Osmotic and cytotoxic study of vitrification of immature bovine oocytes

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Four experiments were conducted to determine the composition of a solution suitable for vitrification of immature bovine oocytes. The osmotic and cytotoxic effect of different concentrations (0, 0.25, 0.5 and 1 mol l⁻¹) of nonpermeating solutes (sucrose versus trehalose) were examined. In addition, the effect of permeating cryoprotectants such as glycerol, dimethyl sulfoxide, and propylene glycol (40% w/v) on the viability of oocytes was studied to determine the optimal time of exposure and the most suitable cryoprotectant. Exposure of bovine oocytes to trehalose was less harmful than exposure to sucrose ($P < 0.01$), and high normospermic fertilization (70%) was achieved after exposure to 0.25 mol trehalose l⁻¹. Propylene glycol was chosen as the cryoprotectant for the vitrification of immature bovine oocytes because of its fast permeating rate and its low cytotoxic effect. The composition of this solution (40% propylene glycol and 0.25 mol trehalose l⁻¹ in PBS containing 4% w/v BSA) appeared to be suitable for vitrification, as the fertilization rate of the vitrified oocytes was 37% (36 of 97).

Introduction

There is increasing interest in the development of gamete banks for embryo production by *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). Effective cryopreservation of oocytes would greatly enhance their availability at every time and from low cost sources such as abattoir animals or from high genetic value animals (Pieterse *et al.*, 1991).

Cryopreservation of oocytes and embryos has been attempted by vitrification (Rall and Fahy, 1985; Nakagata, 1989). Vitrification is the solidification of a liquid by an extreme increase in viscosity during very rapid cooling (Fahy *et al.*, 1984). The solid, called glass, has the molecular and ionic distribution of the liquid state, and thus avoids the potentially detrimental effects of extracellular and intracellular crystallization. In addition, the oocytes are exposed to a range of temperatures from 15 to 0°C, to which they are particularly sensitive (Heyman *et al.*, 1986; Parks and Ruffing, 1992), for short periods only, as high cooling rates are used in this technique.

Successful vitrification has been reported for mouse (Rall, 1987), rabbit (Smorag *et al.*, 1989), rat (Kono *et al.*, 1988), pig (Rubinsky *et al.*, 1992), ovine (Schiewe *et al.*, 1990) and bovine (Massip *et al.*, 1986) embryos and for mouse (Nakagata, 1989) and pig (Arav *et al.*, 1990) oocytes. Vitrification requires the presence of a high concentration of cryoprotectant. It is therefore important to minimize the damage to cells by the osmotic stress or chemical toxicity caused by multimolar concentrations of the cryoprotectant used, which strictly depends on the kind of cell used. No ideal cryoprotectant that meets the requirements of all different species and developmental embryonic stages has been found; vitrification studies should therefore be preceded by osmotic and cytotoxic studies.

Bovine oocytes have been cryopreserved either at the germinal vesicle or at the metaphase II stage. Lim *et al.* (1992) reported that bovine oocytes showed a greater fertilization ability when they were frozen at the metaphase II stage rather than at the germinal vesicle stage. However, many studies on mature (metaphase II) bovine and mouse oocytes have shown that specific structures such as the meiotic spindles are very sensitive to exposure to cryoprotectant (Van der Elst *et al.*, 1988; Shaw and Trounson, 1989; Richardson and Parks, 1992) or to low temperatures (Magistrini and Szollosi, 1980; Pickering and Johnson, 1987) and undergo tubulin depolymerization. Immature bovine oocytes were therefore studied. We examined the osmotic behaviour of immature bovine oocytes exposed to high concentrations of different cryoprotectants and investigated their cytotoxic effects, to establish an optimal combination of cryoprotectants that permits successful vitrification.

Materials and Methods

Source of oocytes

Ovaries from cows and heifers were collected at a local abattoir immediately after slaughter and transported to the laboratory in PBS medium (Whittingham, 1971) at 20°C. Immature oocytes at the germinal vesicle stage were recovered by aspiration through a 15-gauge needle from small follicles (3–6 mm diameter). Only the oocytes enclosed in compact multilayer cumulus cells and with evenly granulated cytoplasm were used.

