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Cryotolerance of Day 2 or Day 6 *in vitro* produced ovine embryos after vitrification by Cryotop or Spatula methods ☆

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ABSTRACT

This study was conducted to evaluate the cryotolerance of *in vitro* produced ovine embryos submitted to vitrification at different developmental stages using two methods of minimum volume and rapid cooling rate. Embryos were vitrified at early stage (2 to 8-cells) on Day 2 or at advanced stage (morulae and blastocysts) on Day 6 after *in vitro* fertilization. Vitrification procedure consisted of the Cryotop (Day 2, $n = 165$; Day 6, $n = 174$) or the Spatula method (Day 2, $n = 165$; Day 6, $n = 175$). Non vitrified embryos were maintained in *in vitro* culture as a control group ($n = 408$). Embryo survival was determined at 3 h and 24 h after warming, development and hatching rates were evaluated on Day 6 and Day 8 after fertilization, and total cell number was determined on expanded blastocysts. Embryo survival at 24 h after warming increased as the developmental stage progressed ($P < 0.05$) and was not affected by the vitrification method. The ability for hatching of survived embryos was not affected by the stage of the embryos at vitrification or by the vitrification method. Thus, the proportion of hatching from vitrified embryos was determined by the survival rate and was lower for Day 2 than Day 6 vitrified embryos. The percentage of blastocysts on Day 8 was lower for the embryos vitrified on Day 2 than Day 6 ($P < 0.05$), and was lower for both days of vitrification than for non-vitrified embryos ($P < 0.05$). No interaction of embryo stage by vitrification method was found (P = NS) and no significant difference was found in the blastocyst cell number among vitrified and non-vitrified embryos. In conclusion, both methods using minimum volume and ultra-rapid cooling rate allow acceptable survival and development rates in Day 2 and Day 6 *in vitro* produced embryos in sheep. Even though early stage embryos showed lower cryotolerance, those embryos that survive the vitrification–warming process show high development and hatching rates, similar to vitrification of morulae or blastocysts.

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Introduction

Since the first successful vitrification of bovine embryos [21], relevant achievements have been reported related to offspring of vitrified oocytes and embryos in several species by different methods. Initially, the best embryo survival using vitrification was obtained in advanced stages of development through the use of concentrated cryoprotectant solutions or rapid cooling rate [33]. The use of concentrated cryoprotectant is responsible for cell damage due to osmotic stress and chemical toxicity affecting embryo survival [26]. This phenomenon which is more frequently observed in oocytes and early stages embryos can be reduced through the most rapid cooling and warming rates, reducing toxicity of cryoprotectant and decreasing the time of exposure of the cell to critical temperatures [2]. Minimum volume vitrification methods (i.e. <1 µl) as Cryotop [16] have improved the probability of success of cryopreservation in some mammalian embryos such as human and murine, reporting over 90% of survival rate [13,36]. In ruminant species the Cryotop method has shown acceptable embryo survival rates (cattle [22]; goats [23]). However, in sheep embryos little information about Cryotop method is available [15]. As an alternative, the novel Spatula method using minimal volume and ultra-rapid cooling rate has been reported only in mouse [32]. Spatula method has been slightly modified in our Lab (i.e. so-called Spatula MVD) and is used routinely in mouse embryos with survival rates greater than 90%, always higher than slow frozen techniques, and development rates similar to non-cryopreserved embryos [8]. To our knowledge, no information about this method in ovine embryos has been reported yet.

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Consensus exists that late embryonic stages as morulae and blastocyst resist the cryopreservation process better than early stages [19,14]. However, for *in vitro* embryo production system the main losses occur during the culture period from 2-cell embryos to blastocyst stage (e.g. approximately 80% of cleavage rate, and 30% of blastocyst rate is expected). This failure in embryo development is due in part to *in vitro* culture conditions and is partially overcome when the embryos are transferred to recipients soon after fertilization [35]. For this reason it would be useful to improve cryopreservation techniques allowing acceptable survival rates of early stages to transfer the embryos into the oviduct, avoiding *in vitro* culture. This alternative acquires more interest in sheep and goats where conventional embryo transfer is performed into the uterine horn by surgery, with similar complexity to oviduct transfer. However, little information is available about the survival rate after vitrification of early stages of ovine embryos produced by *in vitro* fertilization [28].

The aim of this study was to evaluate the embryo survival and subsequent development following vitrification performed on Day 2 or Day 6 after *in vitro* fertilization, using the Cryotop and Spatula methods in ovine embryos.

