Effects of Vitrification on Immature and in vitro Matured, Denuded and Cumulus Compact Goat Oocytes and Their Subsequent Fertilization

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Abstract

Background: Vitrification has proven to be more effective than slow freezing methods to cryopreserve mammalian oocytes. The objectives of this study were to evaluate the effects of vitrification on immature and in vitro matured, denuded and cumulus compact goat oocytes and their subsequent fertilization.

Methods: Oocytes were either cryopreserved as immature cumulus compact (IMCC) (n=98 Exp 1; 102 Exp 2) and immature denuded (IMDN) (n=127 Exp 1; 109 Exp 2) or were first matured in vitro for 28 *h* and then cryopreserved as mature cumulus compact (MCC) (n=109 Exp 1; 89 Exp 2) or mature denuded (MDN) (n=112 Exp 1; 110 Exp 2) oocytes in four groups. The vitrification solution comprised of Dulbecco's phosphate buffered saline supplemented with 0.5% sucrose, 0.4% bovine serum albumin and 8 *M* propylene glycol. After 7 days of cryopreservation in liquid nitrogen, oocytes in all groups were evaluated for normal morphologic survival and in vitro maturation (Experiment 1) and fertilization in vitro using epididymal buck spermatozoa (Experiment 2).

Results: The number of oocytes retaining normal morphology was significantly higher ($p < 0.05$) for cumulus compact oocytes (IMCC: 94.12% *vs*. IMDN: 89.22%, experiment 1 and MCC: 87.80% *vs*. MDN: 82.17%, experiment 2) compared to the denuded oocytes. The in vitro maturation of oocytes was highest for non-vitrified control oocytes. The maturation of vitrified IMCC oocytes was significantly higher than IMDN and their fertilizability was higher than MCC and MDN oocytes.

Conclusion: The results suggest that immature cumulus compact goat oocytes better tolerate cryopreservation stress by vitrification in terms of fertilization rate.

Keywords: Goat, In vitro fertilization, In vitro maturation, Oocytes, Vitrification.
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Introduction

Vitrification has proven to be more effective than slow cooling methods to cryopreserve mammalian oocytes (1–6). Vitrification technique is a cryoprotectant system involving the addition of higher concentrations of cryoprotectants and ultra rapid cooling (7, 8) and has been tested in various species with good results (9–14). Exposure of oocytes to high concentrations of CPAs[1] causes the oocytes to undergo osmotic dehydration prior to cooling; that treatment coupled with extremely high cooling rates prevent the formation of intracellular ice crystals within oocytes; thus, reducing disruption and damage to cellular architecture. The vitrification treatment does not affect the proportion of oocytes with intact morphology after warming although it has been reported that mammalian oocytes are very sensitive to high concentration of CPAs (15).

A large number of variables affect the outcome of vitrification of oocytes including the type and concentration of cryopreservation, stage of devel-

1- Cryoprotectant agents

opment of oocytes and the presence or absence of cumulus cells (10, 11, 16, 17). 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 counterpart. Reports on the vitrification of caprine oocytes are less frequent (6, 13, 18–24) and few of these studies point out severe damage to in vitro matured oocytes when vitrified and subsequently fertilized in vitro (18, 24). The optimum concentration of cryoprotectants suggested for caprine oocytes appears to be 8 *M* of ethylene glycol, glycerol or propanediol (13, 24); however, it remains to be seen whether these concentrations of cryoprotectants work equally both on immature and mature oocytes and also on cumulus compact and denuded oocytes. The present study examined the effects of vitrification on subsequent fertilization of immature and mature cumulus compact and denuded goat oocytes.

Methods

Cumulus oocyte complexes (n=1041) were collected by aspiration of surface follicles present over goat ovaries (n=460) collected from a local abattoir. Oocytes were either cryopreserved as immature cumulus compact (IMCC) and immature denuded (IMDN) oocytes or they were first matured in vitro for 28 *h* and then cryopreserved as mature cumulus compact (MCC) or mature denuded (MDN) oocytes in four groups. Cumulus compact oocytes were mechanically denuded by repeated pipetting in warm DPBS[1] till the cluster of cumulus cells was completely separated.

