



Effect of vitrification and slow freezing on *in-vitro* matured prepubertal goat oocytes

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Cryopreservation of oocytes has important role in the preservation and management of genetic resources, rapid dissemination of germplasm via *in-vitro* embryo production, genetic engineering and nuclear transfer procedures. It is successfully used in livestock including cattle, goats, sheep, and other model animals, but has met with limited practical success in goat. Oocytes are intrinsically very sensitive to cooling, since cryopreservation can alter the meiotic spindle assembly, microtubules, cortical granule distribution, zona pellucida characteristics, and cause chromosomal aberrations (Larman *et al.* 2006), hence it is important to determine the development ability of oocytes by vitrification or slow cooling procedures. The vitrification technique prevents cell damage caused by intracellular ice crystal formation. It is faster, cheaper, and more effective (Al Yacoub *et al.* 2010), in slow freezing the cooling rate is slow and uses cryoprotectant agents. It allows solution exchange between the intracellular and extracellular compartments without inducing serious osmotic effects. The present study was designed to determine the effect of conventional slow freezing and vitrification on post-thaw morphology and cleavage rate of prepubertal matured goat oocytes.

In the present experiment, goat ovaries were collected from a local slaughterhouse and transported to the laboratory within 3–4 h in a thermos flask containing sterile warm normal saline solution supplemented with antibiotics (100g/ml streptomycin and 100IU/ml penicillin). Each ovary was rinsed with warm saline fortified with antibiotics for 5–6 times and transferred into laminar flow. The oocytes were collected from each ovary by slicing technique into a petriplate containing Dulbecco's phosphate buffered saline (DPBS). Each petri dish containing oocytes was evaluated under a stereo zoom microscope and graded as per Kharche *et al.* (2008a). Granulosa cell monolayer and granulosa cell co-culture was established (Kharche *et al.* 2008a, b); half of the medium was replaced with fresh medium for every

48 h. After isolation and selection, oocytes were matured in tissue culture medium 199 supplemented with sodium pyruvate (0.25 mM), L- glutamine (1mg/10ml), gentamycin sulphate (50 µg/ml), 10% Fetal bovine serum and no hormones. The oocytes were cultured in GCC layer and incubated in 5% CO₂ under humidified air at 38.5°C for 27 h. The matured prepubertal goat oocytes were assigned for freezing in following groups after denudation.

Group 1- Vitrification: Good quality oocytes (125) were equilibrated at one step in vitrification solution for 10 min. The vitrification solution comprises propylene glycol (40% v/v) and trehalose (0.25mol/l) in TCM 199-HEPES, supplemented with (0.4% w/v) BSA. After equilibration oocytes were immediately loaded into 0.25 ml French mini straws having 10–12 oocytes per straw with 1M sucrose/l solution in a minimal fluid (100µl). The straws were sealed with polyvinyl chloride powder (PVC) and stored for 1 year in liquid nitrogen. For thawing, straws were removed from liquid nitrogen and placed horizontally in a water-bath at 37°C for 20 sec, wiped straws allowed into thawing media for washing (5–6 times) comprising 1M sucrose/l in BSA free TCM to remove the cryoprotectant from the cells. The oocytes were allowed for 1 min equilibration in fresh TCM-199 then in 1.25 M sucrose solution for 1 min, after which the oocytes were exposed to decreasing concentrations of sucrose (0.62, 0.42 and 0.31 M) for 30 sec (Gasparrini *et al.* 2007). Oocyte survival, post-warming was evaluated on the basis of integrity of oocyte membrane and zona pellucida together with homogeneity of cytoplasm. The survived oocytes were washed for 5–6 times in the maturation medium and cultured in 50 µl drop of embryo development medium containing 10% FBS and 3 mg/ml BSA in CO₂ incubator (at 5% CO₂, high humidity and 38.5°C) for further development.

Group 2- Slow cooling: Good oocytes (120) were equilibrated in freezing solution for 10 min at room temperature. The freezing solution consisted of 1, 2 propanediol (1.5M) in TCM-199 supplemented with 10% fetal bovine serum (FBS). After 1 min equilibration 6–8 oocytes were loaded into 0.25 ml French mini straws and open end was sealed with polyvinyl powder (PVC). The oocytes were frozen in embryo freezer from room

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temperature (25°C) to -7°C @ 4°C/min, seeded at -7°C for 10 min. The oocytes were subsequently cooled from -7°C to -35°C @ 0.3°C/min and then plunged into liquid nitrogen. The straws were stored for 1 year. For thawing, straws were removed from liquid nitrogen and placed horizontally in a water-bath at 37°C for 20 sec, wiped straws allowed into thawing media (0.25M sucrose in TCM 199 without any supplements) and equilibrated for 10 min. The oocytes were washed for 5–6 times in the maturation medium then cultured in 50 µl drop of embryo development medium containing 10% FBS and 3 mg/ml BSA in CO₂ incubator (at 5% CO₂, high humidity and 38.5°C).

