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Embryo survival and birth rate after minimum volume vitrification or slow freezing of *in vivo* and *in vitro* produced ovine embryos

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ABSTRACT

The objective was to evaluate pregnancy outcomes and birth rate of *in vivo* derived *vs. in vitro* produced ovine embryos submitted to different cryopreservation methods. A total of 197 *in vivo* and 240 *in vitro* produced embryos were cryopreserved either by conventional freezing, or by vitrification with Cryotop or Spatula MVD methods on Day 6 after insemination/fertilization. After thawing/warming and transfer, embryo survival rate on Day 30 of gestation was affected by the source of the embryos (*in vivo* 53.3%, *in vitro* 20.8%; $P < 0.05$) and by the method of cryopreservation (conventional freezing 26.5%, Cryotop 52.0%, Spatula MVD 22.2%; $P < 0.05$). For *in vivo* derived embryos, survival rate after embryo transfer was 45.6% for conventional freezing, 67.1% for Cryotop, and 40.4% for Spatula MVD. For *in vitro* produced embryos, survival rate was 7.3% for conventional freezing, 38.7% for Cryotop, and 11.4% for Spatula MVD. Fetal loss from Day 30 to birth showed a tendency to be greater for *in vitro* (15.0%) rather than for *in vivo* produced embryos (5.7%), and was not affected by the cryopreservation method. Gestation length, weight at birth and lamb survival rate after birth were not affected by the source of the embryo, the cryopreservation method or stage of development (average: 150.5 ± 1.8 days; 4232.8 ± 102.8 g; 85.4%; respectively). This study demonstrates that embryo survival and birth rate of both *in vivo* and *in vitro* produced ovine embryos are improved by vitrification with the minimum volume Cryotop method.

1. Introduction

Embryo cryopreservation by slow freezing technique originally developed for *in vivo* derived embryos and worldwide spread in farm animals, in general results in low survival rates when applied to *in vitro* produced embryos [25]. For this reason, vitrification methods have been further studied in parallel with the development of *in vitro* embryo production (IVEP) technology, and currently is the technique of choice in other species such as mice [32] and human [1,21]. Several methods of vitrification consisting of different cryoprotectants, devices and protocols have been reported for sheep embryos produced *in vivo* [6] and *in vitro* [41] with different success. Nevertheless, the slow freezing method continues to be the most widely used technology for cryopreservation of ovine and bovine *in vivo* and *in vitro* produced embryos.

Novel concepts related to vitrification have supported the development of minimum volume methods, reducing thus the volume for cryopreservation and increasing the cooling and warming rates, improving embryo survival [3,35]. The advantages of this new method of vitrification and the rapid dissemination in human clinics and mice facilities around the world have triggered the interest for application in livestock embryos requiring further information and more investigation. Although some reports have studied these novel methods in the laboratory on *in vitro* conditions [5], in sheep, very few studies [10] have been performed to compare the outcomes of different vitrification methods using minimum volume on pregnancy and lamb survival rates.

Recently, minimum volume vitrification methods such as Cryotop and Spatula MVD were evaluated in our Laboratory in ovine IVEP, allowing acceptable survival and development rate in *in vitro* conditions until Day 8 [12]. However, no information about pregnancy and birth rates after vitrification of *in vitro* produced embryos with these two methods has been reported in sheep. Furthermore, no studies comparing minimum volume vitrification methods *versus* conventional slow freezing technique, nor the

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effect of the embryonic stage at time of vitrification are available in this species. Since the effectiveness of vitrification depends on many factors as the species, the embryo source (*in vivo* or *in vitro*), the stage of embryonic development, cryoprotectant media, volume and cooling rate, among others [2], it would be useful to know the embryo survival rate in different conditions to choose the better approach to be applied in large scale programs.

The aim of this study was to compare the success obtained with sheep embryos from different sources (*in vivo vs. in vitro*) and stages (morulae *vs.* blastocysts) submitted to cryopreservation with conventional slow freezing or minimum volume vitrification methods (Cryotop and Spatula MVD) after embryo transfer into recipient females.

2. Materials and methods

2.1. Experimental design

The study was conducted during breeding season (March–August) at Fundación IRAUy and Transgenic and Experimental Animal Unit of Institut Pasteur de Montevideo, Uruguay (34° S). Ovine embryos were produced by superstimulation and intrauterine insemination or by follicular aspiration and *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC). A total of 437 embryos were frozen or vitrified on Day 6 after insemination and transferred into recipient females. One hundred ninety seven *in vivo* derived embryos were cryopreserved by conventional freezing ($n = 68$), or were vitrified by Cryotop ($n = 82$) or Spatula MVD methods ($n = 47$). Two hundred forty *in vitro* produced embryos were cryopreserved by conventional freezing ($n = 68$), or were vitrified by Cryotop ($n = 93$) or Spatula MVD methods ($n = 79$). For both i*n vivo* and *in vitro* produced embryos, only morulae to expanded blastocysts classified as excellent and good Grade 1 quality were assigned to each experimental group. Synchronized ewes were used to transfer the embryos on Day 6–7 after estrus. Embryo survival rate (viable embryos on Day 30/transferred embryos), pregnancy rate (pregnant ewes/transferred ewes), fetal loss from gestation to birth (lambs born accessed at birth/viable embryos on Day 30), and lamb survival rate (live lambs one week after birth/lambs born) were compared among experimental groups. Fetal losses and gestation length were just evaluated for those recipients present at delivery. Sixteen ewes (5 for Slow freezing, 5 for Cryotop, and 6 for Spatula MVD) were removed from the flock during pregnancy for reasons beyond the experiment. During the parturition period the recipients were checked twice daily for assistance and lamb identification.