A total of 133 (68 in experiment 1 and 65 in experiment 2) cumulus compact oocytes were matured and fertilized without vitrification in experiment 1 and 2, respectively and kept as controls. A total of 446 oocytes (98,127,109 and 112 oocytes in groups ImCC, ImDN, MCC, and MDN, respectively) were vitrified in experiment 1, and 410 oocytes (102,109, 89 and 110 oocytes in groups ImCC, ImDN, MCC, and MDN, respectively) were vitrified in experiment 2. Subsequent to vitrification of oocytes, only morphologically normal oocytes were included for in vitro maturation in experiment 1 and in vitro fertilization in experiment 2.

1- Dulbecco's phosphate Buffered Saline

Cryopreservation of oocytes: Oocytes were cryopreserved by ultra rapid cooling as previously described methods (13) with some modification. The vitrification solution (*vs.*) comprised of DPBS +0.5% sucrose +0.4% BSA[2] and 8 *M* concentration of propylene glycol (13). The 50% *vs.* was prepared by diluting the *vs.* in DPBS. The oocytes to be used for cryopreservation were pre-equilibrated in 50% of the *vs.* for 3.5 *min* and then kept in *vs.* for 2–3 *min*. The oocytes were loaded in the 0.25 *ml* straw, by attaching the straw to an embryo exhauter (IMV, France). Oocytes (4, 5) placed in a small volume (50 *µl*) of *vs.* were filled in the straws with minimum possible volume of *vs.* and keeping air bubbles on both the sides of the *vs.* containing the oocytes. The straws were heat sealed and pre-cooled by keeping the straw over the LN2 (Subscript 2) vapors for 2 *min*, at the height of about 5 *cm* from the LN2 (Subscript 2) level. The straws were then plunged in LN_2 for storage and kept for at least 7 days after which they were taken out warmed and evaluated (14, 24). After evaluation of morphological damage, normal oocytes were put to in vitro maturation and fertilization as previously described methods (13, 25, 47).

Warming and evaluation of morphology: Frozen straws containing the oocytes were thawed in a water bath at 38 *°C* for 30 *sec*. The contents of the straws were emptied in 35 *mm* petri dishes and the cryoprotectant was removed by placing oocytes in DPBS with 0.5M sucrose and then IVM[3] media for 20 *sec* each. Oocytes were considered abnormal when there was a change in shape, breakage of zona pellucida, uneven granulation or leakage of oocyte contents as described previously (14).

The number of morphologically damaged oocytes was recorded for each replicate in each group. The oocytes were further subjected to in vitro maturation and fertilization as methods described previously (26). In two separate experiments nearly the same number of oocytes were used and evaluated for in vitro maturation and in vitro fertilization.

In vitro maturation: Warmed oocytes were separately cultured in TCM-199 supplemented with 5 *µg/ml* FSH, 5 *µg/ml* LH, 1 *ng/ml* estradiol, 25 *mM* Hepes, 0.25 *mM* pyruvate and antibiotics in 50–100 *µl* maturation media (5–8 oocytes per

2- Bovine Serum Albumin
3- In-Vitro Maturation

drop) for 28 *h* at 38±1 *°C* and 5% CO_2 in humidified air in a CO_2 incubator. The maturation of oocytes was evaluated as a previous method (27). At the end of experiment 1, oocytes were fixed and stained with 1% aceto-orcein to record the nuclear status using previously described methods (14). Briefly, the surrounding cumulus cells were removed by vortexing for 1 *min*. The cumulus-free oocytes were placed in the center of an area delineated by two paraffin wax bars on a clean grease-free glass slide. They were compressed gently with a cover slip to hold and fixed by keeping them in acetic methanol (1:3, *v/v*) for 24 *h*. Oocytes were stained with 1% aceto-orcein (1% orcein in 45% glacial acetic acid). The nuclear status of oocytes was evaluated under a microscope and considered to be matured if they were at metaphase II stage (reduced number of chromatin, metaphase plate and extrusion of the 1st polar body).

