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Developmental competence of goat oocytes vitrified at immature and mature stage in comparsion to fresh oocytes after *in vitro* fertilization using cauda epididymal spermatozoa

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ABSTRACT

The oocytes were cryopreserved at 2 developmental check points, viz. immature and metaphase II stage, and the outcome of *in vitro* fertilization (IVF) was assessed as compared to fresh oocytes. The oocytes were cryopreserved by solid surface vitrification technique using equilibration media (4% ethylene glycol) followed by vitrification media (0.5 M sucrose + 35% ethylene glycol + 0.5% polyvinyl pyrollidone). The mature oocytes were pre-treated with cytochalasin B for 30 min before vitrification. The vitrified-thawed oocytes of both groups as well fresh oocytes were fertilized with buck epididymal sperm. The results revealed a significantly higher rate of embryonic development in vitrified-thawed immature oocytes as compared to that in vitrified-thawed mature oocytes at all the embryonic stages. The embryonic developmental rate under fresh oocyte group was significantly higher than both vitrified groups. Results indicated that the immature goat oocytes could be a better candidate for long term storage of female germplasm as well as dissemination into distant places.

Key words: Embryos, *In vitro* fertilization, Metaphase spindle, Oocytes, Vitrification

Oocytes are very susceptible to cryopreservation damage because of its structural integrity that differs from normal cells and sperm. Oocytes are generally cryopreserved at immature or mature/metaphase II (MII) stage. Cryopreservation of mature oocytes can result in damage to the metaphase spindle due to the temperature sensitivity of microfilaments and microtubules. Further, other morphological complexities may result into damage of meiotic spindle, actin filaments, chromosomal dispersal and microtubule depolymerisation (Baka *et al*. 1995). Cryopreservation of immature oocytes can circumvent this problem because these structures have not yet formed and the genetic material is enclosed within a nuclear envelope. However, on contrary the immature oocytes are thought to be more sensitive to anastomotic stress and have lower cell membrane stability than MII stage oocytes (Bogliolo *et al.* 2007). The success of cryopreservation of immature oocyte largely depends on the ability to preserve the structural and functional integrity of the entire oocyte. The cumulus cells play an important role in the maturation process by providing nutritive substances that have a supportive role during IVF (Massip and Donnay 2003). However, cumulus cells can also be an obstacle for penetration of cryoprotectants (Vajta 2000).

A large number of variables affect the process of vitrification of oocytes including type and concentration of protectants, stage of development of oocytes and the presence or absence of cumulus cells (Dattena *et al.* 2000). It has been observed that the immature germinal vesicle stage oocytes tolerate the cryopreservation damage more efficiently compared to oocytes at metaphase II and cumulus compact oocytes are less vulnerable to cryo-injuries compared to their denuded counterparts. Reports on the vitrification of caprine oocytes are less frequent and few of these studies have indicated the damage to *in vitro* matured oocytes when vitrified and subsequently fertilized *in vitro* (Sharma *et al.* 2006, Garg and Purohit 2007).

MATERIALS AND METHODS

All plasticwares were of tissue culture grade purchased from Becton Dickinson Labware (NJ, USA) and the chemicals, reagents and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

Collection of ovaries and cauda epididymis: Goat ovaries and cauda epididymes were collected from small animal slaughterhouse, Jabalpur, Madhya Pradesh. The biological samples were carried in sterile warm normal saline (37°C) and transported to the laboratory within 3 h. Ovaries in growth phase with neither corpus luteum nor follicles larger than 8 mm in diameter were selected for oocyte

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collection.The ovaries and cauda epididymis were trimmed and washed 5–6 times with sterile Dulbecco's phosphate buffer saline (DPBS).

In vitro *maturation of oocytes:* Cumulus oocyte complexes (COCs) were aspirated from 3–8 mm sized ovarian follicles. The COCs were matured *in vitro* as per standard protocol (Kumar *et al.* 2016). In brief, the good quality COCs were washed and incubated in maturation media consisted of TCM-199, 7.5% (v/v) FBS, 2.5 unit/ml follicle stimulating hormone, 2.5 unit/ml leuteinizing hormone, 1 µg/ml oestradiol, 0.8 mM/ml sodium pyruvate. The droplets containing oocytes were overlaid with sterile pre-equilibrated mineral oil and incubated under humidified atmosphere in 5% CO_2 at 38.5°C for 27 h.

