Anim. Reprod., v.4, n.3/4, p.113-118, Jul./Dec. 2007

Effect of different cryoprotectant concentrations for ultrarapid freezing of immature goat follicular oocytes on their subsequent maturation and fertilization *in vitro*

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Abstract

The *in vitro* maturation, fertilization, and morphological changes of goat cumulus–oocyte complexes (COCs) cryopreserved by ultrarapid freezing using DPBS + 0.5% sucrose + 0.4% BSA and 4 M, 6 M, 8 M, or 10 M concentrations of glycerol (G) or ethylene glycol (EG) were studied. COCs were cryopreserved by ultrarapid freezing by holding them over LN_2 vapor for 2-3 min and then plunging them into LN_2. After 7–10 d of storage, the COCs were rewarmed and used for *in vitro* maturation or fertilization in 2 experiments to record morphological damage due to ultrarapid freezing, nuclear maturation 28 h after culture, and fertilization 24 h after insemination. Freshly collected COCs were matured and fertilized simultaneously and kept as controls. The proportions of COCs that were recovered in morphologically normal form were highest for oocytes vitrified in 6 M concentrations of both G (90.8%) and EG (95.0%). The proportions of oocytes in morphologically normal form increased with increasing concentrations of both G and EG up to 8 M but at 10 M, the proportion of normal oocytes decreased significantly (70.7% for G and 72.0% for EG; $P < 0.05$). At the end of experiments 1 (n = 566) and 2 (n = 1019), nuclear maturation and fertilization of oocytes was significantly higher ($P < 0.05$) for fresh oocytes (M-II: 65.62%; Fert: 40.8%) than for oocytes frozen by ultrarapid freezing (M-II:8.0%,21.1%, and 25.0% for 4MG, 6MG and 8MG and, 22.6%, 34.3% and 41.9% for 4MEG, 6MEG and 8MEG respectively; Fert: 5.4%, 14.6% and 17.2% for 4MG, 6MG and 8MG and, 15.2%, 25.7% and 31.5% for 4MEG, 6MEG and 8MEG respectively). Nuclear maturation and fertilization of vitrified oocytes increased with increasing concentration of both G and EG up to 8 M concentration, but at 10 M concentration, the proportion of oocytes matured (6.4% for G, 8.4% for EG) or fertilized (4.2% for G, 5.6% for EG) decreased significantly ($P < 0.05$). EG was found to be a better cryoprotectant for ultrarapid freezing of goat oocytes as evident by significantly higher proportions ($P < 0.05$) of oocytes maturing and fertilizing in EG compared to G at all concentrations tested. It was concluded that ultrarapid freezing causes morphological damage to oocytes. The optimum ultrarapid freezing cryoprotectant is up to 8 M concentration of G and EG, with EG exceeding G.

Keywords: ethylene glycol, goat, glycerol, oocytes, ultrarapid freezing.

Introduction

There are innumerable protocols that have been used to cryopreserve cells and tissues of many types, including vitrification. During the cryopreservation process, cells experience several changes in their milieu: water is removed from the solution in the form of ice; consequently solutes become more concentrated and can precipitate; the cell responds by losing water. Researchers have debated whether changes in temperature, several solution effects, or both are the cause of cellular damage and cell death during cryopreservation (Mazur, 1970; Karrow and Critser, 1997; Fuller *et al*., 2004). Luyet was the first to describe the use of vitrification for the preservation of tissues (Luyet, 1937). The methods of vitrification involve the use of high concentrations (5-7 M) of cryoprotectants (CPAs) and ultrarapid cooling (Purohit *et al*., 2003). Cells are suspended in CPA and plunged directly into LN_2 forming a glass-like suspension of cells. This technique completely eliminates intracellular ice formation (Porcu, 2001). However, cells may be damaged by exposure to such high concentrations of CPA (Eroglu *et al*., 1998). Vitrification of immature oocytes may circumvent some problems of vitrifying mature oocytes. This would also help in establishment of oocyte banks from oocytes recovered from dead/slaughtered animals. In spite of a large number of reports on the vitrification of oocytes (Kasai *et al*., 1979; Lim *et al*., 1992; Vieira *et al*., 2002; Kharche *et al*., 2005), the results obtained so far have been modest, and ultrarapid freezing procedures need substantial improvement. The objectives of the study were to cryopreserve goat follicular oocytes by ultrarapid freezing and determine their morphological survival and subsequent *in vitro* maturation and *in vitro* fertilization.