2.2. In vivo derived embryos

Forty-five multiparous Australian Merino donors received an FSH treatment for superstimulation of the first follicular wave with the Day 0 Protocol described by Ref. [28] with minimal modifications [9] and routinely used by our group. Briefly, this protocol consisted of FSH treatment beginning at the emergence of the first follicular wave (i.e. soon after ovulation on Day 0 of the cycle). Six days after insemination, embryo recovery was performed under general anesthesia induced with 7.5 mg of diazepam (Unizepan; Unimedical, Montevideo, Uruguay) and 100 mg of ketamine (Vetanarcol; Konig, Buenos Aires, Argentina) iv, and maintained by inhalation with isoflurane (Isoforine; Cristalia, Sao Paulo, SP, Brazil). Ova/embryos were surgically collected by laparotomy using 30 mL flushing media (ViGro, Bioniche, Canada) injected in the cranial portion of the uterine horn and collected with a Foley catheter inserted at the base of each horn. Embryos were recovered in a 90 mm Petri dish and maintained in holding media to be morphologically evaluated under a stereomicroscope (40× magnifications) following International Embryo Transfer Society (IETS) recommendations [37], using only Grade 1 morulae and blastocyst stages.

2.3. In vitro embryo production (IVEP)

The IVEP followed the protocol routinely used in our Laboratory and further described previously [8,27]. Briefly, ovaries from multiparous ewes were collected from a local slaughterhouse and transported within 2 h to the laboratory in saline solution at 35 °C. Cumulus oocyte complexes (COCs) were mechanically aspirated and a total of 804 oocytes with three or more layer of cumulus cells with homogeneous cytoplasm were selected and incubated for 24 h in maturation medium (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 humidified atmosphere with 5% CO_2 at 39 °C. Groups of 25–30 matured oocytes per 100 μL drops were inseminated with frozen semen using 1×10^6 spermatozoa/drop after selection by swim up method [34] with slight modifications [27]. Fertilization was performed in 5% CO_2 at 39 °C for 22 h. Presumptive zygotes were denuded and placed in *in vitro* culture (IVC) medium under mineral oil with 5% CO_2, 5% O_2 and 90% N_2 in humidified atmosphere at 39 °C until Day 6. The culture medium consisted of 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. The culture medium was replaced on Day 3 with fresh SOFaa BSA [27]. Morulae and blastocysts were randomly assigned to three experimental groups to be frozen/vitrified and thawed/warmed and further transferred to synchronized recipient ewes.

2.4. Embryo cryopreservation

2.4.1. Conventional freezing

The protocol used was first reported by Ref. [43] with few modifications and routinely used in our Laboratory for *in vivo* derived embryos in sheep. Day 6 embryos were subjected to increasing concentrations of ethylene glycol (EG) (0.5, 1.0 and 1.5 M diluted in PBS with 4% BSA at room temperature), maintained during 5 min each in the first two concentrations and during 10 min in 1.5 M. Then, the embryos were loaded into 0.25 mL plastic straws and sealed before being placed in the controlled freezer equipment (CL-5500TC, Cryologic, Australia) at room temperature. The cooling curve was as follows: drop 1 °C/min from 20 °C to −7 °C, hold for 10 min (seeding at 3 min), drop 0.3 °C/min to −30 °C, hold for 15 min, and then, the straws were plunged into liquid nitrogen. For thawing, straws were exposed at room temperature for 10 s and were immerged in a water bath at 32 °C for 20 s. The embryos were discharged in a drop of PBS with 4% BSA and 0.5 M sucrose, and were maintained for 5 min at room temperature to be washed two times in holding solution for 5 min prior to transfer.

2.4.2. Minimum volume vitrification methods

Embryos were vitrified either with the Cryotop or Spatula MVD methods on Day 6 after fertilization. The two methods of vitrification differ in the type of device for loading the embryos, in the media composition for handling, equilibration and vitrification, as well as in vitrification and warming procedures. All media were used at room temperature except for the warming solution which was used at 37 °C for both, Cryotop and Spatula MVD methods. Embryo manipulations were performed at 37 °C on heat stage in a warm room (25–27 °C). The handling medium (HM) used during the vitrification and warming was TCM 199 Hepes buffered plus