Immature and in vitro matured cumulus compact and denuded goat oocytes were vitrified and after 7–10 days of storage in LN_2 they were taken out and warmed for further processing. The immature oocytes were matured in vitro for 28 *h* in CO_2 incubator whereas mature oocytes were evaluated for nuclear status immediately after warming. Immature oocytes were also evaluated for nuclear status after their in vitro maturation. The oocytes were considered mature if they showed extrusion of polar body or were at M-II stage.

In experiment 2, the procedures were similar to experiment 1 except that the oocytes were fertilized in vitro after their in vitro maturation using epididymal spermatozoa.

Sperm preparation and in vitro fertilization: Epididymal sperms were recovered as previously described methods (28). Briefly, testicles from bucks were obtained from a local slaughter house in warm DPBS reaching the laboratory within 0.5 *h*. Testicles with epididymis attached were isolated from the scrotum. Epididymal spermatozoa were collected by giving several incisions on each cauda epididymis with a surgical blade and placing the sperm suspension in 1 *ml* phosphate buffered saline (PBS; pH=7.5). After retrieval of sperm their quality was assessed and sperm was prepared for in vitro fertilization.

Semen was prepared by centrifugation in sperm TALP[1] medium as described previously (29) with some modification. Briefly, 0.5 *ml* of the sperm suspension was placed in a centrifuge tube. Four *ml* of HEPES[2]–TALP medium was added to the tube and the tube was centrifuged at 2000 x *g* for 10 minutes. After discarding the supernatant, an aliquot of the pellet was resuspended (1:1) with heparin containing (100 *µl/ml*; Heparin sodium salt) HEPES–TALP medium and incubated for 45 *min* at 38.5*°C* in CO2 incubator. The actively motile spermatozoa were allowed to swim up and used for insemination. After maturation, the oocyte complexes of all groups used for in vitro maturation were transferred to 95 *µl* micro drops of TALP fertilization medium supplemented with 1 *mg/ml* heparin (Sigma, USA). After heparin treatment, sperm concentration was assessed in a haemocytometer and further dilution was made before addition of 5 *µl* of the sperm suspension to the fertilization drops in order to provide a final concentration of an approximately 4×10^6 sperm *cells/ml*. Culture was done in 100 *µl* micro drops (≤ 5–8 oocytes per drop) under paraffin oil and humidified 5% CO_2 atmosphere at 38.5 *°C*.

Following co-incubation for 24 *h* with sperm, the oocytes were evaluated for fertilization under phase contrast microscope as a group (200× magnification). Oocytes from each group were washed with fresh medium and vortexed for 1–2 minutes to separate the cumulus mass. They were processed for fixing and staining in the same way as oocytes were fixed after IVM. If oocytes showed sperm head in the vitellus along with M-II chromosomes or swollen sperm head along with M-II chromosome or both male and female pronuclei, then oocytes were considered fertilized (30).

Statistical analysis: The data related to each replicate were recorded separately for two end–points in vitro maturation and in vitro fertilization in experiment 1 and 2, respectively. The proportion of morphologically normal oocytes, in vitro matured and fertilized over the various groups was compared by Duncan's New Multiple Range Test (DNMR test) on arcsine transformed data.

