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Original article

Evaluation of cryopreservation techniques for goat embryos

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Summary — A total of 410 goat embryos were divided at random into 9 groups. Cryoprotectants (glycerol, ethylene glycol or dimethylsulfoxide) were added by a 3-step procedure using increasing concentrations of cryoprotectant (0.5 M; 1 M; 1.5 M) in PBS at 10 min intervals. After freezing and thawing, each cryoprotectant was removed by 3 methods: the classic 3-step procedure (cryoprotectant 1 M–10 min; 0.5 M–10 min; PBS alone–10 min); the same procedure, but with sucrose added to the first 2 steps (sucrose 0.25 M and cryoprotectant 1 M–10 min; sucrose 0.25 M and cryoprotectant 0.5 M–10 min; PBS alone–10 min); and a 2-step procedure with sucrose alone (sucrose 0.25 M–10 min; PBS alone–10 min). Each removal protocol was performed for embryos in each cryoprotectant. The viability of the embryo was evaluated by its capacity to subsequently develop during 48 h *in vitro* culture. For morulae the development rate of the embryos was significantly higher when they were frozen with ethylene glycol than when dimethylsulfoxide or glycerol was used ($P < 0.05$). For blastocysts the development rate was the same whether they had been frozen with ethylene glycol or dimethylsulfoxide, and was significantly lower when they were frozen with glycerol ($P < 0.05$). Among the 3 removal procedures tested, the 3-step procedure with sucrose gave the best development rate and differed significantly from the classic 3-step procedure ($P < 0.05$).

cryopreservation / goat / embryo

Résumé — Étude des modalités de congélation de l'embryon caprin. Comparaison de 3 cryoprotecteurs et de 3 techniques d'extraction de ces cryoprotecteurs. *Cette étude de la congélation de l'embryon caprin a pour objet de comparer, d'une part, 3 cryoprotecteurs et, d'autre part, 3 procédures d'extraction de ces cryoprotecteurs après congélation. Au total 410 embryons sont divisés aléatoirement en 9 lots. Trois cryoprotecteurs, le glycérol, l'éthylène-glycol et le diméthylsulfoxyde sont incorporés aux embryons par bains successifs de 10 min de concentration croissante (0,5 M ; 1 M ; 1,5 M). Après congélation et décongélation, chaque cryoprotecteur est extrait par 3 procédures diffé-*

rentes : 3 bains successifs de 10 min de concentration décroissante du cryoprotecteur (1 M ; 0,5 M ; 0 M) dans du PBS; la même procédure avec addition de 0,25 M de sucrose ; 2 bains successifs de 10 min de concentration décroissante de sucrose (0,25 M ; 0 M) dans du PBS. La viabilité embryonnaire après congélation est évaluée par leur aptitude à se développer en culture in vitro *pendant 48 h. Les morules congelées avec de l'éthylène-glycol présentent un taux de viabilité significativement plus élevé que celles congelées avec le diméthylsulfoxide ou le glycérol (P < 0,05). Pour les blastocystes, le taux de viabilité est équivalent après congélation avec l'éthylène-glycol ou le diméthylsulfoxide, mais significativement supérieur à celui obtenu par congélation avec le glycérol (P < 0,05). Parmi les 3 procédures d'extraction du cryoprotecteur, c'est l'association cryoprotecteur à concentration décroissante et sucrose qui donne les meilleurs résultats avec une différence significative vis-à-vis de l'utilisation du cryoprotecteur seul à concentration décroissante (P < 0,05).*

cryoconservation / embryon / chèvre

INTRODUCTION

In domestic animals, embryo cryopreservation allows a time and/or space dissociation between embryo collection and transfer. In addition, embryo cryopreservation facilitates a variety of applications such as international transport of embryos and the preservation of the gene pool of valuable or endangered breeds or species.

The procedure of cryopreservation includes initial exposure to and equilibration with a cryoprotectant, cooling to subzero temperatures, storage, thawing and finally dilution and removal of the cryoprotectant with return to a physiological environment that will allow further development. Cell-to-cell junctions must maintain structural integrity throughout the cryoprotectant exposure and cryopreservation procedures. Major factors (Schiewe, 1991) known to affect survival of cryopreserved embryos include the species, the development stage (Li *et al,* 1990; Heymann, 1992), the origins (produced *in vivo* or *in vitro*) (Greve *et al,* 1993; Tervit *et al,* 1994), the methods of cryopreservation and the nature of the cryoprotectant.

