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Technical note: Vitrification of goat embryos by the open pulled-straw method¹

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ABSTRACT: The suitability of the open pulled-straw (OPS) method for vitrifying bovine embryos was tested for goat embryos. Of 14 does receiving OPS-vitrified embryos, all became pregnant and 13 (93%) kidded. The corresponding values for an established conventional freezing program were 58% pregnant and 50% (6/12)

kidding. Overall embryo survival amounted to 64% (18/28) for OPS-vitrified and 42% (10/24) for conventionally frozen embryos. All differences were statistically significant. It is concluded that OPS vitrification is a suitable method for cryopreserving caprine d-7 blastocysts.

Key Words: Goats, Cryopreservation, Vitrification, Embryo Transfer

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Introduction

The first successful transfer of vitrified goat embryos was reported by Yuswiati and Holtz (1990). Since then, different freezing protocols have been proposed, but few results are available concerning pregnancy rates after the transfer of vitrified embryos (Traldi et al., 1999; Branca et al., 2000; Traldi, 2000). Survival rates were generally substantially lower than with embryos cryopreserved by conventional techniques (see review by Holtz, 1996). Most efforts to improve vitrification focused on media, cryoprotectants, equilibration times, and dilution. Less attention has been paid to cooling and warming rates during freezing and thawing. Recently, good embryo survival was observed in the bovine with the so-called open pulled-straw (OPS)-vitrification technique (Vajta et al., 1997). In this investigation, vitrification of goat blastocysts by the OPS method was attempted. OPS-vitrified and conventionally cryopreserved embryos were transferred to recipients in order to assess their *in vivo* survival.

Materials and Methods

Parous Boer goat does 23 to 70 mo of age and weighing 48 to 91 kg were synchronized during the estrous season using progestagenic ear implants (1.5 mg norgestomet, Crestar, Intervet, Boxmeer, The Netherlands) for 10 d. Two PGF_{2α} injections (5 mg dinoprost, Dinolytic, Pharmacia and Upjohn, Erlangen, Germany) were administered at 12-h intervals on the day of implant removal. In order to obtain multiple ovulations, donors were treated with 16 Armour units (AU) of pFSH supplemented with 40% LH (Nowshari et al., 1995). Beginning 48 h before implant removal, consecutive s.c. injections of 4, 4, 2, 2, 2, and 2 AU were administered at 12-h intervals. Animals showing estrus were hand-mated daily as long as females permitted the male to mount. Embryos were collected transcervically as described in Holtz et al. (2000) and Suyadi et al. (2000) 7 d after the last mating. This involved the induction of luteolysis with prostaglandin F_{2α} followed, 20 h later, by transcervical insertion of a flushing catheter and 10 flushes of each uterine horn. Within 2 to 4 h after collection, morphologically intact blastocysts were vitrified by the OPS method described by Vajta et al. (1998). Briefly, French ministraws (0.25 mL, Minitueb, Landshut, Germany) were softened over a hot plate at 200°C, pulled to approximately half the original diameter and wall thickness, and cut at the thinnest point. One embryo at a time was equilibrated in holding medium (10% TCM 199 [catalog No. M-0650, Sigma, Steinheim, Germany], 2 mM sodium bicarbonate, 2 mM sodium pyruvate, 25 mM Hepes-sodium salt, 1 mM L-glutamine, adjusted to pH 7.4 and 280 mOsm, and supplemented with 20% goat serum) containing 10% ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO) at 39°C for 1 min before being transferred, in 1 to 2 μL of solution, to a 20-

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Table 1. Transfer results of d-7 goat blastocysts cryopreserved either by conventional freezing or by vitrification applying the open pulled-straw (OPS) method

Method of cryopreservation	Number of embryos thawed	Number of recipients	Recipients pregnant		Recipients kidding		Average litter size	Embryo survival % ^c
			n	%	n	%		
Conventional	24	12	7	58 ^a	6	50 ^a	1.7	42 ^a
Vitrification (OPS)	28	14	14	100 ^b	13	93 ^b	1.4	64 ^b

^{a,b}Within a column, values lacking a common superscript letter differ ($P < 0.01$, χ^2 -test).

^cKids born per embryo transferred, taking all recipients into account.