Materials and Methods

Cumulus oocyte complexes (COCs) were collected by aspiration of surface follicles (2-8 mm) present on goat ovaries (n = 929) collected from a local abattoir. COCs with an evenly granulated cytoplasm and 3-4 or more layers of cumulus cells attached were selected (n = 2239) for further work.

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Received: June 11, 2007
Accepted: January 11, 2008

Oocyte cryopreservation

Freshly collected COCs were cryopreserved by ultrarapid cooling as described previously (Yadav *et al*., 2007) with some modifications. The ultrarapid freezing solution (VS) comprised of Dulbeccos phosphate buffered saline (DPBS) supplemented with 0.5 M sucrose, 0.4% bovine serum albumin (BSA) and different molar (M) concentrations of the cryoprotectant glycerol (G) and ethylene glycol (EG) was tested as an ultrarapid freezing cryoprotectant. The chemicals used were from Sigma Chemical Co (St. Louis, USA) unless mentioned otherwise. The concentrations tested were 4 M, 6 M, 8 M and 10 M of both G and EG. COCs were randomly allocated to one of the ultrarapid freezing solutions for cryopreservation. The oocytes were pre-equilibrated in 50% of VS in DPBS for 3-5 min and then kept in VS for 1 min and loaded (4-5 oocytes per straw) in pre-sterilized 0.25 cc straws (IMV, France). The straws were heat sealed and pre-cooled by keeping the straws over LN2 vapor for 2 min at the height of about 5 cm from LN_2 level. The straws were then plunged in LN_2 and stored for 7-10 days.

Warming and evaluation

Frozen straws containing the COCs were warmed in a water bath at 38°C for 5 sec. The contents of the straw were emptied in a 35 mm petri dish. The cryoprotectant was removed by placing oocytes in 50% VS and then transferring to DPBS. The oocytes were evaluated for morphological damage within 30 min of warming. The oocytes were considered abnormal when there was change in shape, breakage of zona pellucida, uneven granulation or leakage of oocyte contents. The morphologic survival percent was calculated by the proportion of oocytes seen to be normal against the total number vitrified. The morphologically normal oocytes were matured and fertilized *in vitro* in two experiments to record their *in vitro* maturation and fertilization. In experiment 1, morphologically normal oocytes (n = 566) were matured *in vitro* (9 replicates with 5-10 oocytes per replicate) for 28 h and evaluated at the end of the experiment for nuclear maturation. Freshly collected oocytes were simultaneously matured *in vitro* and kept as control (n = 64).

In experiment 2, oocytes were cryopreserved by ultrarapid freezing and those recovered in normal form were matured in vitro and then fertilized *in vitro* (n = 1204). Fertilized oocytes were evaluated for fertilization by fixing and staining (n = 1019). Freshly collected oocytes were also evaluated for fertilization (n = 115) and kept as control.

In vitro maturation

Oocytes were matured *in vitro* in 50 µl of TCM-199 (5-10 oocytes/drop) supplemented with 5 µg/ml FSH, 5 µg/ml LH and 1 ng/ml oestradiol, 25 mM Hepes, 0.25 mM Pyruvate and antibiotics (Streptomycin 50 µg/ml and penicillin 500 IU/ml) and by keeping them in an incubator with 5% CO_2 and 39.5ºC temperature as previously described (Kumar and Purohit, 2004; Purohit *et al*., 2005). After 28 h of *in vitro* culture, all oocytes from different groups and replicates were fixed and stained (Purohit *et al*., 2005) and evaluated.