Osmotic response to nonpermeating cryoprotectants

Denuded oocytes were obtained by incubation at 20–22°C for 1–1.5 min in PBS containing 1 mg hyaluronidase ml⁻¹ (Sigma Chemical Co., St Louis, MO) and transferring them back

to fresh PBS medium. Complete removal of cumulus cells was achieved by repeatedly aspirating the oocytes through a small bore pipette. Denuded oocytes were used in osmotic studies because the presence of the foot processes between cumulus cells and the oocyte causes an irregularly shaped shrinkage of the oolemma. The oocytes were then exposed to PBS containing 0, 0.25, 0.5 and 1 mol sucrose l^{-1} (Sigma) or trehalose (Sigma) at 22°C for 10 min. A telecamera connected to a Leitz inverted microscope (Fluovert, Germany) was used to record the oocytes for the whole time of exposure. Each oocyte was monitored continuously. The volume of the oocytes was determined from the cross-sectional area and the assumption of sphericity as described by Jackowsky *et al.* (1980). Oocytes contracting in a nonsymmetrical manner were not used.

In vitro development of germinal vesicle oocytes after exposure to nonpermeating cryoprotectants

After exposure to PBS solution containing 0.25, 0.5 and 1 mol sucrose or trehalose l^{-1} for 10 min at 22°C, cumulus enclosed oocytes were matured and fertilized *in vitro*. The oocytes were placed in TCM-199 medium (pH 7.4; Gibco Laboratories, New York) supplemented with 5 μg LH ml^{-1} (NIH-S20) and 5 μg FSH ml^{-1} (LER441-2) as well as granulosa cells at the concentration of 5×10^4 cells ml^{-1} , and incubated at 38°C under 5% CO_2 in air for 26 h. The oocytes were then washed, denuded and placed in the fertilization medium (Brackett and Oliphant, 1975) supplemented with 1.9 mg caffeine ml^{-1} (Sigma). Capacitated bull spermatozoa were prepared from frozen bull semen in straws by the method of Fukui *et al.* (1990). The oocytes were collected 16 h after exposure to spermatozoa and fixed in acetic acid:alcohol (1:3 v:v). Twenty-four hours later, the oocytes were stained with Lacmoid (Sigma) and observed under a phase contrast microscope. The viability of oocytes was evaluated by their ability to undergo normal *in vitro* fertilization after *in vitro* maturation. An oocyte was considered normospermic if two pronuclei and the second polar body were observed. Fertilized oocytes could be distinguished from activated (parthenogenetic) oocytes by the presence of the remnant tail of the spermatozoon. The criteria for distinguishing digyny from dispermy was the presence of the second polar body.

Osmotic response to concentrated solutions of permeating cryoprotectants

Cumulus-free oocytes were directly introduced to PBS containing 40% w/v glycerol (BDH, Poole), dimethyl sulfoxide (DMSO, BDH), propylene glycol (Fluka Chemie, AG, Buchs) or mixtures of them (20:20 w:v) at 22°. Video recordings were taken of the oocytes for the whole equilibration time (i.e. during which the oocyte regains the isotonic volume as a consequence of cryoprotectant penetration inside the cell) in each of these solutions. Determination of volume was performed as described above.

In vitro development of immature oocytes exposed to permeating cryoprotectant

The deleterious effect of the permeating cryoprotectants was evaluated by exposing the cumulus-enclosed oocytes to PBS

Fig. 1. Osmotic behaviour of oocytes when exposed to (Δ) sucrose and ($\square$) trehalose solutions plotted as a Boyle Van't Hoff function. Mean values of the fractional volume in 0, 0.25, 0.5 and 1 mol sucrose l^{-1} (Δ) and trehalose l^{-1} ($\square$) in PBS are shown. Each point represents the mean of five determinations; SEM are too small to be shown.

solutions of propylene glycol, DMSO or glycerol (40% w/v) for the time required for equilibration found in Expt 3. The oocytes were then transferred to a culture dish containing 1 mol sucrose l^{-1} in PBS to remove the cryoprotectant from the cells (Leibo, 1984). Fresh PBS was added drop by drop to reach a final sucrose concentration of 0.125 mol l^{-1} within 10 min and the oocytes were then transferred to fresh PBS. The oocytes were matured and fertilized *in vitro* and their viability was assessed as described above.

Vitrification of immature bovine oocytes

On the basis of Expts 1, 2, 3 and 4, propylene glycol and trehalose were selected as the cryoprotectants for use in the vitrification solution.