Since the report by Bilton and Moore of the first kid born after transfer of a frozen-thawed embryo, variable embryonic survival rates (26–53%) have been obtained after transferring morulae or blastocysts frozen with glycerol (Bilton and Moore, 1976; Chemineau *et al,* 1986; Rao *et al,* 1988; Wang *et al,* 1988; Baril *et al,* 1989; Li *et al,* 1990; Le Gall *et al,* 1993), ethylene glycol (Bousseau *et al,* 1989; Le Gall *et al,* 1993) and dimethylsulfoxide (Bilton and Moore, 1976; Rao *et al,* 1988; Li *et al,* 1990). To determine the best way for freezing goat embryos the present *in vitro* study compared the 3 cryoprotectants commonly used in the goat (*eg,* glycerol, ethylene glycol and dimethylsulfoxide), as well as 3 methods of cryoprotectant removal and return to physiological conditions.

MATERIALS AND METHODS

Embryo production

Eighty non-lactating dairy female goats (42 Alpines and 38 Saanens) were each superovulated once during a 6 month period from April to September. Synchronisation or induction of oestrus was achieved by injecting 100 μg of cloprostenol (a PGF2α analogue, Estrumate, Pitman-Moore, Meaux, France) on the ninth day of an 11-day progestagen treatment (regimen: vaginal sponge impregnated with 45 mg of fluorogeston acetate (Intervet, Angers, France). To induce superovulation, 16 Armour Units of p-FSH prepared by one of the authors (JPB) were administered in 6 decreasing doses (4–4, 2–2, 2–2 AU), injected intramuscularly at 12 h intervals during the last 3 d of the progestagen treatment. Asso-

ciated with the FSH injection, porcine pure LH (prepared by JPB) was administered in 6 increasing doses (5–5, 20–20, 50–50 µg). Each female was hand-mated at the onset of oestrus and 12 h later. One male was used per 3 females. The embryos were recovered surgically 7 d after the onset of oestrus. The female goats were anaesthetised with 5 mg/kg of tiletamine-zolazepam in solution (Zoletil, Reading, Nice, France). The reproductive track was exteriorised through a mid-ventral incision. A paediatric Foley catheter was inserted in the uterine horn next to the bifucation and its bulb was inflated with air. A 7/10 Venofix needle (Bruneau, Boulogne-Billancourt, France) was inserted at the top of the uterine horn near the utero-tubal junction. Sixty millilitres of modified Dulbecco's phosphate-buffered saline (PBS) combined with 4 g/l of bovine serum albumin and 1 g/l glucose (PBS, Eurobio, Paris, France) were injected through the Venofix needle and collected through the Foley catheter into a sterile glass bottle.

The collection bottles were allowed to stand, at room temperature, for approximately 10 min. The supernatant was then siphoned off leaving 20 ml in each bottle which was transferred to sterile plastic Petri dishes for examination under a stereomicroscope. Embryos were easily seen with this method, and were transferred immediately into fresh PBS.

Embryo classification

Ova and embryos were evaluated microscopically (magnification x 100). They were first scored as morulae and blastocysts (Amoroso *et al,* 1942). The quality of embryos was evaluated on morphological criteria, according to Eldsen *et al* (1978), and classified as *grade 1*, *2* and *3*. *Grade 3* embryos were discarded; *grades 1* and *2* were pooled for the cryopreservation experiment. The embryos were washed in 10 successive baths of PBS.

Embryo distribution

Morulae and blastocysts were allocated at random into 9 groups for cryoprotectant exposure (3 cryoprotectants: glycerol, ethylene glycol and dimethylsulfoxide) and for cryoprotectant removal (3 removal protocols for each cryoprotectant).

Cryoprotectant exposure and equilibration

The cryoprotectants were added in 3 steps. The embryos were transferred successively into PBS solution containing 0.5 M, 1.0 M and 1.5 M cryoprotectant. The embryos were left to incubate for 10 min, between solution changes, at room temperature (20°C). The embryos were then loaded into 0.25 ml French insemination straws (Ref-A201–IMV, L'Aigle-France). Generally each straw was loaded with 3 embryos in order to create an easily used number for the 3 methods of cryoprotectant removal after thawing.

Freezing and thawing procedure

The cooling procedure was the same for the 3 groups of embryos. The straws were placed horizontally in the cooling chamber of a programmable biological freezer (Hubert-Unisat, France), precooled to –7°C. After an equilibration time of 5 min, ice crystallisation ('seeding') was induced by touching the extremity of the straws with a pair of forceps cooled in liquid nitrogen. After seeding, this temperature was maintained for 5 min. The temperature was then reduced at the rate of 0.4°C/min until –35°C. After a 5 min holding period, the straws were plunged directly into liquid nitrogen (–196°C) for storage.