Results

Survival and morphological evaluations of thawed oocytes: After vitrifying immature (CC or DN) and in vitro matured (CC and DN) oocytes they were warmed as per method described previously. The

1- Tyrode's Albumin Lactate Pyruvate

2- Hydroxyethyl Piperazineethanesulfonic Acid

Table 1. Morphological survival of immature and in vitro matured cumulus compact and denuded goat oocytes after vitrification in experiment 1 and experiment 2

	Group	Oocytes vitrified	Oocytes recovered	Morphologically Normal Oocytes	Abnormal Oocytes
Experiment 1					
	ImCC	98	85(86.73%)[ab]	82(94.12%)[b]	5(5.88)[a]
	mDN	127	102(80.31%)[a]	91(89.22%)[a]	11(10.78)[b]
	MCC	109	100(91.74%)[b]	95(95.0%)[b]	5(5.00)[a]
	MDN	112	98(87.5%)[ab]	92(93.87%)[b]	6(6.12)[a]
	Total	446	385(86.32%)	358(92.98%)	27(7.01)
Experiment 2					
	ImCC	102	90(88.23%)[b]	82(91.11%)[b]	8(8.88)[a]
	ImDN	109	89(81.65%)[b]	76(85.39%)[b]	13(10.78)[a]
	MCC	89	82(92.13%)[b]	72(87.80%)[b]	10(12.20)[a]
	MDN	110	101(91.81%)[b]	83(82.17%)[a]	18(17.82)[b]
	Total	410	362(88.29%)	313(86.46%)	49(13.53%)

Number of replicates in each treatment=10; Proportions with different superscripted letter in the same column are significantly different (p <0.05). (DNMRT on arcsine transformed data). IMCC=Immature cumulus compact; IMDN=immature denuded; MCC=mature cumulus compact and MDN=mature denuded

morphological survival of immature and mature denuded and cumulus compact oocytes evaluated in experiments 1 and 2 showed that the morphologically normal survival rate was significantly higher (p <0.05) for IMCC oocytes compared to IMDN oocytes in experiment 1 and also significantly higher (p <0.05) for MCC oocytes compared to MDN oocytes in experiment 2 (Table 1).

In vitro maturation of warmed oocytes: A significantly higher (p <0.05) proportion of oocytes were matured in the non–vitrified control goat oocytes compared to vitrified immature (ImCC and ImDN) and mature (MCC and MDN) oocytes. Within group comparison revealed that immature cumulus compact oocytes (ImCC) had significantly higher maturation rates compared to their denuded counterparts (ImDN). Mature cumulus compact oocytes, however, showed non-significantly higher in vitro maturation rates (MCC: 43.15% *vs*. MDN: 31.52%). Denuded oocytes evidenced lowest maturation rate (Table 2).

In vitro fertilization of warmed oocytes: The highest fertilization was seen in the control group and significantly lower fertilization was seen in ImDN, MCC and MDN groups. The immature cumulus compact oocyte (IMCC) group evidenced significantly higher (p <0.05) fertilization compared to mature cumulus compact (MCC) and mature denuded (MDN) oocytes (Table 2). Between the immature oocyte group, significantly higher (p <0.05) proportion of fertilized oocytes were seen for ImCC group compared to ImDN group. The proportion of fertilized oocytes was non–significantly different between the control and ImCC groups.

Table 2. In vitro maturation and fertilization statuses of immature and mature cumulus compact and denuded vitrified goat oocytes

Group	Proportion of in vitro matured oocytes	Proportion of fertilized oocytes
Control	61.53[c]	42.64[c]
ImCC	41.25[b]	31.70[bc]
ImDN	27.48[a]	25.0[ab]
MCC	43.15[b]	19.44[a]
MDN	31.52[ab]	16.86[a]
Total	39.71	26.77

Number of replicates in each treatment=8; Proportions with different superscripted letter in the same column are significantly different (p <0.05). Comparison on arcsine transformed data of proportion by ANOVA and DNMRT. IMCC=Immature cumulus compact; IMDN=immature denuded; MCC=mature cumulus compact and MDN=mature denuded

Discussion

During the present study, the proportion of morphologically normal oocytes recovered after vitrification at the end of experiments was significantly lower in mature denuded oocytes in experiment 1 and mature denuded oocytes in experiment 2. This reflects that the cumulus cells attached to oocytes partly offer some protection from cryodamage due to vitrification. One study (31) has previously shown that the mechanical removal of cumulus cells affected the maturational competence of bovine oocytes.