Immature cumulus-enclosed oocytes were introduced at one-step into a vitrification solution containing propylene glycol (40% w/v) and trehalose (0.25 mol l^{-1}) in PBS supplemented with 4% w/v BSA until equilibrium was reached (10 min). Some of the oocytes were immediately placed in 1 mol sucrose l^{-1} , diluted and cultured for IVM and IVF (solution control). Others were loaded in 5 μl glass straws without being cooled and the contents were emptied into a culture dish containing 1 mol sucrose l^{-1} . The oocytes were then diluted and cultured as already described. The remaining oocytes were loaded in 5 μl glass straws in a volume of 1 μl and cooled in a directional solidification cryomicroscope (DCS Galcore, Mitzpe Aviv, IS) using a cooling and warming rate of $9600^\circ\text{C min}^{-1}$ between predetermined temperatures (20°C to -140°C). The principle of directional solidification and the mathematical calculations of the cooling and warming rate were described by Rubinsky *et al.* (1991). A video camera was used in conjunction with the microscope to evaluate changes in morphology of the cells and the

Table 1. Effect of different types and concentrations of nonpermeating cryoprotectants on *in vitro* fertilization of immature bovine oocytes

Treatment	Total number of oocytes	Number of oocytes fertilized (%)		Number of oocytes unfertilized (%)	Number of oocytes degenerated (%)
		Normospermic	Polyspermic		
Sucrose (0.25 mol l ⁻¹)	58 ^a	31 (54) ^b	11 (20)	10 (17)	6 (10)
Sucrose (0.5 mol l ⁻¹)	69 ^a	30 (44) ^b	21 (30)	10 (14)	8 (11)
Sucrose (1 mol l ⁻¹)	60 ^a	19 (32)	19 (32)	6 (10)	16 (26)
Trehalose (0.25 mol l ⁻¹)	60 ^a	42 (70) ^c	9 (15)	7 (11)	2 (3)
Trehalose (0.5 mol l ⁻¹)	66 ^a	41 (62) ^c	17 (26)	2 (3)	6 (9)
Trehalose (1 mol l ⁻¹)	69 ^a	43 (63)	19 (28)	3 (4)	4 (5)
Control	74 ^a	52 (70) ^b	6 (8)	13 (17)	3 (4)

^aResults were pooled from three replicate experiments.

^{b,c}Values with different superscripts within a column are significantly different ($P < 0.01$).

Fig. 2. Volumetric changes of oocytes during one-step exposure to concentrated solutions (40% w/v) of (○) propylene glycol, (□) dimethyl sulfoxide and (△) glycerol. Each point represents the mean of five determinations; SEM are too small to be shown.

Fig. 3. Transient volume changes of oocytes exposed to mixtures (20% w/v + 20% w/v) of (●) propylene glycol + dimethyl sulfoxide, (■) propylene glycol + glycerol, and (▲) dimethyl sulfoxide + glycerol. Each point represents the mean of five determinations; SEM are too small to be shown.

physical state of the solution as described by Rubinsky *et al.* (1991).

Immediately after warming, the oocytes were placed in PBS containing 1 mol sucrose l⁻¹ to remove the cryoprotectant, diluted with fresh PBS within 10 min as already described and then transferred into fresh PBS. The viability of oocytes was assessed after IVM and IVF as described above.

Statistical analysis

Statistical comparisons were made using χ^2 analysis. A probability of less than or equal to 0.01 ($P < 0.01$) was considered significant.

Results

Effects of nonpermeating cryoprotectants

The oocytes responded as a perfect osmometer over this range of osmolality (0.285–1.335) (Fig. 1). The regression analysis yielded the Boyle Van't Hoff function $FV_{eq} = 0.166 + 0.2^* (\text{osmol}^{-1})$, $r = 0.96$ (SD = 0.008) for the sucrose solutions and $FV_{eq} = 0.212 + 0.285^* (\text{osmol}^{-1})$, $r = 0.98$ (SD = 0.006) for the trehalose solutions, where FV_{eq} is the equilibrium fractional volume. No statistical differences were found between the sucrose and trehalose Van't Hoff plots ($P > 0.01$). The non-osmotic volume was similar to that of immature bovine oocytes found by Myers *et al.* (1987), but was much lower than that found by Ruffing *et al.* (1990). Fertilization rate is given as

Table 2. Effect of high concentrations (40%) of permeating cryoprotectants on *in vitro* fertilization rate of immature bovine oocytes

Type of cryoprotectant	Total number of oocytes	Number of oocytes fertilized (%)		Number of oocytes unfertilized (%)	Number of oocytes degenerated (%)
		Normospermic	Polyspermic		
PG	50 ^a	26 (52) ^b	4 (8)	8 (16)	12 (24)
DMSO	50 ^a	4 (8) ^c	2 (4)	2 (4)	42 (84)
GLY	50 ^a	6 (12) ^c	4 (9)	6 (12)	34 (68)
Control	54 ^a	30 (55) ^b	6 (11)	16 (29)	2 (3)

^aResults were pooled from two replicate experiments.

