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Short communication

A simple vitrification technique for sheep and goat embryo cryopreservation

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ABSTRACT

The aim of this study was to evaluate pregnancy and embryo survival rate of vitrified in vivo produced Merino sheep and Criolla goat (morulae and blastocysts) embryos, using the plastic tips of micropipettes, as containers (Cryo-tips). The embryos were exposed, at room temperature, to two successive equilibration solutions for a period of 5 min and then to a vitrification solution (VS) for 30 s. Then embryos were then loaded in 1 μl VS, into a plastic micropipette tip, and plunged into liquid nitrogen. On thawing, the embryos were warmed (37 °C) and placed into cryoprotectant dilutions (three-step-process). In the ovine, the morula and blastocyst pregnancy rates (47.1% vs 50%) and embryo survival rates (41.2% vs 50%) recorded were similar for both embryonic stages. Unlike the sheep, no pregnancies were recorded in goat vitrified/thawed morulae embryos, following transfer. However, in contrast, goats receiving blastocysts recorded high rates of pregnancy and embryo survival (64% and 64%, respectively). This technique allows for easy handling of cryopreserved embryos, is simple and efficient in both ovine embryo stages and also for goat vitrified blastocysts. The technique has definite potential application.

1. Introduction

The vitrification of small ruminant embryos, by direct plunging into liquid nitrogen has eminated following several cryobiological investigations (Schiewe et al., 1991; Ali and Shelton, 1993; Brown and Radcievic, 1999). The first kids born following embryo transfer of vitrified goat embryos in 1990 were reported by Yuswiati and Holtz (1990) and the first lamb born following transfer of a vitrified embryo, reported by Széll et al. (1990).

Sheep and goat embryos have generally been treated with different relevant cryoprotectants. So for example ethylene glycol has been shown to have a high penetration rate and low toxicity, and is currently the most extensively used permeating agent (Dochi et al., 1995). In association with glycerol, this cryoprotectant also normally records high embryo survival and pregnancy rates (Martínez et al., 2002; Guignot et al., 2006). Another recommendation has been the use of very small vitrification volumes (0.6–2 μl), to avoid osmotic injury to the embryos, during cryopreservation (Kasai and Mukaida, 2004). However, nowadays the method of low volume vitrification using thin straws is supported and named the Open Pulled Straw (OPS) technique (Vajta et al., 1997).

Various methods for embryo vitrification have been utilised in different species in the past e.g. straws (Baril et al., 2001), electron microscopy grids (Martino et al., 1996), fine capillaries (Vajta et al., 1997), cryo-tops (Kuwayama and Kato, 2000), cryo-loops (Lane et al., 1999), or the tips of micropipettes (Cremades et al., 2004). Literature has shown, independently of the method used, the rate of

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embryo survival following slow freezing or for vitrified embryos obtained following in vivo or in vitro embryo collection, are generally similar (Vajta, 2000; Isachenko et al., 2003; Guignot et al., 2006; Martínez et al., 2006; Bettencourt et al., 2009; Green et al., 2009). Even though there are practical benefits and economical advantages in the cryopreservation of embryos, acceptable results to date have been limited. Furthermore, embryo vitrification procedures have not been extensively used, as no standard protocol exists for a specific specie. Due to the limited literature regarding, especially embryo vitrification in small ruminants, the objective of this trial was to evaluate the pregnancy success of vitrified ovine and goat embryos, using a simple cryopreservation method, utilising plastic micropipette tips.