Vitrification and thawing of immature oocyte: Immature oocytes were vitrified by solid surface vitrification (SSV) based on the protocol as standardized in our laboratory (Nema *et al.* 2007). Briefly, the oocytes were washed in DPBS + polyvinyl alcohol (PVA), followed by vortexing, so that only 2–3 cumulus layer remain intact. The oocytes were treated with equilibration media (EM) that consisted of 4% ethylene glycol (EG) in TCM-199 with 20% FBS for 15 min and subsequently in vitrification media (VM) (TCM-199 medium supplemented with 0.5 M sucrose, 0.5% polyvinyl pyrollidone, 35% EG and 20% FBS). The thawing/warming procedure of vitrified oocytes was performed on warming plate and all warming solutions were equilibrated in 5% CO_2 at 38.5°C temperature. The vitrified oocytes were thawed in to thawing media (TM) in 2 steps, 0.4 M and 0.2 M sucrose subsequently. The thawed oocytes were kept in holding media (TCM-199 supplemented with 20% FBS) for 5 min. After warming, the oocytes were matured *in vitro* under *in vitro* maturation media for 27 h as per the standard protocol.

*Vitrification and thawing of matured oocytes:*The matured oocytes were vitrified and thawed by using similar protocol as described for immature oocytes. However, the matured oocytes were pre-treated with Cytochalasin B (2.5 µM dissolved in TCM-199 with 20% FBS) for 30 min before vitrification.

Oocytes preparation for IVF: The matured oocytes (Fresh and Vitrified-thawed) were collected from IVM drops and transferred into fertilization drops overlaid with mineral oil. The oocytes were washed 3 times in the fertilization drops and finally incubated in groups of 15–20 oocytes/drop in CO_2 incubator at 38.5ºC and 5% CO_2 for at least 30 min before IVF.

Sperm processing for IVF: The sperms were harvested by multiple incisions of slaughtered buck (3–5 years old) cauda epididymis and the sperm were collected in a 15 ml centrifuge tube with 10 ml DPBS. Immediately after sperm harvesting, the concentration and gross motility of sperm were assessed. The sperm suspension was centrifuged at 1,000 rpm for 8 min. The supernatant was discarded and pellet was dissolved in 10 ml Bracket and Olliphant (BO) media (Parrish 2014). The suspension was recentrifuged at 1,000 rpm for 8 min. The pellet was again washed with 10 ml BO media having 0.3% Bovine serum albumin (BSA) with same centrifugation parameters. The sperm was resuspended in 5 ml of fertilization medium having 50 µg/ml heparin and 0.6% BSA in BO stock, followed by centrifugation @ 1,000 rpm for 5 min. Supernatant was discarded and the sperm pellet was transferred in 2 ml tube and made final volume up to 1.5 ml with fertilization media. The suspension was centrifuged @ 1,000 rpm for 1 min and sperm pellet was loosened with a fine bore of glass pasteur pipette and kept in incubator for 30–40 min. In the meantime, the matured oocytes of each group (Fresh and Vitrified-thawed) were transferred into fertilization medium @ 15–20 oocytes/drop overlaid with mineral oil. The oocytes were incubated in CO_2 incubator at 38.5ºC and 5% CO_2 for 30 min. From the top layer of sperm samples, aliquots of sperm suspension were added into the droplets of oocytes to achieve a final concentration of 10^6 sperm/ml. The oocytes and sperm together were co-incubated with sperm for 18 h under humidified atmosphere of 5% CO_2 at 38.5°C temperature.

In vitro embryo culture: After 18 h of co-incubation, presumptive zygotes were denuded by using 0.1% hyaluronidase and cultured sequentially in commercially available embryo culture media, viz. Research vitro cleave (RVCL, Cook®, Queensland, Australia) and Blastocyst medium (Cook®, Queensland, Australia) supplemented with 1% fatty acid free BSA as described by Kumar *et al.* (2014). The embryonic growth of each embryo group was assessed at different intervals of time under inverted microscope.

Statistical analysis: In the present study, total 15 IVF experimental trials were conducted employing five tests each for three oocyte group, viz. oocytes vitrified at immature stage, oocytes vitrified at mature stage and fresh oocytes (control). Each trial consisted of 30 oocytes of each respective group. Data generated from the study was analysed using One-way ANOVA at $P \leq 0.05$ level of significance (Snedecor and Cochoran 1994).