After variable periods of storage in liquid nitrogen for 2 to 16 months, depending on research programming, the straws were thawed rapidly by immersion in a water bath at 37°C for 1 min.

Removal of the cryoprotectant

Immediately after rewarming the straws were opened and the embryos were transferred to freshly prepared PBS containing the 1.5 M concentration of the same cryoprotectant in which the embryos were frozen.

All 3 cryoprotectants were removed according to the following 3 different dilution procedures in PBS at room temperature (≈ 20°C):

– *Dilution method 1:* classic 3-step procedure (cryoprotectant 1 M–10 min; 0.5 M–10 min; PBS alone–10 min);

– *Dilution method 2:* dilution method 1 with sucrose added to the first 2 steps (sucrose 0.25 M

and cryoprotectant 1 M–10 min; sucrose 0.25 M and cryoprotectant 0.5 M–10 min; PBS alone–10 min);

– *Dilution method 3:* 2-step procedure with sucrose alone (sucrose 0.25 M–10 min; PBS alone–10 min).

In vitro culture

After the final removal of cryoprotectant, the embryos were placed individually in a multiwell culture (16 Nunc ROSHILDE, Denmark) containing 0.5 ml of B2 MENEZO medium (Api-system, France). The culture was performed in an incubator in an atmosphere of 5% CO_2 in air at 37°C for 48 h. The medium was changed every 12 h.

Evaluation of embryo viability

During the culture period the embryos were evaluated every 12 h, with a stereomicroscope to determine the embryos' development from 'morula' to 'young blastocyst' or from 'blastocyst' to 'expanded blastocyst' (Fiéni *et al,* 1991a). The embryos that changed their development stage during the 48 h of culture were considered viable.

Statistical analysis

The cryoprotectant effect was tested using the χ^2 test. Cryoprotectant removal procedures were compared with a Mantel–Haenszel test which allows us to take into account the variability due to the cryoprotectant and developmental effects (Mantel, 1963). Differences of $P < 0.05$ were considered to be significant.

RESULTS

A total of 568 ova or embryos were recovered, *ie* 7.1 per goat. Due to the high fertilisation rate (78%) and the low number of grade 3 embryos (8%), 410 embryos (281 morulae and 129 blastocysts) were available and were separately allocated into the 9 experimental groups.

The proportion of thawed embryos that developed in culture was primarily influenced by the cryoprotectant used. When glycerol was used at 1.5 M concentration, morulae as well as blastocysts had a low survival rate after thawing. In contrast ethylene glycol and dimethylsulfoxide gave higher survival rates. The best results were obtained with ethylene glycol, which showed a statistically significant difference in the *in vitro* development rates of morulae and blastocysts between ethylene glycol and glycerol ($P < 0.05$) (table I). The comparison of the results between dimethylsulfoxide and glycerol was a somewhat different; the blastocysts frozen with dimethylsulfoxide had a level of *in vitro* development signifi-

Table I. Effects of cryoprotectants used at various embryo stages on *in vitro* embryonic development.

Cryoprotectant	*Morulae*	*Blastocyst*
Glycerol	2/84 (2) ^{x}	4/43 (9) ^{x}
Ethylene glycol	15/93 (16) ^{y,a}	19/35 (54) ^{y,b}
Dimethylsulfoxide	9/104 (9) ^{a}	21/51 (41) ^{y,b}

In the same column, results with superscripts (x, y) not in common are significantly different ($P < 0.05$). In the same line, results with superscripts (a, b) not in common are significantly different ($P < 0.05$). In parentheses: percentage of embryo development.

Table II. Effects of cryoprotectants and dilution removal methods used at different embryo stages on *in vitro* embryonic development.

Cryoprotectant	*Morulae (n = 281)*			*Blastocysts (n = 129)*		
	Dilution method			*Dilution method*		
	1	*2*	*3*	*1*	*2*	*3*
Glycerol	0/32 (0)	1/29 (3)	1/23 (4)	0/16 (0)	3/16 (19)	1/11 (9)
Ethylene glycol	4/33 (12)	8/33 (24)	3/27 (11)	7/12 (58)	6/10 (60)	6/13 (46)
Dimethylsulfoxide	2/35 (6)	4/36 (11)	3/33 (9)	5/16 (32)	10/18 (55)	6/17 (35)

In parentheses: percentage of embryo development.

cantly higher than the blastocysts frozen with glycerol ($P < 0.05$) but the 2 cryoprotectants had no statistically significant effect on the development of the morulae. Finally, there were no statistically significant difference between development rates for embryos frozen with either ethylene glycol or dimethylsulfoxide.