2. Materials and methods

This experiment was conducted during the natural breeding season, at the Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA), Bariloche, Argentina (latitude 41°07′S, longitude 71°15′W), at 786 m above sea level. Multiparous sheep and goats, maintained on natural pastures were utilised. During the experimental period the ewes and does were group-housed in pens, with ad libitum access to alfalfa hay and daily supplemented with 400 g concentrate per fermal (corn 10%, oats 30%, alfalfa pellets 60%). Unless stated otherwise, all chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. In vivo embryo production

The estrous cycles of 36 Criolla adult does were synchronized for use as embryo donors ($n = 10$) and embryo recipients ($n = 26$), by the insertion of intravaginal progestagen sponges containing 60 mg MAP (Progespon®, Syntex, Argentina), for a period of 18 days (day 0, sponge insertion). At sponge removal, embryo recipients received 200 IU eCG im (Novormon®, Syntex, Argentina). The embryo donors were superovulated using a protocol with a total of 80 mg pFSH (Folltropin V®, Bioniche, Canada) im – every 12 h in 6 decreasing doses (18, 18, 14, 14, 8, 8 mg), during the last 3 days of intravaginal progestagen treatment (days 16–18). The onset of estrus was detected with the aid of an adult teaser buck (24–36 h after sponge removal), and the donors were inseminated using laparoscopy, using frozen/thawed semen (200×10^6 sperm per doe), 12–14 h after the onset of the induced estrus.

Similarly the estrous cycles of 48 adult Merino ewes were synchronized for use as embryo donors ($n = 10$) and embryo recipients ($n = 38$) by insertion of progestagen intravaginal sponges containing 60 mg MAP (Progespon®, Syntex, Argentina), for a period of 14 days (day 0, sponge insertion). At sponge removal, all embryo recipients received 200 IU eCG im (Novormon®, Syntex, Argentina). The sheep embryo donors were superovulated using a total of 80 mg pFSH (Folltropin V®, Bioniche, Canada), injected im every 12 h – in 6 decreasing doses (18, 18, 14, 14, 8, 8 mg), during the last 3 days of intravaginal progestagen (days 12–14) treatment. The onset of estrus was detected with the aid of an adult teaser ram (24–36 h after sponge removal). All embryo donors were inseminated using laparoscopy with frozen/thawed semen (100×10^6 sperm per ewe), 12–14 h after the onset of the induced estrus.

All sheep and goat embryos were surgically flushed 8 days after intravaginal sponge withdrawal, implementing the prepubic laparotomy technique and under general anesthesia, using with xylazine (sheep: 8 mg im; goats: 7 mg im, Kensol®, König, Montevideo, Uruguay) and ketamine (sheep: 150 mg im; goats: 88 mg im, Ketalar® Parke Davis, Buenos Aires, Argentina). Local anesthesia was also administered in the surgical area (2 ml of lidocaine hydrochloride, Frankaina® 2%; FatroVonFrankel, Buenos Aires, Argentina).

All embryos were collected with the aid of laparotomy, flushing each uterine horn, with 20 ml commercial embryo recovery medium (Bovipro®, Minitube, USA), pre-warmed to 38 °C and supplemented with 10% fetal bovine serum (FBS®, 013/07; Internegocios, Buenos Aires, Argentina). The embryo flushing medium was directed from the uterine horn toward the utero-tubal junction, where a catheter was inserted. General antibiotic was administered in the form of oxytetracycline (1 ml/10 kg im), and local antibiotic (gentamicine) at the site of the abdominal incision. Ethical concerns were always taken into account – according to local animal welfare regulations and practices.

Only acceptable and viable ovine and caprine morulae and blastocysts recovered, were used in the trial. The flushed embryos were classified, based on the morphological criteria and using the guidelines of the International Embryo Transfer Society (1998).

2.2. Vitrification, thawing and embryo transfer procedures

Vitrification procedures in this trial were based on the method as described by Mermillod et al. (1999). Following flushing, the collected embryos were stored for 20 min at room temperature (20–25 °C), in an embryo incubation medium (Syngro®, Bioniche, USA). Thereafter the basic medium (BM) for vitrification and the thawing procedures comprise of commercial flushing medium (Bovi-pro®, Minitube, USA), supplemented with 20% FBS was implemented.