Material and methods

Experimental design

The study was conducted during breeding season (January–July) at Fundación IRAUy and Transgenic and Experimental Animals Unit of Institut Pasteur de Montevideo, Uruguay (34° S). Ovine embryos were produced by *in vitro* fertilization from a total of 1749 oocytes performed in 8 replicates. Day 0 was defined as the day of insemination. Embryos at early stage on Day 2 (i.e. 2–8 cell embryos) or advanced stages on Day 6 (i.e. morulae to expanded blastocysts) were cryopreserved by both vitrification methods, Cryotop and Spatula. Cleaved embryos on Day 2 were assigned to 5 experimental groups to be maintained in culture medium without undergoing vitrification (Control group, $n = 408$); or were vitrified with the Cryotop method on Day 2 ($n = 165$) or Day 6 ($n = 174$); or were vitrified with the Spatula method on Day 2 ($n = 165$) or Day 6 ($n = 175$). The quality of embryos was evaluated by morphology following the criteria recommended by the International Embryo Transfer Society (IETS) [30] and those excellent and good Grade 1 embryos were assigned to each experimental group. The survival rate at 3 h and 24 h after vitrification–warming, development rate on Day 6 and Day 8, hatching rate on Day 8, and total cell number in expanded blastocysts on Day 7, were compared among experimental groups. Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company (St Louis, Missouri, USA).

Oocyte collection

Ovaries from multiparous ewes were collected from a slaughterhouse and transported within 2 h to the laboratory at 35 °C in saline solution with 50 UI/ml of Penicillin and 50 μg/ml of Streptomycin. Cumulus oocyte complexes (COCs) were aspirated from 2 to 6 mm follicles using 21 gauge needle and 5 ml syringe containing 0.5 ml of collection medium consisted of Tissue Culture Medium (TCM) 199 buffered Hepes (25 mM) supplemented with 5 UI/ml of heparin, 1% fetal bovine serum, 50 UI/ml of Penicillin and 50 μg/ml of Streptomycin. The COCs with three or more layer of cumulus cells and containing an oocyte with homogeneous cytoplasm were selected for maturation.

In vitro maturation (IVM)

Selected COCs were washed three times in holding medium containing Hepes buffered TCM 199 supplemented with 10% (v/v) fetal bovine serum (FBS), 50 UI/ml Penicillin and 50 μg/ml of Streptomycin. Groups of 25–30 COCs were placed into 100 μl drops of maturation medium and cultured for 24 h in 35 mm Petri dish covered with embryo tested mineral oil in 5% CO_2 in humidified air atmosphere at 39 °C. Maturation medium consisted of TCM 199 supplemented with 10% estrous sheep serum (ESS), 10 μg/ml FSH, 10 μg/ml LH, 100 μM cysteamine, 50 UI/ml and 50 μg/ml of Penicillin and Streptomycin, respectively.

In vitro fertilization (IVF)

After IVM oocytes were washed three times in drops of fertilization medium containing synthetic oviduct fluid (SOF), 2% ESS, 10 μg/ml heparin and 10 μg/ml hypotaurine. Frozen-thawed semen was used from a single ram, previously frozen in our Lab in 0.25 ml straws containing 70×10^6 sperm in a Tris-based egg yolk extender supplemented with glycerol. Motile spermatozoa were obtained with a swim up method [27] with slight modifications. An aliquot of sperm suspension containing 1×10^6 sperm was added to each 100 μl drop of fertilization medium including 25–30 oocytes, covered with mineral oil. Fertilization was carried out by co-incubation of sperm and matured oocytes in fertilization medium in 5% CO_2 with humidified atmosphere at 39° C.

In vitro culture (IVC)

Approximately 22 h after insemination presumptive zygotes were denuded by gentle pipetting and received three washes in drops of development medium (SOFaaBSA bicarbonate buffered) containing SOF supplemented with BME essential amino acids 5%, MEM nonessential amino acids 2.5% and 0.4% of bovine serum albumin. Zygotes were transferred in groups of 25–30 to 100 μl culture droplets of development medium under mineral oil. Embryonic development was conducted in 5% CO_2, 5% O_2 and 90% N_2 in humidified atmosphere at 39 °C. The cleavage rate was recorded on Day 2 after fertilization (2–8 cell embryos/total oocytes). The embryos were randomly assigned to 5 experimental groups to be vitrified and warmed for further development on *in vitro* culture until Day 8. The culture medium was replaced on Day 3 and Day 6 with fresh SOFaa BSA [34].

Vitrification and warming procedure

Embryos were vitrified with the Cryotop and Spatula MVD methods on Day 2 or Day 6. The handling medium (HM) used during the vitrification and warming was TCM 199 Hepes buffered plus 20% (v/v) fetal bovine serum (FBS). The two methods of vitrification differ not only in the device used to place the embryos, but also in terms of basic, equilibration, and vitrification media, as well as the procedure for vitrification and warming. All manipulations were performed at 37 °C on heat stage in a warm room (25–27 °C) and all the media were used at room temperature, except for the first warming solution which was used at 37 °C for both methods. Vitrified embryos were warmed within 30 min after vitrification to continue the development under the same conditions as the control group.

Cryotop method

This method was performed according to the published method for human oocytes and blastocysts with few modifications [23]. Embryos were submerged individually in 300 μl of equilibration solution containing HM supplemented with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO) for 15 min to allow initial shrinkage and recovery. Following equilibration, they were transferred to the vitrification solution containing HM supplemented with 15% (v/v) EG, 15% (v/v) DMSO and 0.5 mol/l sucrose. This procedure was performed in two steps within 90 s. Then, the embryos were individually loaded on to the Cryotop

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