

Video Article

Cryopreservation of Preimplantation Embryos of Cattle, Sheep, and Goats

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Abstract

Preimplantation embryos from cattle, sheep, and goats may be cryopreserved for short- or long-term storage. Preimplantation embryos consist predominantly of water, and the avoidance of intracellular ice crystal formation during the cryopreservation process is of paramount importance to maintain embryo viability. Embryos are placed into a hypertonic solution (1.4 – 1.5 M) of a cryoprotective agent (CPA) such as ethylene glycol (EG) or glycerol (GLYC) to create an osmotic gradient that facilitates cellular dehydration. After embryos reach osmotic equilibrium in the CPA solution, they are individually loaded in the hypertonic CPA solution into 0.25 ml plastic straws for freezing. Embryos are placed into a controlled rate freezer at a temperature of -6°C. Ice crystal formation is induced in the CPA solution surrounding the embryo, and crystallization causes an increase in the concentration of CPA outside of the embryo, causing further cellular dehydration. Embryos are cooled at a rate of 0.5°C/min, enabling further dehydration, to a temperature of -34°C before being plunged into liquid nitrogen (-196°C). Cryopreserved embryos must be thawed prior to transfer to a recipient (surrogate) female. Straws containing the embryos are removed from the liquid nitrogen dewar, held in room temperature air for 3 to 5 sec, and placed into a 37°C water bath for 25 to 30 sec. Embryos cryopreserved in GLYC are placed into a 1 M solution of sucrose for 10 min for removal of the CPA before transfer to a recipient (surrogate) female. Embryos cryopreserved in EG, however, may be directly transferred to the uterus of a recipient.

Video Link

The video component of this article can be found at <http://www.jove.com/video/2764/>

Protocol

1. Evaluating Suitability of Embryos for Cryopreservation¹

1. Specimens harvested from the reproductive tract of individual donor females should be placed into an isotonic embryo holding medium for 10 minutes prior to evaluation using a stereomicroscope. Be certain to keep embryos from each donor female separate from one another to enable identification of parentage of embryo transfer offspring.
2. Using low magnification ($\leq 10X$), specimens from an individual donor female should be segregated into separate groups consisting of unfertilized ova, degenerate embryos, or transferable quality embryos. Only quality grade 1 or 2 embryos at the compact morula through expanded blastocyst stages of development are suitable for cryopreservation, and all others should be discarded (unless synchronous recipient females are available for quality grade 3 embryos and a post-transfer pregnancy rate of approximately 25-30% is deemed acceptable).
3. Inspect quality grade 1 and 2 compact morula through expanded blastocyst stage embryos at a magnification $\geq 50X$ to ensure that the zona pellucida of the embryo is intact and that there is no material (e.g., cells, mucus) adhering to the zona pellucida. Any embryo with a cracked or missing zona pellucida or with a zona pellucida having adherent material should be segregated from other embryos and either be discarded or processed separately.

2. Washing Embryos to Reduce Likelihood of Disease Transmission²

1. Move zona pellucida-intact embryos in a minimal volume of isotonic embryo holding medium into the first well of embryo washing medium (e.g., phosphate buffered saline augmented with antibiotics and bovine serum albumin, newborn calf serum, or polyvinyl alcohol). Keep embryos from each donor female separate, and do not attempt to wash more than 10 embryos at one time. Move embryos in a minimal volume of medium into each successive wash to achieve a serial dilution $\geq 1:100$, and be certain to use sterile embryo handling tips that are changed between each successive wash.
2. Move embryos through 4 more washes as described in 2.1.
3. If transmission of viral diseases is of concern, wash embryos two times in a 0.25% trypsin solution, for a total combined exposure time to trypsin of no more than 90 seconds.
4. After the two trypsin washes, wash embryos 5 times more in embryo washing medium as described in 2.1.