The stage of development of the goat embryo at the time of freezing was thus particularly important. When ethylene glycol or dimethylsulfoxide were used, morulae and blastocysts differed significantly in terms of survival in culture after thawing ($P < 0.05$) (table I).

The results in tables II and III indicate the effects of the 3 dilution methods described in the *Materials and methods*. In this experiment, because of the number of variables such as 2 embryo development stages and 3 cryoprotectants, the specific effect of the cryoprotectant removal dilution method did not show a statistically significant difference in a χ^2 test (table II). The use of a Mantel–Haenszel test with adjustment for morphological stages (morulae and blastocysts) and cryoprotectants showed a significantly higher proportion of surviving embryos ($P < 0.05$) when the cryoprotectant was removed with *Dilution method 2* than with *Dilution method 1* (22 *versus* 12%) (table III).

Table III. Effects of cryoprotectant dilution removal methods on *in vitro* embryonic development.

Dilution method for removal of the cryoprotectants	*Development rate after 48 h of culture*
1	18/144 (12) ^{x}
2	32/142 (22) ^{y}
3	20/121 (16)

Results with superscripts (x, y) not in common are significantly different ($P < 0.05$). In parentheses: percentage of embryo development.

DISCUSSION

The aim of this experiment was to compare the cryoprotectant properties of 3 substances in frozen goat embryos. Our embryos show that the cryoprotectant properties of ethylene glycol were significantly better than those of glycerol, for both the morulae as well as the blastocysts.

Dimethylsulfoxide seemed better than glycerol for freezing the goat embryos, but we could only demonstrate this for blastocysts. The poor qualities of glycerol as a cryoprotectant of embryos are known in the ewe (Tervit and Goold, 1984; Cocero *et al,* 1988). They have also been recently demonstrated *in vitro* and *in vivo* in the goat (Le Gall *et al,* 1993).

The blastocysts frozen with ethylene glycol or dimethylsulfoxide showed an *in vitro* survival rate significantly better than that of the morulae (54 *vs* 16% with ethylene glycol; 41 *vs* 9% with dimethylsulfoxide). Li *et al* (1990) found a similar development-dependent difference *in vitro* between frozen–thawed morulae or blastocysts with glycerol or dimethylsulfoxide. However, comparing *in vitro* development and kidding rates for frozen-thawed embryos with ethylene glycol and glycerol Le Gall *et al* (1993) found no significant difference in kidding rates whether the embryos were frozen as morulae (55% with ethylene glycol *vs* 32% with glycerol) or blastocysts (47% with ethylene glycol *vs* 29% with glycerol). Their observations of *in vitro* development also indicated that in both cryoprotectants morulae survived freezing-thawing less well than did blastocysts (23 *vs* 45% with ethylene glycol, 0 *vs* 35% with glycerol). These authors considered that the conditions of the *in vitro* cultures were probably the cause of these differences. We agree with this hypothesis because in a previous work (Fiéni, 1991) studying the culture techniques of fresh goat embryos we showed that *in vitro* development rates for blastocysts were higher than morulae after 48 h in the same medium (respectively 90 *vs* 47%). The utilisation of embryo co-cultivation with oviduct cells will perhaps permit us to resolve the problem of poor morulae development in acellular media.

The procedure used for cryoprotectant removal influenced the results observed; the lower rates of embryonic development were obtained with the removal procedure without sucrose (*Dilution method 1*). Tervit and Goold (1984) in ewes and Le Gall *et al* (1993) in goats reported similar observations; the best results were obtained when both cryoprotectant and sucrose were used in the removal procedure. If thawed embryos with a high intracellular concentration of solutions are directly subjected to an isotonic medium, the embryo inflates to obtain the osmotic balance and sometimes even the zone pellucid cannot prevent the rupture of the embryo (Rall *et al,* 1980). The addition of sucrose (a non-permeating solute) in the dilution solution increases the extra embryonic osmotic pressure and minimises osmotic damage to the embryo cells.

In conclusion, this study shows that ethylene glycol and dimethylsulfoxide (1.5 M in PBS) are convenient for cryopreservation of goat embryos and that under the same conditions glycerol appears to give lower survival rates. In addition, our data indicate that the introduction of sucrose (0.25 M) in the dilution solution facilitates the cryoprotectant removal process and increases the survival rate of embryos. In these aspects the goat embryo seems to have a behaviour similar to that of the ovine embryo in regards to cryoprotectant exposure.

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