Sperm preparation and in vitro fertilization (IVF)

Frozen-thawed buck semen was prepared for IVF using previously described methods (Palomo *et al*., 1999) with some modifications. Briefly, two frozen semen straws were thawed in a water bath at 38°C for 30 sec and emptied in a centrifuge tube. Four milliliters of Hepes-TALP medium was added to the tube and the tube was centrifuged at 200 x g for 10 min. After discarding the supernatant, an aliquot of sperm pellet was resuspended (1:1) with Hepes-TALP medium. Swim-up in sperm-TALP medium was achieved using 50 µl aliquots of spermatozoa placed at the bottom of a conical tube containing 2 ml of Hepes-TALP medium. After 1 h of placing them at 38.5°C when the actively motile sperms swam up, 0.5 ml of the sperm suspension was collected from the upper part of the tube and centrifuged at 200 x g for 10 min. After discarding the supernatant, the resulting sperm pellet was re-suspended with heparin containing (100 µg/ml) Hepes-TALP medium and incubated for 45 min at 38.5°C.

The sperm concentration was assessed in a haemocytometer and the sperm pellet was resuspended in TALP to give a final concentration of 3 x 10^9 sperms/ml. The COCs were transferred to another dish containing Fert-TALP medium (TALP supplemented with 30 µg penicillamine/ml, 15 mM hypotaurine/ml) under paraffin oil. This was inseminated with prepared sperm in a volume, so as to give a final concentration of 4 x 10^6 sperms/ml. Following co-incubation for 20-24 h with sperm, oocytes from each treatment group were washed with fresh medium and vortexed for 1 min to separate the cumulus mass. They were prepared for fixing and staining in the same way as oocytes were fixed after IVM. The fertilization was evaluated as per previous method (Purohit *et al*., 2005).

Statistical analysis

The proportion of morphologically normal oocytes recovered between various groups was compared by Chi-square test. The arcsin transformed data of the proportion of oocytes matured and fertilized over various treatment groups were compared by Duncans New Multiple Range test (Purohit *et al*., 2005).

Results

Morphologic survival

In experiments 1 and 2, the morphologic survival of oocytes was 84.9% (649/764) and 84.6%

(1019/1204), respectively. The highest proportions of morphologically normal oocytes were seen in 6 M EG (95.0%) and 8 M EG (94.0%) and the lowest were in 10 M G, in both experiment 1 and 2. Nonsignificant (P > 0.05) differences were seen between the COCs recovered in morphologically normal form for equal concentrations of both G and EG. Pooled data of morphologic survival of oocytes in the various concentrations of G and EG or their combination are shown in Table 1.

Table 1. Morphologic survival of goat oocytes after ultrarapid freezing in varying concentrations of glycerol and ethylene glycol.

Vitrification solution	Percentage of morphologically normal oocytes
4 M G	82.10^{abd}
6 M G	90.74^{ac}
8 M G	86.40^{ac}
10 M G	70.78^{bd}
4 M EG	87.50^{ac}
6 M EG	95.0^{ce}
8 M EG	94.0^{ce}
10 M EG	72.04^{d}

Values in same column with different superscripts differ significantly.
Chi-square test (P < 0.05).
Pooled data of two experiments.

In vitro maturation

In experiment 1, the oocyte maturation was significantly lower (P < 0.05) in all the ultrarapid freezing cryoprotectants compared to control. The proportion of oocytes attaining M-II increased significantly (P < 0.05) up to 8 M G in the G treatments compared to 4 M G. In the EG treatments, the proportion of oocytes attaining M-II was significantly (P < 0.05) higher at 8 M concentration compared to 4 M, and there was no difference at 6 M compared to 4 M concentration. However, at 10 M concentration of both G and EG, the nuclear maturation rates decreased significantly (P < 0.05). EG was found to be a better cryoprotectant compared to G as evident by significantly higher (P < 0.05) proportion of oocytes reaching M-II at equal concentration of EG (4 M, 6 M, 8 M and 10 M) as compared to G.