5. After the final embryo wash, place embryos into isotonic embryo holding medium.

3. Dehydrating Embryos Prior to Cryopreservation³

1. Embryos should be transferred in a minimal volume of isotonic embryo holding medium into a hypertonic solution (1.4-1.5 Molar concentration) of a cryoprotective agent (CPA) such as ethylene glycol or glycerol.
2. Allow embryos to sit in freezing medium (hypertonic CPA solution) until they reach osmotic equilibrium. Because ethylene glycol permeates embryonic cells more rapidly than does glycerol, equilibration times are typically less for embryos in ethylene glycol (5 min) than in glycerol (10 min). Part of this equilibration time may be achieved during and after embryos are loaded into straws (described in next step).

4. Loading Embryos into a 0.25 ml Plastic Straw for Cryopreservation

1. Attach the cotton plug end of a 0.25 ml plastic straw (11.5 cm working length) to an embryo loading device, and aspirate approximately 1.3 cm of hypertonic CPA solution into a properly labeled straw. A variety of labeling procedures exists, including use of printed labels attached to either a straw sealing plug or to another straw connected with a straw adapter.
2. Remove the straw from the CPA solution, and aspirate approximately 0.15 cm of air to create a tiny air bubble within the straw.
3. Aspirate a single embryo in approximately 0.8 cm of hypertonic CPA solution into the straw.
4. Remove the straw from the CPA solution and aspirate approximately 0.15 cm of air to create a second tiny air bubble within the straw (air bubbles serve to physically isolate the embryo within the straw).
5. Aspirate approximately 9.1 cm of CPA solution to completely fill the straw, being certain to draw in a sufficient volume of CPA solution to moisten the polyvinylchloride (PVC) powder that exists within the cotton plug end of the straw. The CPA solution causes the PVC powder to gel and seal that end of the straw.
6. Seal the other end of the straw with PVC powder, plastic straw sealing plugs, or a heat sealer.

5. Placing Embryos into a Controlled Rate Embryo Freezing Machine

1. Either methanol bath or liquid nitrogen vapor freezing machines may be programmed for cryopreservation of preimplantation embryos.
2. Load the straws containing the embryos into a freezing machine whose temperature has been cooled from ambient temperature to -6°C. Load straws cotton plug end up (unless plastic straw sealing plugs or straw adapters are being used, in which case cotton plug end is placed down).
3. Allow embryos to sit at this temperature for at least 2 minutes before proceeding.

6. Seeding

1. Once embryos have cooled to -6°C, use a pair of tongs supercooled in liquid nitrogen (or a cotton-tipped stick immersed in liquid nitrogen) to induce ice crystal formation in the CPA solution inside the straw by touching the tongs (or cotton tipped stick) to the column of solution either above or below the embryo.
2. The water in the CPA solution will crystallize in the region exposed to liquid nitrogen, and ice crystals will spread to the column of CPA solution immediately surrounding the embryo.
3. Hold embryos at seeding temperature for 10 minutes before further cooling.

7. Continuing Dehydration of Embryos

1. Cool embryos at a rate of 0.5°C/min down to a temperature of -34°C. This cooling rate is important to ensure continued dehydration of the embryo.
2. Hold embryos at -34°C for 10 minutes before plunging embryos into liquid nitrogen (-196°C).
3. Place cryopreserved embryos into an appropriately labeled goblet (filled with liquid nitrogen) attached to an appropriately labeled cane, and place the cane into a canister of a liquid nitrogen dewar for short- or long-term storage.

8. Thawing Cryopreserved Embryos

1. Pull the canister into the neck of the liquid nitrogen dewar, ensuring that the canister remains below the frost line in the dewar.
2. Locate the cane containing the embryo to be thawed, and quickly and carefully remove the straw from the goblet, being certain not to warm other straws in the goblet.
3. Hold the straw in air for 3-5 seconds (to reduce the incidence of a cracked zona pellucida⁴), and then submerge into a 37°C water bath for an additional 25-30 seconds.
4. Remove the straw from the water bath, wipe the straw (being careful not to smudge the embryo identification on the straw), use a straw cutting device to snip off the non-cotton plug end of the straw (if sealed using heat or PVC powder), or carefully remove the plastic sealing plug, and either:
 1. load the straw into an embryo transfer device and transfer as quickly as possible to a synchronous embryo recipient (but ONLY if the embryo was cryopreserved using ethylene glycol as the CPA), or
 2. hold the open end of the straw over a dish containing a 1.0 Molar concentration of sucrose (a non-permeating compound), and use a pair of scissors to cut off the cotton plug end of the straw. Contents of the straw should freely flow into the sucrose solution, but pushing a small column of air through the straw may be necessary if any residual medium exists inside the straw. Allow embryos to remain in

sucrose solution for 10 minutes, and then transfer embryos into isotonic embryo holding medium for 10 minutes. Evaluate embryos post-thaw, load into a new straw, and transfer to suitable recipient females.

