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Technical Note: Transfer of caprine blastocysts vitrified by the open pulled straw (OPS) or the solid surface procedure and warmed in sucrose-free medium

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ABSTRACT

The present paper outlines ways of non-surgical collection and semi-laparoscopic transfer of caprine embryos. Two different ways of embryo cryo-preservation by way of vitrification are described; the open pulled straw (OPS) procedure, known to be well suited and the solid surface procedure called for in situations where contact between embryos and non-sterile liquid nitrogen is to be avoided. Based on 13 transfers of OPS-vitrified and 9 transfers of solid surface-vitrified blastocysts (2 blastocysts/recipient) it was shown that either procedure is applicable (54% vs. 56% pregnancy- and 39% vs. 44% kidding rate). Furthermore the experiment showed that warming of vitrified embryos may be accomplished by one-step procedure (88% transferable post-warming embryos), opening up the possibility to transfer vitrified embryos under field conditions.

1. Introduction

Cryopreservation of embryos has become an established component of assisted reproduction in various mammalian species. In farm animals conventional slow freezing is most commonly applied. An extremely rapid cooling procedure, called vitrification, was introduced by Rall and Fahy in 1985. The procedure comprises brief exposure of embryos to highly concentrated permeating cryoprotectants, immediately followed by cooling at an ultra-rapid rate of 16,700 °C/min (Criado-Scholz, 2012) accomplished by direct submersion in liquid nitrogen. This approach circumvents ice crystal formation and dehydration of embryonic cells. A major breakthrough was achieved by the invention of the "open pulled straw" (OPS) procedure by Vajta et al. (1997). A point of criticism with this procedure is the potential risk of contamination resulting from direct contact of the embryos with non-sterile liquid nitrogen (Bielanski et al., 2000). The European Directive on Tissue Storage and the U.S. Food and Drug Administration call for hermetically closed aseptic systems precluding contact of embryos with liquid nitrogen (Abdelhafez et al., 2011). For that reason the "solid surface" vitrification technique was invented, an approach by which the embryo, suspended in a tiny drop of vitrification medium, is brought in contact with the surface of a metal block cooled with liquid nitrogen. With this procedure the cooling rate approaches 10,000 °C/min (Dinnyes et al., 2000; Begin et al., 2003; Somfai et al., 2010; Beebe et al., 2011). Once vitrified, embryos are sealed in a plastic sleeve and stored submerged in liquid nitrogen (Lindemans et al., 2004; Beebe et al., 2011).

The first successful vitrification of caprine embryos was reported by Yuswiati and Holtz (1990). When comparing transfer of caprine blastocysts cryopreserved by conventional freezing vs. vitrification by the OPS procedure, El-Gayar and Holtz (2001) and Al-Yacoub et al. (2010) achieved higher pregnancy rates with the latter. The objective of the present investigation was to determine whether it is possible to employ the solid surface vitrification procedure instead of the OPS procedure in goats. An additional aspect addressed in the present study was the warming of vitrified caprine blastocysts in a single step in sucrose-free holding medium as reconstitution diluent.

2. Material and methods

2.1. Superovulation and embryo collection

Embryos were obtained from 31 pluriparous Boer goat does from the breeding flock of the Department of Animal Science at Goettingen University in Germany (51°46′N, 9°41′E). Does were, on average, 3.7 (2–7) yrs. of age and weighed 60 (46–79) kg. They were synchronized by providing them with progestogen releasing intravaginal pessaries that remained in place for 7d. Beginning 48 h before pessary removal

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porcine Follicle Stimulating Hormone (pFSH) supplemented with 40% porcine Luteinizing Hormone (pLH) (Nowshari et al., 1995) was administered by six i.m. injections at 12 h intervals of 4, 4, 2, 2, 2 and 2 Armour Units (1 AU corresponds to 10 µg purified FSH). Along with the last two FSH injections, 5 mg dinoprost (1 mL Dinolytic; Zoetis, Berlin, Germany) was administered. Does were tested for estrus with an aproned adult male at 8 h intervals and, when exhibiting standing estrus, mated once daily. Seven days after the last mating embryos were collected transcervically as described by Pereira et al. (1998) and Suyadi et al. (2000). Briefly, 16 h before collection 5 mg dinoprost was administered to induce luteolysis. With the aid of a duckbill speculum and pen light the lip of the external os cervix was grasped with a 255 mm long sharp-pointed uterine tenaculum forceps (Possi; Aesculap, Tuttlingen, Germany) and gently pulled caudally almost to the vulvar orifice. A flushing catheter (Ruesch, Nelaton-Robinson, Art NR. 220500, Ch 12, Kernen, Germany) with a pliable steel stylet inserted was passed through the cervical canal. The stylet was removed and the catheter was further advanced into one uterine horn directed by a finger in the vaginal fornix. After infusing 20 mL Dulbecco's phosphate buffered saline (PBS), supplemented with 0.06% bovine serum albumin (BSA; A9647-50G, Sigma-Aldrich, Steinheim, Germany), 100 IU/mL penicillin and 100 µg/mL streptomycin (PAA P11-010, Darmstadt, Germany), the reflux was collected via embryo filter (75 µm Em Con Embryofilter, Albrecht, Aulendorf, Germany). To maintain an uninterrupted flow the catheter had to be gently moved to and fro. This procedure was repeated 8 times. Then the catheter was partially withdrawn and directed into the other uterine horn which was also flushed 8 times. Occasionally cellular debris was encountered in the first of a series of flushings. In these cases the flushing was diluted by adding more diluent. Embryos that were recovered were assessed for developmental stage and morphological intactness under a stereoscope equipped with a warming stage at 20 to 40x. Blastocysts with intact zona pellucida and uniform blastomeres without visible cellular damage were vitrified.

