Small Ruminant Research 95 (2011) 61–64

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Short communication

A simple vitrification technique for sheep and goat embryo cryopreservation

A. Gibbons*, M.I. Cueto, F. Pereyra Bonnet

Laboratorio de Reproducción de Rumiantes Menores, Estación Experimental Bariloche, Instituto Nacional de Tecnología Agropecuaria, San Carlos de Bariloche, Río Negro, Argentina

ARTICLE INFO

Article history:
Received 2 March 2010
Received in revised form 10 August 2010
Accepted 16 August 2010
Available online 15 September 2010

Keywords:
Cryopreservation
Vitrification
Embryo transfer
Ovine
Caprine

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

* Corresponding author at: Laboratorio de Reproducción de Rumiantes Menores, Estación Experimental Agropecuaria Bariloche, Instituto Nacional de Tecnología Agropecuaria (INTA), Av. Modesta Victoria 4450, San Carlos de Bariloche CP 8400, Argentina.
Tel.: +54 2944 22731; fax: +54 2944 422990.
E-mail address: agibbons@bariloche.inta.gov.ar (A. Gibbons).

0921-4488/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.smallrumres.2010.08.007

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-