9. Representative Results:

A variety of factors may influence the pregnancy and live birth rates resulting from the transfer of frozen-thawed preimplantation embryos to synchronous recipient females⁵. Embryos at developmental stages less advanced than compact morula or more advanced than expanded blastocyst often do not survive the cryopreservation process as well as embryos at the compact morula, early blastocyst, blastocyst, and expanded blastocyst stages of embryonic development. Embryos of quality grade 1 and 2 yield higher post-thaw pregnancy rates than do quality grade 3 embryos (which typically are not cryopreserved due to the low post-thaw pregnancy rates). Embryos mishandled during the embryo freezing or embryo thawing procedures are likely to exhibit reduced pregnancy rates. Embryos transferred to recipient females whose estrous cycles are not synchronized with that of the donor female typically produce lower pregnancy rates. Embryos produced *in vitro* or manipulated in some manner (e.g., biopsied or bisected) usually yield lower pregnancy rates. Under optimal conditions, pregnancy rates obtained after transfer of frozen-thawed *in vivo* derived embryos is typically 60-70% in cattle^{6,7}, 65-75% in sheep^{8,9,10,11}, and 60-70% in goats^{12,13,14,15}. Similarly, pregnancy rates obtained after transfer of frozen-thawed *in vitro* produced embryos is typically 40-50% in cattle^{6,16}, 25-35% in sheep¹⁷, and 30-40% in goats¹⁸.

Type of Embryo	Species		
	Cattle	Sheep	Goats
<i>In vivo</i> derived	60-70%	65-75%	60-70%
<i>In vitro</i> produced	40-50%	25-35%	30-40%

Table 1. Expected pregnancy rates following transfer of frozen-thawed embryos from domestic ruminant livestock species to synchronous recipient females.

Discussion

Because embryos consist predominantly of water, it is crucial that preimplantation embryos be adequately dehydrated and slowly cooled in accordance with the protocol described herein to avoid intracellular ice crystal formation. Once the cooling process has begun, it is important to maintain unidirectional temperature change and to avoid temperature fluctuations. Initiation of ice crystal formation ("seeding") at an appropriate temperature for the specific CPA solution is critical.

Modifications to this slow cooling/rapid thawing method for preimplantation embryo cryopreservation include the one-step method (where the last column of medium loaded into the straw is sucrose instead of CPA solution)¹⁹ and the direct transfer method (where embryos frozen in ethylene glycol are thawed and transferred directly to the uterus of a recipient female without first removing the ethylene glycol from the embryo)²⁰. To reduce the volume of ethylene glycol deposited into the uterus of a direct transfer (DT) recipient female, it is advisable to fill the 0.25 ml straw with 6 cm holding medium before continuing as described previously. It should be noted that a voluntary standard exists within the commercial embryo transfer industry that preimplantation embryos cryopreserved for DT should be frozen in yellow-colored straws and stored in yellow-colored goblets in the liquid nitrogen dewar. The commercial industry also has specific labeling requirements for all cryopreserved embryos that should be followed.

One alternative to this method is vitrification²¹, a non-equilibrium method of cryopreservation where a more highly concentrated (6-8 M) CPA solution is cooled ultra-rapidly causing the CPA solution to change from liquid to a "glassy" state without ice crystal formation. Vitrification is likely better suited for cryopreservation of embryos that are less developmentally advanced than compact morula due to their increased temperature sensitivity.

This technique has application for preservation of unique germplasm resources²², as well as to the international commercial embryo transfer industry where more than 55% of approximately 500,000 bovine preimplantation embryos transferred in calendar year 2008 were cryopreserved.²³

Disclosures

No conflicts of interest declared.

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