μL droplet of holding medium containing 20% EG and 20% DMSO. Within 20 s thereafter, the droplet containing the embryo was picked up by the narrow end of the modified straw by capillary action. The straw was submerged into liquid nitrogen in a vertical position with the thin end first. For thawing, the end of the straw containing the embryo was immersed in 1.2 mL of holding medium containing 0.33 M sucrose at 39°C while the opening on the other end was occluded by the tip of a finger. Within 1 s, the vitrification medium liquefied and the embryo slid out of the straw. After 1 min, the embryo was transferred to fresh medium of the same composition. The embryo was then transferred to holding medium containing 0.2 M sucrose for another minute and, eventually, to holding medium devoid of sucrose for 5 min. Until transfer, about 2 h after thawing, embryos were kept in M16 medium (Hogan et al., 1986) in an incubator at 39°C under a humidified gas atmosphere of 5% CO₂ in air. Two embryos at a time were surgically transferred (Nowshari and Holtz, 1993) to the tip of the uterine horn of d-6 recipients (parous Boar goats) ipsilateral to the ovary with at least one prominent corpus luteum. By way of comparison, 24 d-7 blastocysts were cryopreserved by the conventional freezing procedure described elsewhere (Nowshari and Holtz, 1993). Briefly, embryos were equilibrated for 20 min in M2 medium (Hogan et al., 1986) containing 1.4 M glycerol and loaded individually into 0.25 mL straws. Straws were transferred to an alcohol bath (Haake, Karlsruhe, Germany) precooled to -6°C. After 10 min, they were seeded and after another 10 min they were cooled to -32°C at a rate of 0.3°C/min. Ten minutes later, straws were immersed in liquid nitrogen. For thawing, embryos were immersed in a water bath at 30°C for 40 s. The cryoprotectant was removed in three steps by decreasing concentrations of glycerol (0.94, 0.47, 0.0 M) in M2 medium containing 0.3 M sucrose. Pregnancy was confirmed echographically on d 40 of gestation (Aloka SSD 500 with 7.5-MHz linear-array transducer), and animals were permitted to carry to term.

Differences between means were tested for significance by the χ^2 -test.

Results

In both groups, all embryos were recovered after thawing. It was noted that, within the 2-h period from

thawing until transfer, the OPS-vitrified embryos had the appearance of flawless, expanded blastocysts, whereas the conventionally frozen-thawed embryos had not reached full reexpansion. As shown in Table 1, of the 12 recipients receiving conventionally frozen-thawed embryos, 7 (58%) were found to be pregnant by d 40. Six of these (50%) kidded with an average litter size of 1.7. Of the 14 recipients receiving vitrified embryos, all were found to be pregnant by d 40. One of these did not kid, resulting in a kidding rate of 93%, with an average litter size of 1.4. The survival rate for conventionally frozen-thawed embryos was 42%, whereas for vitrified embryos it was 64%. All differences, except litter size, between the two methods were significant ($P < 0.01$). The sex ratio was biased in favor of male kids (3.5:1 and 2.3:1 for vitrification and conventional freezing, respectively). All kids born were viable, their birth weight being within the range typical for the population.

Discussion

The results indicate that the open pulled-straw method for vitrifying caprine blastocysts is a good alternative to well-established conventional freezing regimes (Bilton and Moore, 1976; Chemineau et al., 1986; Tsunoda et al., 1987; Holm et al., 1990; Li et al., 1990; Puls-Kleingeld et al., 1992; Nowshari and Holtz, 1995; Baril et al., 1989, 2000). Earlier attempts to vitrify goat embryos yielded low (Yuswiati and Holtz, 1990) or modest (Traldi et al., 1999; Traldi, 2000) pregnancy rates. The OPS method is based on ultrathin straws derived from normal French straws drawn to about half their original diameter and wall thickness. According to Vajta et al. (1998) freezing and thawing rates of the minute amount of medium (0.5 μL) enveloping the embryo in the thin-walled straw are enhanced to about 20,000°C/min compared with 2,500 with the original straw. Therefore, the potentially damaging temperature zone is traversed rapidly, minimizing chilling injury. Due to the open ends of the straw, no pressure changes occur during the process of freezing, avoiding fracture damage (Vajta et al., 1997). The rapid freezing process implies minimal de- and rehydration of cells, reducing the degree of strain placed on the cell membrane. From a practical point of view, the OPS method is simple, rapid, and inexpensive to perform. The embryos

can be loaded by capillary action and remain in place without the necessity to seal or plug the straw. For freezing, no special equipment is required. Recovery from the straw is accomplished by simply dipping its thin end into thawing medium at 39°C, utilizing the expansion force of the air contained. In our opinion, the OPS method is particularly suited for use under field conditions.

Implications

The open pulled-straw vitrification method devised for bovine embryos can be recommended as a practical and effective alternative to the conventional slow-freezing of caprine blastocysts.

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