In vitro fertilization

In experiment 2, the proportion of total oocytes fertilized and the proportion of normally fertilized oocytes at the end of experiment was significantly higher (P < 0.05) for control oocytes as compared to oocytes frozen by ultrarapid freezing (Table 2). A dose dependent significant increase (P < 0.05) in the proportion of total oocytes fertilized was seen up to 6 M concentration in both G and EG. However, at 8 M concentration of both G and EG, the proportion of cryopreserved total oocytes that fertilized was not different. Conversely, at 10 M concentration of both G and EG the proportion of cryopreserved oocytes that fertilized decreased significantly. At equal concentrations (4 M, 6 M, 8 M, and 10 M), significantly higher proportion of oocytes were fertilized in EG compared to G. The proportion of oocytes that evidenced normal fertilization was not significantly different between the various treatment groups.

During the present study, the highest proportions of morphologically normal oocytes were seen in the 6 M EG cryoprotectant treatment. The proportion of oocytes in morphologically normal form increased as the concentration of CPA increased, but at 10 M G and 10 M EG the proportion of morphologically normal oocytes decreased significantly (P < 0.05).

Table 2. *In vitro* maturation and fertilization of goat oocytes cryopreserved by ultrarapid freezing in different concentrations of glycerol (G) and ethylene glycol (EG).

Ultrarapid freezing solution	*In vitro* maturation (%)^{A}	*In vitro* fertilization (%)^{B}
Control	65.62^{e} (42/64)	40.86^{d} (47/115)
4 M G	8.0^{a} (6/75)	5.46^{a} (7/128)
6 M G	21.17^{b} (18/85)	14.61^{b} (19/136)
8 M G	25.0^{bc} (17/68)	17.21^{b} (21/122)
10 M G	6.42^{a} (4/62)	4.23^{a} (5/118)
4 M EG	22.66^{bc} (17/75)	15.26^{b} (20/131)
6 M EG	34.32^{cd} (23/67)	25.71^{c} (36/140)
8 M EG	41.89^{d} (31/74)	31.49^{c} (40/127)
10 M EG	8.33^{a} (5/60)	5.69^{a} (7/123)

Values in same column with different superscripts differ significantly, DNMR-test (arcsin transformed data; P < 0.05).
Data pooled from 9 replicates (A) and 10 replicates (B).
Figures in parantheses represent number of oocytes at M-II in column 2 and total fertilized oocytes in column 3/total oocytes fixed.

Discussion

The present study indicated that a high proportion of oocytes retain their normal morphology after a short exposure to high concentrations of different CPAs, except 10 M concentrations. The proportion of oocytes recovered in morphologically normal form after 7-10 days of their cryo-storage are similar to previous findings of Agarwal (1999) who found only 304 oocytes exhibiting damage changes from the 323 vitrified goat oocytes recovered. Studies by Begin *et al*. (2003) have shown that the oocytes and embryos vitrified by the solid surface vitrification (SSV) had a significantly lower survival rate than control whereas, the survival rate of cryo-loop vitrification (CLV) oocytes and embryos did not differ significantly from the control. The present results are also similar to previous findings on buffalo oocytes (Dhali *et al*., 2000b; Wani *et al*.,2004a; Yadav *et al*., 2007). The damage to oocytes during cryopreservation may be because of the large lipid-like material found in oocytes of many species, since lipid removal or lipid polarization reduces chill and cryo-injury (Otoi *et al*., 1997). The mechanisms of cell damage have been explained widely. Mazur *et al*. (1972) indicated that hyperosmotic stress might cause a net leak/influx of non-permeating solutes. When cells are returned to iso-osmotic conditions, they swell beyond their isotonic volume and lyse.