Group 3– Control (without freezing): *In vitro* matured oocytes (172) were subjected to *in vitro* fertilization. The frozen, thawed matured oocytes (expanded cumulus and presence of first polar body) were treated with PBS containing 0.1% hyaluronidase and gently pipette through a fine bore pipette so as to remove cumulus cells. The denuded oocytes were washed 8–10 times in fertilization TALP medium containing 20% estrus goat serum, 10 µg/ml heparin, pre-equilibrated in CO₂ incubator at 38.5°C for 2 h. After washing 10–15 oocytes were placed in 50 µl drops of Fert-TALP medium in a small culture dish and the drops were covered with sterile mineral oil (Kharche *et al.* 2011). After capacitation, an aliquot of 50 µl of sperm suspension was added to each droplet of the Fert-TALP having matured oocytes and subsequently incubated for 18 h at 38.5 °C, 5% CO₂ in humidified atmosphere (Kharche *et al.* 2008b). After 18 h of sperm-oocyte co-incubation, the oocytes were washed in embryo development medium (deprived of FBS and BSA) and then cultured in 50 µl drop of embryo development medium containing 10% FBS and 3 mg/ml BSA at 5% CO₂, high humidity and 38.5°C. After 48 h of post insemination, oocytes were observed for cleavage and half of the culture medium was replenished with fresh medium. Embryos were morphologically evaluated under inverted phase contrast microscope to separate retarded embryos from normally developing embryos.

The viability of *in-vitro* matured prepubertal goat oocytes was checked with Typhon blue (dye). The oocytes were kept for 5 min in 0.05% dye, then washed with 1 ml drop of phosphate buffer saline for 5–6 times. The viable oocytes remained unstained while nonviable took blue colour; 38% viability was reported in fresh prepubertal goat oocytes.

Analysis of the data was carried using chi square test (χ^2). Cleavage rates between the different treatment groups were compared using the Chi-square test. The level of significance was recorded at the 5% level of confidence (Snedecor and Cochran 1989).

The average recovery of good quality prepubertal goat oocytes for IVM was 1.80 / ovary by puncture method. The oocytes recorded 95.0% maturation rate. The post thaw recovery of morphological normal mature prepubertal goat oocytes in vitrification, slow freezing and control groups were 69.67, 85.0 and 100%, respectively (Table 1). The cleavage rates of the *in-vitro* matured oocytes in the different groups (Table 1) were 1.14%, 3.92%, and 13.37% for vitrification, slow freezing and control group, respectively. While viability for control oocytes was 38%. Significant differences ($P < 0.05$) were recorded between treatments.

Oocytes are able to survive ultra-rapid vitrification procedures, but their further development are impaired due to morphological and biochemical alterations. Vitrification solutions have high concentration of cryo-protective agents (CPAs) that cause biological and physico-chemical toxic effects (Berlinguer *et al.* 2007). Vitrification solutions containing trehalose were used to cryopreserve oocytes from goats (Begin *et al.* 2003) and bovine (Abe *et al.* 2005). Trehalose is effective in stabilizing frozen and dried cells, both *in vivo* and *in vitro* it also protects bio structures from damage because of dehydration, heat or cold (Lins *et al.* 2004). Berlinguer *et al.* (2007) suggested that trehalose could behave like an osmolyte to cope with osmotic changes. Other possibilities include exocytosis of trehalose or its inert presence in the cytoplasm until its dilution by cell division. Trehalose could be interconverted by oocyte in glucose then metabolized particularly after 2-cell stage. We obtained 69.67% normal oocytes after thawing with intact zona pellucida. Morphological evaluation of the vitrified cumulus enclosed oocytes after thawing indicated that the oocytes had a normal morphological appearance with intact zona pellucida and expanded cytoplasm. These results can be compared to the previous reports in oocytes of bovine (Otoi *et al.* 1998), prepubertal sheep (Succu *et al.* 2007a) and in mouse (Men *et al.* 1996). However, the existing results are lower than that reported by Gasparrini *et al.* (2007), Hou *et al.* (2005) and Sharma *et al.* (2006), may be due to freezing procedure used and the steps involved therein, as well as the osmotic shock. In our study