RESULTS AND DISCUSSION

Comparison of in vitro *cytoplasmic and nuclear maturation rate between vitrified-thawed and fresh oocytes:* The immature oocytes were cryopreserved by SSV method and after thawing their degree of *in vitro* maturation were assessed on the basis of cumulus expansion (cytoplasmic maturation) and first polar body extrusion (nuclear maturation). The fresh mature oocytes were taken as control to compare the maturation rate of solid surface vitrified oocytes. Total 15 experiments of *in vitro* maturation were conducted by including 20 oocytes from both vitrified-thawed and fresh groups. It was evident from Table 1 that the cytoplasmic and nuclear maturation was significantly lower ($P \leq 0.05$) in vitrified thawed oocytes group as compared to fresh oocytes (Table 1)

Developmental potency of vitrified thawed (immature and mature) and fresh oocytes after IVF: The present study assessed the developmental competence of oocytes vitrified-

Table 1. Comparison of *in vitro* cytoplasmic and nuclear maturation rates between vitrified thawed and fresh goat oocytes.

Type of oocytes	No. of oocytes set for IVM	Cumulus expansion A + B (%)	Polar body (%)
Vitrified-thawed immature COCs	300	203 (67.67± 2.58[a])	212 (70.67±1.08[a])
Fresh immature COCs	300	249 (83±1.94[b])	259 (86.33±1.33[b])

Mean having different superscript within columns are significantly different at P≤0.05.

thawed at immature and mature stages as compared to fresh oocytes control (Table 2).

Each group comprised 30 oocytes each under 5 experimental trials of IVF. On statistical analysis, it was observed that the rate of embryo production in vitrified-thawed immature oocytes (Figs 1A, B, C) was significantly higher (P≤0.05) as compared to matured oocytes (Figs 1D, E, F) in terms of 2–4 cells, 8–16 cells, morula and blastocysts. However, in fresh oocytes it was significantly higher (P≤0.05) as compared to both vitrified immature and matured oocytes (Figs 1G, H, I).

The present study revealed a significantly lower rate of *in vitro* maturation of vitrified-thawed immature oocytes as compared to fresh mature oocytes in terms of both cytoplasmic and nuclear maturation. The low maturation rates might be due to the adverse effect of cryoprotectants on immature oocytes resulting in failure of meiotic resumption (Moawad *et al*. 2012). Bogliolo *et al*. (2007) observed that the vitrification of immature ovine oocytes induced significant changes in spindle and chromatin configuration after IVM. Purohit *et al*. (2012) reported that immature cumulus compact goat oocytes survived better to vitrification than mature ones. Their study showed that the proportion of fertilized oocytes after vitrification was higher in immature oocytes. Most of the studies suggested that the structural integrity of mature oocytes is one of the crucial factors for low success rate of fertilization and embryonic development after *in vitro* fertilization of vitrified-thawed matured oocytes. The cryoinjury and severe malfunctions can adversely affect the oocytes at MII stage causing spindle disorganization (Mandelbaum *et al*. 2004), loss or clumping of microtubules resulting in some scattering of chromosomes (Sathananthan *et al*. 1988), increased polyploidy after fertilization (Dike 2009) and subsequent decrease in fertilization rates (Somfai *et al*. 2007). Conversely, vitrification of immature oocytes at the germinal vesicle stage might circumvent these problems due to fact that the genetic material is held within the contours of a nuclear envelope (Somfai *et al*. 2010). Reports on the vitrification of caprine oocytes are less frequent (Garg and Purohit 2007) and some of these studies pointed out severe damage to *in vitro* matured oocytes when vitrified and subsequently fertilized *in vitro* (Sharma *et al*. 2006). The reports of above studies were in accordance to our observations which revealed that the rate of embryonic developments in vitrified thawed immature oocytes were significantly higher than vitrified-thawed mature oocytes. However, the embryo production rate in fresh oocyte group was significantly higher in comparison to either group of vitrified oocytes. Similarly, Purohit *et al*. (2012) reported the reduced *in vitro* fertilization ability of vitrified goat oocytes compared to fresh oocytes which could be due to the toxic effects of cryoprotectants and osmotic injuries. One of the reasons for low fertilization rate of vitrified oocytes might be due to post-vitrification zona hardening of the oocytes (Vincent *et al*. 1990).