The proportion of morphologically normal oocytes recovered after 7–10 days of cryo-storage were similar to findings of Agarwal (32) who found only 32 oocytes exhibiting morphological changes from 304 vitrified goat oocytes recovered. The rates of morphologically normal survived oocytes obtained during the present study were similar to those recorded previously (81.4% to 95.0%) in vitrified goat oocytes (13, 23).

Studies by Begin (6) have shown that caprine oocytes and embryos vitrified by solid surface vitrification (SSV) had significantly lower survival rates than the controls, whereas the survival rate of cryoloop vitrified (CLV) oocytes and embryos did not differ significantly from the controls.

The cumulus cell removal prior to in vitro maturation or vitrification have shown to have a detrimental effect on oocyte morphology for both immature and mature vitrified equine (33), mouse (34) and bovine (31) oocytes. However, Zhang (35) observed no difference in the survival rate of vitrified mature ovine oocytes with or without cumulus cells. Cumulus cell removal increases the MPF[1] activity and accelerates the transition to metaphase stage and the redistribution of cortical granules (36). The effect of nuclear stage at cryopreservation appears not to be fully understood. Some workers suggested that GV[2] stage is more resistant to cryo–damage due to their smaller size, lack of cortical granules and a longer period to recover from cryoinjury (37). Other workers, however, have concluded that GV oocytes are more sensitive to cryopreservation (38–40).

During the present study, significantly higher proportion ($p < 0.05$) of fresh (Non–vitrified control) oocytes matured in vitro (reached M-II stage) compared to vitrified oocytes. Moreover, denuded oocytes showed lower maturation compared to cumulus compact oocytes. The freezability of unfertilized oocytes has been reported to be low as embryo development proceeds to the blastocyst stage freezability is increased (41, 42), possibly due to difference in cytoskeleton elements. Metaphase-II oocytes are known to better tolerate cryopreservation compared to GV oocytes (43, 44).

Agarwal (32) had previously shown that lesser number of vitrified goat oocytes reached M-II stage compared to fresh oocytes. One of the major concerns caused by oocyte freezing is the possible effect on cytoskeleton structures. It is generally reported in the literature that GV oocytes are more sensitive to cryopreservative injury than any other nuclear stages (38–40) for reasons poorly known.

The proportions of oocytes fertilized were significantly higher in the control group compared to vitrified groups except for the immature cumulus compact vitrified oocytes. Fertilization rates obtained for fresh oocytes in previous studies on goat oocytes in our laboratory varied between 17.03% to 40.86% (11, 25). The normal fertilization rate of goat oocytes can be increased by supplementation of media by cysteamine (45, 46). The reduced in vitro fertilization ability of vitrified oocytes compared to fresh oocytes could be due to the toxic effects of cryoprotectants and osmotic injuries. In addition, the possibility of ultra structure damage to the oocytes and deleterious effect on chromosomes and other cytoplasmic structures cannot be ruled out since such effects have been demonstrated during cryopreservation of mouse and human oocytes (40–42, 48).

Conclusion

The matured vitrified oocytes in the present study showed significantly lower fertilization rates compared to immature compacted vitrified oocytes and controls. The reasons for such an effect are similar to those explained previously for in vitro maturation of oocytes. As far as results of denudation of oocytes is concerned the presence of cumulus cells is helpful in many processes of oocyte growth and higher blastocyst rates were found for oocytes denuded at the start of vitrifycation compared to those denuded later (49). The results of the present study suggest that immature cumulus compact goat oocytes better tolerate cryopreservation by vitrification.

1- Maturation Promoting Factor
2- Germinal Vesicle

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