Fert-TALP medium and centrifuged for 5 min at 700 g. Afterwards, the pellets were resuspended in Fert-TALP and a final concentration of 1 x $10^6$/ml sperm was added to each fertilization drop. After 20 h of IVF, COCs were vortexed to remove the cumulus cells and excess sperm, washed once in Synthetic oviductal fluid culture medium (SOF) and transferred into culture medium drops (500 µl) under mineral oil under a controlled atmosphere (5% CO2, 5% O2 and 90% $N_2$) at 38.8°C. Cleavage rates were observed from day 2 and embryo development rates were observed from day 7 of the culture period. All blastocyst stage embryos were used for cryopreservation.

### *In vivo embryo production*

*In vivo* embryos were collected from superstimulated Angus cows according to the procedure described by Bó *et al*. (2008). Donor cows were artificially inseminated 12 and 24 h after pLH (Lutropin-V, Bioniche Animal Health, Canada) administration. The frozen/thawed semen used was from the same Angus bull used for *in vitro* embryo production. Embryos were collected on day 7 after pLH administration by flushing the uterine horns with phosphate-buffered saline (PICTOR-PBS, Biogen Argentina SA, Argentina). Total ova/embryos, fertilized ova and Grades 1 (Excellent or Good), 2 (Fair) and 3 (Poor) embryos were classified according to the International Embryo Transfer Society (Robertson and Nelson, 2011). Grade 1 morula and blastocysts were considered suitable for cryopreservation and consequently transfer into Holding media (Vigro Plus®, Bioniche Animal Health, Pullman, WA, USA) to be randomly distributed into the different cryopreservation groups.

### *Vitrification procedure*

Embryos produced *in vivo* or *in vitro* were vitrified with the solid surface procedure as described by Dinnyes *et al*. (2000), with modifications using a commercial vitrification system (CVM®, Cryologic, Victoria, Australia). This method allowed for the

embryos to be vitrified with no contact to liquid nitrogen using a metal solid surface cooled at -196ºC. For vitrification, embryos were exposed to an equilibration solution (15% EG + 0.25 M trehalose) for 1 min and then placed into the vitrification solution (30% EG + 1 M trehalose) for 30 sec at room temperature. For vitrification, a 0.6 µl droplet of the vitrification solution containing an embryo was placed in a hook attached to a straw plug (Fyberplugs™) using a pipette and immediately exposed to a solid metal surface cooled at -196ºC. Then the Fiberplugs were inserted into a short plastic straw under liquid nitrogen. Vitrified embryos were stored in liquid nitrogen for at least one week.

*Freezing procedure*

Embryos produced *in vivo* or *in vitro* were exposed to 1.5 M EG cryoprotectant solution (Vigro Ethylene Gycol®, Bioniche Animal Health, Pullman, WA, USA) for 5 min and loaded into 0.25 ml yellow plastic straws, sealed and directly placed into a Freeze Control 5500 machine (Cryologic®, Australia) at -6.5ºC. After 1 or 2 min at -6.5ºC, straws were seeded, equilibrated for 10 min at -6.5°C and cooled at -0.6ºC/min until -35ºC. Frozen embryos were also stored in liquid nitrogen for at least one week.

*Thawing procedure*

After at least one week storage in liquid nitrogen, embryos in both cryopreservation procedures were thawed at the same time in 10 replicates. Vitrified embryos were thawed in a 0.25 M sucrose solution for 5 min at 37ºC then washed and cultured in SOF medium. Frozen embryos were thawed in a water bath at 30ºC for 12 sec then equilibrated in holding medium at 30ºC for 5 min and transferred into SOF culture medium.

*Assessment of in vitro development*

Cryopreserved embryos (frozen or vitrified) were cultured in 500 µl droplets of SOF medium supplemented with 0.4% BSA under oil at 37ºC, 5% $CO_2$, 5% $O_2$ and 90% $N_2$ under saturated humidity. The re-expansion and hatching status of the embryos were examined at 24 h intervals for 72 h.

*Statistical analysis*

Proportional data were transformed by square root and then analyzed by ANOVA, with type of embryo and cryopreservation procedure as main effects, using Infostat software (UNC, Argentina, 2010). The Protected Least Significant Difference (LSD) test was used for subsequent multiple comparisons when ANOVA revealed statistically significant differences ($P < 0.05$).

## Results

Re-expansion and hatching rates for *in vivo* and *in vitro* produced embryos vitrified by slow freezing or solid surface vitrification are shown in Table 1. *In vivo*-produced embryos had higher ($P < 0.01$) re-expansion and hatching rates than *in vitro*-produced embryos, regardless of cryopreservation method. However, re-expansion and hatching rates were higher ($P < 0.01$) for *in vitro*-produced vitrified embryos than *in vitro*-produced embryos cryopreserved by slow freezing.

Although *in vivo* produced embryos cryopreserved by the two systems had similar re-expansion rates, hatching rates tended to be lower ($P = 0.09$) for vitrified embryos compared to embryos cryopreserved by slow freezing.

Table 1. Re-expansion and hatching rates of *in vivo* and *in vitro* produced bovine embryos cryopreserved by slow freezing or solid-surface vitrification.

| Embryo production | Cryopreservation system | Embryos (n) | Re-expansion n (%) | Hatching n (%) |
|---|---|---|---|---|
| *In vivo* | Slow-freezing | 100 | 86 (86)[c] | 81 (81)[c] |
| | Vitrification | 110 | 93 (85)[c] | 78 (71)[c] |
| *In vitro* | Slow-freezing | 222 | 89 (40)[a] | 45 (20)[a] |
| | Vitrification | 223 | 155 (69)[b] | 132 (59)[b] |

[a,b,c] Rates with different superscripts in the same column differ ($P < 0.05$).