Briefly, all embryos were exposed to three different solutions at room temperature, according to the procedures regarding the equilibration solutions: (i) BM + 10% glycerol (G) for 5 min; (ii) BM + 10% G + 20% ethylene glycol (EG) for 5 min, and the vitrification solution; (iii) BM + 25% G + 25% EG for 30 s (VS). Embryos were then aspirated using an automatic 10 ± 0.1 μl micropipette (Eppendorf, USA) and loaded in 1 μl vitrification solution (VS), into the lumen of plastic tips with a long and soft extremity (2 embryos/tip) (Eppendorf pipette tips, Inc., USA; 10 ± 0.1 μl, Ref: RZ-07936-97 Cat. Cole-Parmer 2009–2010). After the tips were removed from the automatic micropipette, they were introduced into 3.6 ml cryo-tubes (Nunc, Denmark), filled with LN2, identified and stored in a liquid nitrogen container for a period of 1 month.

For thawing, the micropipette tips were warmed between the thumb and middle finger for 10 s and the embryos then immersed in the different basic medium (BM) solutions at 25 °C in three dilutions steps: (i) 12.5% G + 12.5% EG + 0.5 M sucrose; (ii) 0.5 M sucrose and (iii) 0.25 M sucrose, for 5 min each, to allow for the removal of the intracellular cryoprotectant. Finally, the embryos were introduced in a BM solution for 5 min at 25 °C, before the embryos were transferred to the recipients.

In both species, all embryos were transferred in pairs (morulae or blastocysts per recipient), 8 days following sponge withdrawal. The transfer procedure was performed under general anesthesia and using antibiotic administration (same treatment as with embryo recovery). Before embryo transfer, the presence of at least one corpus luteum (CL) was confirmed by laparoscopy. When a normal CL was identified, a small incision (1 cm) was made on the midline of the abdomen, cranial to the udder (laparotomy). The tip of the uterine horn corresponding to the ovary bearing the CL was exposed using a non-traumatic clamp. The embryos were then placed into the lumen of the uterine horn following uterine puncture, using a needle (18 G). A piston pipette for embryo transfer (Assipettor, Minitüb, Germany) was used. The uterine horn was then allowed to return into the abdomen, and the small incision closed by suture. All embryo transfers were performed within 30 min after cryoprotectant removal.

Pregnancy and embryo survival rates were recorded 28 days after embryos were transferred, using transrectal ultrasonography, with a 5 MHz linear array transducer (Aloka 500, Tokyo, Japan).

2.3. Statistical analysis

This study was designed to compare the reproductive success of embryo transfer in sheep and goats, taking into account the stage of embryonic development. Comparisons were performed using the Chi-square test. Significant differences were taken as $P < 0.05$. Data were analyzed using a statistical software package (SAS, 2002).

3. Results

The reproductive efficiency following the transfer of vitrified/thawed embryos in sheep and goats according to the developmental stage of the embryos, is set out in Table 1. The rate of transferable embryos recovered was generally high for the morulae and blastocysts in both species.

In the ovine, the pregnancy and embryo survival rates were similar for both embryo stages (morulae or blasto-

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Table 1
Reproductive efficiency of vitrified sheep and goat embryos, thawed and transferred at different stages of embryonic development.

Species	Embryo stage	Transferable/thawed embryos (%)	Recipients (*n*)	Pregnancy rate (%)	Embryo survival[A] (%)
Sheep	Morula	34/38 (89.5)	17	8/17 (47.1)	14/34 (41.2)
	Blastocyst	36/42 (85.7)	18	9/18 (50.0)	18/36 (50.0)
Goat	Morula	24/30 (80.0)	12	0/12 (0.0)[(a)]	0/24 (0.0)[(a)]
	Blastocyst	22/22 (100)	11	7/11 (63.6)[(b)]	14/22 (63.6)[(b)]

[ab]Values with different superscripts within columns for each species differ significantly ($P < 0.05$).
[A] Defined as live fetuses/transferable embryos.

cysts). Unlike sheep, no pregnancies were recorded in the goat recipients receiving vitrified/thawed morulae. However, the does receiving blastocysts recorded a high rate of pregnancy (64%) and embryo survival (64%) ($P < 0.05$). Only two ovine morulae did not survive until day 28 following embryo transfer.