^{b,c}Values with different superscripts within a column are significantly different ($P < 0.01$).

PG: propylene glycol; DMSO: dimethyl sulfoxide; GLY: glycerol.

Table 3. Effect of vitrification on *in vitro* fertilization of immature bovine oocytes

Treatment	Total number of oocytes	Number of oocytes fertilized (%)		Number of oocytes unfertilized (%)	Number of oocytes degenerated (%)
		Normospermic	Polyspermic		
Vitrification	97 ^a	36 (37) ^b	10 (17)	10 (10)	41 (42)
Solution effect	49 ^a	26 (53) ^d	6 (12)	2 (4)	15 (30)
Exposure to VS in straws	63 ^a	30 (48) ^d	7 (11)	9 (17)	17 (26)
Control	120 ^a	80 (69) ^c	13 (10)	13 (10)	14 (11)

^aResults were pooled from three replicate experiments.

^{b,c,d}Values with different superscripts within a column are significantly different ($P < 0.01$).

VS: vitrification solution.

the ratio of the oocytes that showed normospermy to the total number of oocytes treated (Table 1). The normospermic fertilization rate of the oocytes after exposure to 0.25 and 0.5 mol sucrose l^{-1} was significantly different ($P < 0.01$) from 0.25 and 0.5 mol trehalose l^{-1} , respectively (54% versus 70% and 44% versus 62%). The difference was much higher after exposure to 1 mol of sucrose and trehalose l^{-1} . Only 32% of oocytes were normally fertilized after exposure to 1 mol sucrose l^{-1} but 63% showed normospermy after exposure to 1 mol trehalose l^{-1} . The incidence of polyspermy was therefore high after exposure to solutions of 0.5 mol l^{-1} of both sugars; about 30% of polyspermy was found in the two groups.

Effects of permeating cryoprotectants

The oocytes exposed to propylene glycol or DMSO showed maximum shrinkage to 68% of the isotonic volume within 4 min (Fig. 2). During re-expansion, they reached a volume equal to the isotonic volume in 10 min. The same behaviour was observed in the oocytes exposed to the mixture of PG plus DMSO (Fig. 3).

On the contrary, the equilibration time of immature bovine oocytes in glycerol or in its mixtures with PG or DMSO was about 90 min and the minimal volume reached in 2–8 min was lower than 50% of the isotonic volume (Figs 2 and 3). These

results are very similar to the equilibration time reported by Myers *et al.* (1987) for bovine oocytes at the germinal vesicle stage in TL-Hepes plus 1 mol glycerol l^{-1} . The oocytes exposed to propylene glycol showed a fertilization rate not significantly different ($P > 0.01$) from that of the control group (Table 2). Only six of the 50 oocytes exposed to glycerol were fertilized; the viability of the oocytes after exposure to DMSO was very low (8% of them had two pronuclei). In addition, many of the oocytes of these two groups degenerated (34 out of 50 for the glycerol group and 42 out of 50 for the DMSO group). The number of activated oocytes did not exceed 1% in either group.

Vitrification of immature bovine oocytes

The results of examination by microscope of oocytes matured and fertilized *in vitro* after being vitrified in the vitrification solution (VS) containing 40% w/v propylene glycol and 0.25 mol trehalose l^{-1} in PBS supplemented with 4% BSA are shown (Table 3). After vitrification, 54% (46 of 97) of the oocytes were fertilized and 37% showed normospermy. This value was significantly different from that of the control solution group in which 80 of 120 (69%) fertilized normally. Exposure of oocytes to the vitrification solution caused a slight loss of viability and a decrease in fertilization ability. However, there was no significant difference between the fertilization rate

of the oocytes exposed to the vitrification solution for 10 min (solution effect) or for the real time (16 min) needed for the vitrification procedure (exposure to VS in straws).

Discussion

The presence of cryoprotectant in the vitrification solution decreases the probability of intracellular crystallization which is considered to cause most damage when very rapid cooling takes place, but the high concentration of the cryoprotectant required is toxic and causes osmotic injury to the oocytes even without cooling.

Different methods have been used to reduce this 'solution effect': (i) short time of exposure to cryoprotectants (Fahy *et al.*, 1984; Arav *et al.*, 1988), (ii) use of low toxicity cryoprotectants (Rall, 1987) or mixtures of them (Massip *et al.*, 1986), (iii) addition of nonpermeating cryoprotectants (Fahy *et al.*, 1984), (iv) reduction of the cryoprotectant concentration (Rall, 1987), (v) exposure at low temperatures (Rall, 1987).