2.2. Vitrification and warming of blastocysts

Within 30–60 min after flushing, embryos were transferred to 800 µL holding medium consisting of TCM 199 (M0650, Sigma, Steinheim, Germany) containing 2 mM sodium bicarbonate, 2 mM sodium pyruvate, 25 mM Hepes-sodium salt and 1 mM L-Glutamine, supplemented with 20% heat-inactivated male goat serum, at pH 7.4, osmotic pressure 280 mOsm and temperature 37 °C. Each embryo was washed twice in holding medium and, after 3 min equilibration, transferred to holding medium supplemented with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (Me_2SO) (vitrification solution No. 1). After 3 min, embryos were transferred to a 1 µL droplet of holding medium supplemented with 0.5 M sucrose, 16.5% EG and 16.5% Me_2SO (vitrification solution No. 2) with the aid of a 1 µL Eppendorf pipet and, within less than 40 s, they were vitrified either, at random, by the open pulled straw (OPS) or the solid surface (CVM Ring Fibreplug®) procedure.

2.2.1. OPS vitrification

Straws required for OPS vitrification were fabricated by softening French mini-straws (0.25 mL, Minitueb, Landshut, Germany) over a hot plate at 200 °C, pulling them to approximately half the original diameter and cutting them at the thinnest point. Vitrification was conducted according to the procedure of Vajta et al. (1997), slightly modified by El-Gayar and Holtz (2001). By touching the droplet of vitrification solution No. 2 containing the embryo with the narrow end of the drawn-out straw it was drawn into the straw by capillary force. Immediately afterwards the straw was submerged in liquid nitrogen, thin-end-first, where it was stored until transfer. For transfer, straws were removed from liquid nitrogen, held in the air for 10 s before dipping the thin end into 800 µL of holding medium at 37 °C serving as reconstitution diluent, while occluding the wide end with the tip of a finger. Embryos were expelled from the straw into the reconstitution diluent by the air warming inside the straw.

2.2.2. Solid surface vitrification

The equipment used for solid surface vitrification (CVM Ring Fibreplug®, manufactured by CVM™-CryoLogic, Australia) is commercially available. It consists of a metal (aluminum-nickel) block submerged in liquid nitrogen to 20 mm from the top and an embryo carrier consisting of a plastic rod, 80 mm long and 2 mm in diameter, with a ring of 1.2 mm inner diameter of 0.1 mm nylon thread attached to the tip. With the aid of a 1 µL Eppendorf pipet the 1 µL-droplet of vitrification solution No. 2 containing the embryo is deposited on the ring of the carrier. There it adheres due to surface tension and, as soon as the tip of the carrier is lowered onto the metal block, it turns into a vitrified bead. The carrier with the bead is inserted into a plastic sleeve standing in liquid nitrogen. After sealing the top of the sleeve with a special welder, supplied by the manufacturer, it is completely submerged in liquid nitrogen. For warming, sleeves were partially removed from liquid nitrogen, cut open at the top and the carrier, after removal from the sleeve, was held in the air for 10 s before the ring end was dipped into the reconstitution diluent at 37 °C.

With either procedure, after warming embryo morphology was assessed under a stereoscope equipped with a warming stage. Embryos were classified from 1 (very good) to 5 (degenerate) according to the guidelines of the International Embryo Transfer Society (Stringfellow and Givens, 2010). Of 60 vitrified-warmed embryos recovered, 42 that were classified as "very good" or "good" were transferred, two embryos at a time, to 21 recipients. Transfer was carried out within 5–10 min after removal of the embryos from liquid nitrogen.

2.3. Embryo transfer

Recipients were pluriparous Boer goat does of similar origin, weight and age as the donors. They were synchronized during the breeding season by using intravaginal progesterone-impregnated CIDRs. Upon CIDR removal after 7d, two i.m. injections of 5 mg dinoprost were administered at 12 h interval. Estrus detection was carried out at 8 h intervals with the aid of an aproned adult male. Six days after the last standing estrus semi-laparoscopic embryo transfer was performed. Beforehand, does were deprived of feed for 2 d and of water for 1 d. They were anesthetized by i.v. administration of 0.1 mL/10 kg Sedaxylan (20 mg xylazine; Eurovet Animal Health, Bladel, Netherlands) and 0.1 mL/10 kg Ursotamin (0.1 g ketamine; Serumwerke, Bernburg, Germany). After shaving, washing and disinfection of the area cranial to the udder, does were placed on a laparoscopy cradle in dorsal recumbence. The rear of the animal was elevated to an angle of 20°. The skin was nicked with the tip of a scalpel blade about 8 cm cranial to the udder on the mid-line and 8 cm lateral to the mid-line. At these points cannulae of 5 mm diameter with a trocar inserted were punched through the abdominal wall. Trocars were removed and by introducing the endoscope (Panaview-Optic, 5 mm diameter, 25° angle, Wolf, Knittlingen, Germany) through the lateral cannula the reproductive organs could be inspected, assisted by an exploratory probe (400 mm long, 5 mm in diameter; Storz, Tuttlingen, Germany) introduced through the mid-line cannula. To create sufficient intra-abdominal space the abdominal wall was manually lifted up by the lateral cannula. If a well-developed corpus luteum was identified, the punch hole on the mid-line was extended to a 20–30 mm incision with a scalpel blade. Under endoscopic control the tip of the uterine horn ipsilateral to the ovary carrying a corpus luteum was grasped close to the utero-tubal junction with blunted 255 mm tenaculum forceps (Possi; Aesculap, Tuttlingen, Germany) introduced through the small incision. Care was taken not to pinch the organ. A loop of 30–40 mm of uterine horn was exteriorized and, while holding it gently between thumb and index finger, punctured with a blunted 22-gauge