Table 1. Effect of cryopreservation methods on post thaw recovery and IVF of matured prepubertal goat oocytes

Groups	No of oocytes frozen	Warmed/ frozen thawed oocytes showing morphologically abnormal		Proportion of oocytes showing morphologically normal (n)	Proportion of oocytes cleaved / n	Cleavage (%)
		Cracked Zona Pellucida (%)	Shrunk cytoplasm (%)			
Group 1(vitrification)	125	15 (%)	23 (%)	87 ^a (69.60)	1.0	1.14 ^a
Group 2(slow freezing)	120	04 (%)	14 (%)	102 ^b (85.00)	4.0	3.92 ^a
Group 3(control)	172	-	-	172 ^c (100.00)	23.0	13.37 ^b

Values bearing different superscript in the column were significantly different ($P < 0.05$).

1.14% vitrified oocytes were found cleaved *in vitro*. This rate of cleavage was lower than the results reported in oocytes of caprine (Sharma *et al.* 2006) and bovine (Otoi *et al.* 1998). Moreover, the cleavage rates of vitrified oocytes were significantly lower than that of control group. This is similar to the studies of Succu *et al.* (2007b) and Gasparrini *et al.* (2007). The probable reasons for contrasting results could be that vitrified oocytes were less permeable to cryoprotectant. Vitrification in large volumes of solution can damage DNA in cumulus oocyte complexes (COCs) and affect oocyte developmental competence (Succu *et al.* 2007a); thus structural and biochemical damages caused by vitrification cooperate to impair the ability of oocyte to achieve the development stages (Belinguer *et al.* 2007, Purohit *et al.* 2012). Also, the ability of zygotes to cope up the vitrification and warming conditions leads to zona hardening due to cortical reaction which gives the stability to ooplasm membrane to withstand low temperature and osmotic shock. Morphological evaluation of slow freeze matured oocytes after thawing revealed that 85.00% of the oocytes had a normal morphological appearance with intact zona pellucida as compared to control (100%). As they are more sensitive to osmotic shock than cleavage stage embryos because of lower permeability to cryoprotectants due to which they undergo volume change during equilibration with cryoprotectants. Also the rate of volume change and the temperature at which it occurs plays a critical role in the survival of zygotes. Slow-cooling allows cells to be cooled to low temperatures minimizing intracellular ice formation and detrimental effect of increased solute concentrations. Ice should never be allowed to appear and grow inside the cells as this leads to damage and death of cells (Fasano *et al.* 2010). The cleavage rate of slow freeze oocytes were 3.92% significantly lower than the cleavage rate of oocytes of control group (13.37%). The cleavage rate reported in our study is lower to that reported by Otoi *et al.* (1992) and similar to Saunders *et al.* (1999) and Martino *et al.* (1994) might be due to no supplementation of hormones in culture medium.

When the cells are cooled at rapid rate water present inside the cell can not move out fast enough leading to the formation of intracellular ice crystals, which are lethal to the cell. If the cells are cooled too slowly, there may be severe volume shrinkage leading to high intracellular solute concentration, which have deleterious effects on the lipid-protein complexes of cell membranes (Rama Raju *et al.* 2006). In addition, the cells that are cooled slowly are potentially affected by chilling injury. Hence the rate of cooling and cryoprotectant concentration employed in the protocol should avoid the intracellular ice crystallization and high solute concentration, the 2 main events involved in cellular injury during cryopreservation (Rama Raju *et al.* 2006).

SUMMARY

The present study was conducted to study the post thaw recovery rate of morphologically normal matured oocytes

subjected to vitrification and slow freezing method. Oocytes were matured in TCM-199, which comprises sodium pyruvate (0.25 mM), L- glutamine (1 mg/10 ml), gentamycin (50 µg/ml) and supplemented with 10% fetal bovine serum at 38.5°C in CO₂ incubator under humidified air. After 27 h of *in-vitro* maturation oocytes were assigned for freezing, viz. vitrification, slow cooling and control cooling. The percentage of oocytes recovered after thawing was significantly higher for control than those for vitrification (69.67%) and slow freezing (85.00%), respectively. Moreover, the cleavage rate in vitrification, slow freezing and control were 1.14, 3.92 and 13.37%, respectively. Thus, the cleavage rate of oocytes in vitrification and slow freezing method did not differ significantly. However, there was a significant difference in cleavage rates of freezing groups and the control groups.

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