## Discussion

Developmental rates for *in vitro*-derived embryos were lower than *in vivo*-derived embryos, regardless of cryopreservation technique. This effect is generally observed in post-thaw survival rates of *in vitro*-derived embryos compared with *in vivo*-derived embryos, and confirms the lower freezability of *in vitro* produced embryos (Sommerfield and Niemman, 1999; Kaidi *et al*., 2001; Seidel, 2006). The differences between these two types of embryos at the morphological (Abe and Hochi, 2003), ultrastructural (Fair *et al*., 2001), metabolic (Khurana and Niemman, 2000) and genomic levels (Rizos *et al.*, 2002, 2003) are known to be reflected in higher cellular damage or metabolic disturbances of *in vitro*-derived embryos

during cryopreservation procedures than *in vivo*-derived ones (Rizos *et al*., 2003; Yu *et al*., 2010). Cells generally suffer stress during cryopreservation, nevertheless *in vitro* produced embryos have a higher osmotic stress compared with their *in vivo* counterparts due to the higher percentage of lipids in their membrane and less flexibility, which makes them less tolerable of decreased temperatures (Seidel, 2006).

In this study, *in vitro*-derived embryo survival rates were higher for the embryos cryopreserved by the vitrification method than by slow freezing (59 *vs*. 20%), which is consistent with the results reported by other authors (Kaidi *et al*., 2001; Nedambale *et al*., 2004; Peachey *et al*., 2005; Mucci *et al*., 2006; Yu *et al*., 2010). Structural characteristics like swollen blastomeres (Van Soom *et al*., 1992), a more fragile zona pelucida (ZP; Duby *et al*., 1997) and, as mentioned before, the higher content of intracytoplasmic lipids (Abe *et al*., 2004; Barcelo-Fimbres and Seidel, 2011), in *in vitro*-derived embryos make them more sensitive than *in vivo* derived embryos when they are cryopreserved by the conventional slow freezing procedure (Kaidi *et al*., 2001; Assumpção *et al*., 2008; Dinnyes and Nedambale, 2009; Nicacio *et al*., 2011). The lipid droplets have a strong relationship with mitochondrial maturation (Gomez *et al*., 2008). Consequently, when cryopreservation affects lipids, the mitochondrial structure is probably altered and embryo development is impaired (Gomez *et al*., 2008). Slow cooling procedures can be highly detrimental to cells with a high content of lipids, as they involve prolonged exposure time to the temperatures, when most of damage occurs (+15 to 0°C). The use of very rapid cooling rates in vitrification procedures benefits embryos and oocytes by providing a very fast transit through this temperature zone (Visintin *et al*., 2002). Furthermore, vitrification prevents mechanical damage caused by intracellular ice crystal formation (Bagis and Odoman, 2005). Others have shown that the displacement of intracellular lipids by centrifugation, reduction of the cytoplasmic lipid content of embryos with phenazine ethosulfate and *in vitro* embryo culture in serum-free media significantly improves cryosurvival rates of *in vitro*-produced embryos (Abe and Hoshi, 2003; Abe *et al*., 2004; Mucci *et al*., 2006; Seidel, 2006; Pryor *et al*., 2011).

Among the commercially available vitrification systems, the CVM system has a comparative advantage, allowing for the use of a minimum volume and a higher heat transfer rate, improving cooling rates greater than 10,000°C/min. In this study we used precise volumes of 0.6 µl, enhancing not only the cooling/warming rates but also the probability of vitrification. The combination of higher cooling rates with minimum volumes also enables the possibility of using a vitrification solution with lower cryoprotectant concentrations compared with those usually used for vitrification, minimizing the possibility of toxicity and osmotic effects (He *et al*., 2008; Yavin *et al*., 2009).

The CVM system prevents the risk of contamination, using all sterilized material and avoiding the direct contact of samples with $LN_2$ during cryopreservation. Also, it is a simplified system to hold and seal easily by an integrated plug used to cover the sleeve, which avoids any cross contamination during storage (Lindemans *et al.*, 2004; Beebe *et al*., 2011).

Finally, although the expansion rates of *in vivo*-derived embryos were similar between the slow-freezing and CVM vitrification methods, there was a tendency for a lower hatching rate in vitrified embryos compared to those frozen with the slow freezing technique. Other studies have shown that vitrification is a feasible method for *in vivo*-derived embryos, with similar embryo survival rates post-thawing as the conventional slow freezing (Van Wagtendonk-de Leeuw *et al*., 1997; Campos-Chillon *et al*., 2006; Pryor *et al*., 2007; Yu *et al*., 2010). Therefore, there is a tendency for different hatching rates than for recipient cows. Since pregnancy rates after non-surgical transfer are usually lower than hatching rates observed *in vitro* for both *in vivo* and *in vitro* produced embryos, differences in hatching rates may not be that apparent when pregnancy rates are evaluated (Dochi *et al*., 2006; Vieira *et al*., 2008).

In conclusion, the solid surface vitrification system could be used to cryopreserve both *in vitro*-derived and *in vivo*-derived bovine embryos. This method improved survival rates post-thawing of *in vitro*-derived embryos in comparison with the conventional slow freezing, and may be an alternative for *in vivo*-derived embryos.

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