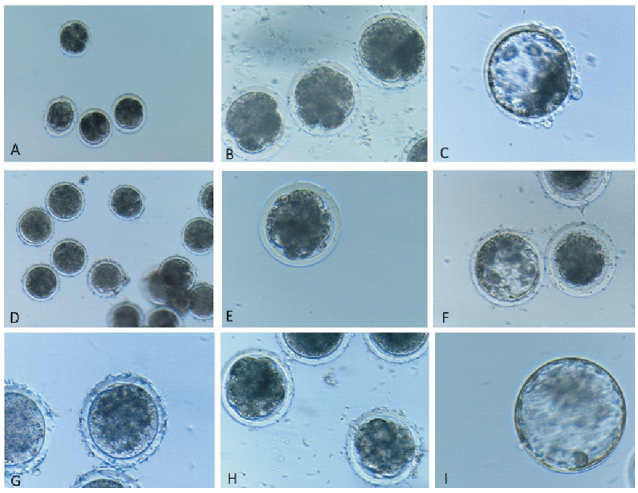

Fig 1. Developmental stages of IVF embryos developed from different groups of goat oocytes: Oocytes vitrified at immature stage (A, B, C), mature stage (C, D, E) and fresh oocytes (G, H, I).

Although the effect of nuclear stage at cryopreservation is not fully understood, some reports suggested that GV stage is more resistant to cryodamage due to their smaller size, lack of cortical granules and a longer period to recover from cryoinjury. To improve the survival and developmental

Table 2. Developmental potential of vitrified thawed (immature and mature) and fresh goat oocytes after IVF

Type of oocytes	No. of oocytes	Developmental stages of IVF embryos (Mean±SE%)			
		2–4 cells	8–16 cells	Morula	Blastocyst
Vitrified immature COCs	150	72 (48±1.70)[a]	63 (41.33±1.7)[a]	33 (21.33±1.7)[a]	13 (8.67±1.33)[a]
Vitrified mature COCs	150	46 (30.65±1.95)[b]	37 (24.67±2.26)[b]	22 (14.67±1.7)[b]	6 (4±1.24)[b]
Fresh COCs	150	95 (63.34±3.09)[c]	85 (56.67±1.49)[c]	62 (41.33±1.7)[c]	39 (26±1.94)[c]

Mean having different superscript within columns are significantly different at P≤0.05.

rate of post-vitrification, the mature oocytes under the present study were briefly exposed to cytoskeletal relaxant, cytochalasin B (CB) to counter the microtubules disruption. Cytochalasin B is known as an inhibitor of cytokinesis by disruption of microfilaments which can reduced microtubule damage during cryopreservation (Silvestre *et al.* 2006). It has been reported that pre-treatment with the CB might reduce the chilling injury to oocytes and embryos thus resulting in improved survival and reduced damage to microtubules and enhance microtubule stabilization during vitrification (Fujihira *et al.* 2004). However, the opinions drawn from many studies suggested that the CB might act differently in immature and mature stages of oocytes of different species (Ledda *et al.* 2001). In vitrification of bovine matured oocytes, CB reduced damage and improved the stability of microtubules (Rho *et al.* 2002). In ovine, Zhang *et al.* (2009) showed that CB pre-treatment of ovine matured oocytes following vitrification and IVF improved embryo developmental rate to blastocyst stage. Shirazi *et al.* (2012) reported that the pre-treatment with CB had no effect on survival rate of adult sheep oocytes after warming.

In conclusion, the present study compared the chilling sensitivity of goat oocytes vitrified at immature and mature stages in terms of post-fertilization and embryonic development rates after IVF. The vitrified-thawed immature oocytes showed a higher cleavage and blastocyst rate as compared to matured oocytes after IVF. The data of our results may provide a significant recognition to vitrified goat immature oocytes for the purpose of conservation or long term storage of female germplasm as well as dissemination of female germplasm into distant places or field condition under "Assisted Reproduction Technology" by adopting solid surface vitrification. However, further studies are necessary to improve the vitrification procedure for oocytes of goats and other small ruminants at various developmental stages.

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