The use of nonpermeating cryoprotectants is very useful either because the shrinkage of the oocyte and consequently the amount of water inside the cell that may crystallize during rapid cooling and warming is lower (Rall, 1987) or because of the reduction of the amount of the cryoprotectant that penetrates the cell thus reducing the possible toxic effect (Szell and Shelton, 1987). In addition, the carbohydrates used as a non-permeating cryoprotectant have a stabilizing effect on membranes (Crowe *et al.*, 1983). In the study reported here trehalose was less harmful than sucrose. Determination of the Boyle Van't Hoff relationship for both sucrose and trehalose produced the same regression line, so it is possible that this beneficial effect could be a consequence of its interaction with the membrane (Crowe *et al.*, 1983). In addition, higher survival rates were obtained after exposure to sucrose or trehalose solutions than to the solution reported by Heyman *et al.* (1986) in which immature bovine oocytes were already sensitive to 0.25 mol sucrose l^{-1} . The exposure to even 0.5 mol sucrose and trehalose l^{-1} and the consequent volume reduction of 50% did not affect the ability of immature bovine oocytes to mature and fertilize normally *in vitro*. An increase in the frequency of polyspermic fertilization was observed; volume changes induced by the cryoprotectant may alter the spatial relationship between cortical granules and the oolemma, thus impairing their exocytosis at fertilization and therefore the block to polyspermy.

Only 10 min of exposure are required for equilibration in propylene glycol and DMSO solutions or mixtures of them. The membrane is very permeable to both of them. The one-step exposure to these cryoprotectants is not likely to cause marked osmotic damage to the cell as the volume is reduced only up to 68% of the isotonic volume. Step-wise addition of the cryoprotectant therefore seems unnecessary.

The exposure to glycerol solution causes injury to the membrane that is probably attributable to the considerable shrinkage during the long period of equilibration. Titration of the glycerol solution seems a good approach to attain major viability after vitrification (data not shown). In addition mixtures of propylene glycol or DMSO with glycerol produced the same osmotic effect and are not suggested.

DMSO was the most harmful of the cryoprotectants used in this study, as few oocytes exposed to a 40% w/v DMSO underwent IVF after IVM. It probably has a toxic effect on the oocytes. In addition, vitrification solutions based on 40% w/v DMSO or glycerol were devitrified upon warming (data not shown). By contrast, the vitrification solution based on propylene glycol (40% w/v) did not show any type of crystallization that could be detected by the cryomicroscope. The results of the vitrification provide evidence that propylene glycol can be used successfully. Indeed, the IVF rate of the oocytes vitrified in a solution containing 40% w/v propylene glycol was 37% and is not different from the results obtained by Schellander *et al.* (1988) using a slow freezing protocol.

Rall *et al.* (1989) successfully increased the viability of vitrified mouse embryos by reducing the concentration of the cryoprotectant (90% of vitrification solution). However, concentrated solutions of permeating cryoprotectant are required for successful cryopreservation of oocytes when rapid cooling and warming rates are used. In earlier reports on immature pig oocytes, we showed that when lower concentrations of cryoprotectant are used, despite apparent vitrification, membrane destruction was unavoidable (Rubinsky *et al.*, 1991). In this study, evaluation of the oocytes by microscope after vitrification did not reveal any membrane damage. Moreover, in the study reported here, no increased incidence of polyspermy was seen in the vitrified oocytes. Exposure of the oocytes to the vitrification solution caused a loss of viability probably because of the initial osmotic shock. Propylene glycol does not seem to have a toxic effect as prolonged exposure to vitrification solution did not cause any further loss of viability. The decrease in fertilization rate was probably due to zona hardening brought about by exposure to cryoprotectants but this needs to be investigated further. No increase in parthenogenetic activation of the oocytes was observed as a result of exposure to cryoprotectants. Rubinsky *et al.* (1992) reported that antifreeze glycoproteins could protect cell membranes from damage caused by low temperatures. The viability of immature pig oocytes and of pig and mouse embryos was increased when antifreeze glycoproteins were added to the vitrification solution at a concentration of 40 mg ml^{-1} .

We suggest that the use of this glycoprotein together with the vitrification solution proposed here could be a great challenge for reaching a successful cryopreservation of immature bovine oocytes.

This work was supported by the National Research Council of Italy, Special Project RAISA, sub-project No. 3, Paper No. 738.

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