The *in vitro* maturation and fertilization of the vitrified immature goat oocytes were significantly lower compared to controls in the present study. Similar to the present findings, Kharche *et al*. (2005) found that goat oocytes vitrified using propanediol (40% w/v) and trehalose (0.25 mol/l) exhibited a lower *in vitro* maturation and fertilization compared to controls or denuded oocytes after warming, but a similar maturation and fertilization rate to oocytes exposed to propanediol without freezing. Wani *et al*. (2004b) recorded that less number of buffalo oocytes reached metaphase-II for oocytes cryopreserved in different concentrations of DMSO, EG, PROH, and glycerol compared to fresh oocytes. Likewise, Sharma and Purohit (2007) found that *in vitro* maturation and fertilization of buffalo oocytes was lower for vitrified oocytes compared to controls. The *in vitro* maturation and fertilization rates of vitrified immature goat oocytes obtained during the present study agree with studies by Agarwal (1999). The *in vitro* fertilization rates obtained in the present study resemble previous reports (Saxena and Maurya, 1999; Begin *et al*., 2003) on goat oocytes.

A dose-dependent increase in the proportion of oocytes that survived morphologic damage and subsequently matured or fertilized *in vitro* suggests that the optimum concentration of both the cryoprotectants is 8 M. Many publications on the problems of mammalian oocyte cryopreservation contain information regarding the negative effects of low temperature, including the cytoskeleton depolymerization effects of permeable cryoprotectants (Pickering and Johnson, 1987; Vincent *et al*., 1989; Aigner *et al*., 1992; Joly *et al*., 1992; Yoon *et al*., 2000). Such effects may be more pronounced with high concentrations of the two permeable cryoprotectants used in the present study. The freeze-thaw process is known to induce an alteration in the physico-chemical properties in the intra-cellular lipids (Isachenko *et al*., 2001; Kim *et al*., 2001) and such damages may render the oocyte incapable of retaining its developmental competence. Despite the protective effects of cryoprotectants during freezing they may impose concentration, time and temperature dependant toxicity (Fahy *et al*., 1990).

The findings of better performance of ethylene glycol as a ultrarapid freezing cryoprotectant, compared to glycerol and the optimum concentration of both the cryoprotectants being 8 M during the present study suggests that there is a limit to the concentration of the cryoprotectant to be used beyond which it may exert toxic effects. This is in part similar to previous findings of Wani *et al.* (2004b). These workers observed that the highest IVM of buffalo oocytes was observed for oocytes vitrified in 7 M solution of all the cryoprotectants and that EG was better compared to glycerol. Ethylene glycol has been found to be an effective cryoprotectant for the ultrarapid freezing of mouse (Miyake *et al*., 1993), cattle (Saha *et al*., 1996) and buffalo (Dhali *et al*., 2000a, b; Wani *et al*., 2004b, Sharma and Purohit, 2007; Yadav *et al*., 2007) oocytes. Ethylene glycol offers advantages over other cryoprotectants in terms of higher permeation into oocytes/embryos for ultrarapid freezing and faster removal during dilution, as its molecular weight is lower than that of glycerol (Dhali *et al*., 2000b). Glycerol is known to induce severe osmotic damage to the cytoplasm of the cell due to its low membrane permeability (Szell *et al*., 1989). Ethylene glycol on the contrary, has been found to be less toxic than glycerol and propylene glycol (PG) to mouse embryos (Kasai *et al*., 1990), and the post-ultrarapid freezing survival of bovine embryos has been found to be much higher in ethylene glycol than in either a combination of DMSO, PG and polyethylene glycol or a combination of glycerol and PG (Mahmoudzadeh *et al*., 1993). The survival of *in vitro* produced ovine embryos vitrified in EG has been reported to be better compared to IVP ovine embryos vitrified in G (Martinez and Matkovic, 1998; Zhu *et al*., 2001). Dattena *et al*. (2004), however, found no difference in the pregnancy rates after transfer of *in vitro* produced or *in vivo* derived ovine embryos vitrified in either EG or G.

It was concluded that ultrarapid freezing brings morphological damage to oocytes. The optimum ultrarapid freezing cryoprotectant is up to 8 M concentration of G and EG, with EG exceeding G.

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