4. Discussion

Results from the current study show that the incidence of transferable embryos was high for morulae and blastocysts in both species. This is in agreement with previous findings in sheep (Baril et al., 2001) and goats (Guignot et al., 2006). In ovine, results show that the cryopreservation procedure used in this trial proves to be efficient for preserving both embryonic stages (morulae and blastocysts). There is however a large variation in pregnancy rates, reported by different researchers. No differences have however been recorded in pregnancy rates between the slow freezing cryopreservation method (38–73%), vitrification (52–79%) and embryos transferred fresh (50–90%) (Ali and Shelton, 1993; Dattena et al., 2000; Baril et al., 2001; Papadopoulos et al., 2002; Isachenko et al., 2003; Bettencourt et al., 2009; Green et al., 2009). Similarly no differences have been reported in embryo survival rate (38–55%) between the slow freezing, the conventional vitrification or OPS vitrification methods (Bettencourt et al., 2009; Green et al., 2009).

The pregnancy rates in sheep at different embryonic developmental stages reported by Green et al. (2009), indicated that the vitrification procedures may increase the viability at the embryo morula stage, compared to the blastocyst stage. However, in the current study, no significant differences in pregnancy or embryo survival rate according to the stage of the vitrified embryos were recorded. This is in agreement with Baril et al. (2001) in sheep, and Lapatarova et al. (2006) in cattle.

In goats, Hong et al. (2007) reported similar results following the transfer of cryopreserved embryos following the conventional slow freezing method (46%), or vitrified method using OPS (Open Pulled Straws) (51.4%), or for fresh embryos (57.1%). In the present study, a high embryo survival rate following blastocyst vitrification in tips was obtained – comparable to preliminary results reported in previous studies, using similar methods (Gibbons et al., 2009; Traldi et al., 2009).

In the current study, the embryo survival rate of the blastocysts in goats (64%) was higher than the results obtained following slow freezing, using ethylene glycol, in a conventional transfer programme (45–53%) (Holm et al., 1990; Baril et al., 2001; Guignot et al., 2006). Also, when compared to the different success rates of the vitrification techniques reported by Traldi (2000) (40%), Branca et al. (2000) (44%) and Guignot et al. (2006) (14–35%).

Current results clearly indicate the method of tip vitrification to be very effective for goat blastocysts, but inadequate for goat morulae. The embryonic developmental stage is considered to be an important factor related to the viability of the embryos following cryopreservation (Cocero et al., 1996). The rate of embryo survival generally increases with advancing stages of development – from late morula to late blastocyst (Széll and Windsor, 1994; Garcia-Garcia et al., 2006). Embryos at the morula stage seem to be more difficult to cryopreserve and this may be due to a high intrinsic sensitivity of morulae to freezing (Massip, 2001). The difference in survivability of blastocysts and morulae in goats may also be attributed to a variation in the sensitivity to the cryoprotective agents or vitrification procedures, and the determination of an optimal concentration of the cryoprotectant used. There were evident differences in the embryo tip vitrification tolerance for goat and sheep morulae, although this was not observed for the blastocyst stages. Rates of permeability and toxicity may thus be related to species and to the developmental stage of the embryo (Bautista and Kanagawa, 1998).

The differences in morula survival rate between the ovine and goat species, indicate that further studies are required, to reduce the damage to the goat morula cells. The most widely used technique for the cryopreservation of sheep and goat embryos is the classic slow freezing technique, probably as the slow method is well established, and gives consistent results. However, the method of vitrification developed in this study, allows for easy handling of embryos prior to vitrification in liquid nitrogen, also for subsequent thawing and rehydration procedures. Moreover, this technique has been proven to be simple and efficient in the cryopreservation of ovine embryos (blastocysts and morulae), and can be utilised successfully for goat blastocysts.

For large scale commercial application, aseptic conditions will certainly be essential. To current knowledge, no reports regarding infection mediated by LN2 have been reported in relation with any vitrification technique in domestic animal embryology. Although these preliminary results are promising, a larger number of vitrified embryo transfers using this technique, need to be performed.

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