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Effect of cryopreservation on *in vitro* maturation and cleavage rate of immature prepubertal goat oocytes

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ABSTRACT

The present study was conducted to evaluate effects of cryopreservation on the morphology, maturation rate and developmental capacity of immature prepubertal goat oocytes subjected to vitrification and slow freezing method. The proportion of immature oocytes recovered in a morphologically normal state was significantly higher for control than those for vitrification and slow freezing method. Following *in-vitro* maturation, the nuclear maturation rate was significantly higher for control (29.70%) than those for vitrified (9.80%) and slow freezing (18.18%) method. The percentage of normal *in vitro* matured oocytes following thawing in vitrification and slow freezing groups were 53.73% and 80.00%, respectively. A significant difference was observed in the cleavage rate 48 h post insemination, among vitrification, slow freezing and control were 2.0, 5.80 and 13.58%, respectively. The results indicated that the cleavage rates of oocytes in vitrification and slow freezing method did not differ significantly but differ signitificantly (P<0.05) with control groups.

Key words: Cleavage, Cryopreservation, *In vitro* maturation, Prepubertal goat oocytes, Vitrification

Successful cryopreservation is a cellular dehydration process that prevents or reduces the damage caused by ice crystal formation. Cryopreservation of embryos, ovarian tissue and gametes is a promising technique/approach to preserve the fertility of mammalian species, including endangered species and young cancer patients. It offers the advantage of storing large numbers of follicles that could be subsequently transplanted or cultured *in vitro* to obtain mature oocytes Recently, it is possible to cryopreserve immature oocytes enclosed in preantral follicles in mouse (Cox *et al.* 1996), woman (Oktay *et al*. 1998), sheep (Amorim *et al*. 2003a, 2003b), goat (Rodrigues *et al*. 2004) and cow (Lucci *et al*. 2004).

It is generally recognized that the developmental competence of cryopreserved mammalian oocytes is extremely limited (Neimann 1991). Cryopreserved oocytes from several species have been fertilized *in vitro*, but live births have been reported only for the mouse, rabbit and human. The highest success rate has been reported in mice with as much as 15–30% frozen oocytes produce variable offspring (Nakagata 1989, Kono *et al*. 1991). Live birth have also been achieved in rabbits (Al-Hassani *et al*. 1989) and cows (Fuku *et al*. 1994). It is observed that only a small percentage of frozen oocytes can be fertilized and develops to term in most species regardless of the developmental stage or method used. According to Parks and Ruffing (1992), better results are possible with mouse oocytes, for which success rates of 15–30% viable oocytes frozen have been obtained with both conventional and vitrification protocols.

Therefore, the present study was designed to determine the effect of the conventional slow freezing and vitrification on in vitro maturation and cleavage rate of immature prepubertal goat oocytes.

MATERIALS AND METHODS

Collection and processing of ovaries: Goat ovaries were obtained from prepubertal goats within 3–4 h of slaughter from the local abattoir and transported to laboratory in a thermos flask containing sterile warm physiological normal saline solution supplemented with antibiotics. All the ovaries were then subjected to washings (5–6 times) with warm saline fortified with antibiotics and then transferred into laminar flow.

Recovery of oocytes: The oocytes were collected from each ovary in to a petri dish containing oocyte collection media (Dulbecco's phosphate-buffered saline with 3 mg/ml BSA) by follicle puncture method using 18-G needle. Each petri dish containing oocytes was evaluated under a stereo zoom microscope and grade A, B and C oocytes were chosen for *in vitro* maturation as it has evenly granulated cytoplasm

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which represents its active physiological state with having bunch of compact cumulus cell mass around them (Kharche *et al*. 2008).

Cryopreservation of oocytes: The selected oocytes were randomly divided in to following groups.

Group 1- Vitrification: Approximately 281 immature oocytes were equilibrated at one step in vitrification solution for 10 minutes. The vitrification solution comprises of propylene glycol (40% v/v) and trehalose (0.25mol/l) in TCM 199-Hepes, supplemented with (4%w/v) BSA. After equilibration oocytes were immediately loaded into 0.25 ml French mini straw having 10–12 oocytes per straw with 1 mol sucrose/l solution. The straws were sealed with polyvinyl powder (PVC) and plunged into liquid nitrogen. The straws were thawed (at 37°C for 20 sec) after storage for a period of 1 year. Then the oocytes were transferred to a culture dish, containing 1mol sucrose/L in TCM to remove the cryoprotectant from the cells, after equilibration (1 min) fresh TCM –199 was added drop by drop to reach a final concentration of 0.125mol/L within 10 min.

Group 2- slow freezing: Approximately 290 immature oocytes 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 equilibration 6–8 oocytes were loaded into 0.25 ml French mini straws and open end was sealed with polyvinyl powder (PVC). The slow freezing consisted of the following steps: oocytes frozen from room temperature (25°C) to –7°C @ 4°C/min, seeded by holding with super cooled forceps 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 for 1 year. Thawing was done as explained above with thawing media (0.25M sucrose in TCM 199).

Group 3- Control (without freezing) Approximately 263 immature oocytes were taken as control.

In vitro *maturation and assessment of nuclear maturation*: Frozen thawed oocytes of group 1, 2 and 3 were taken for *in vitro* maturation. The oocytes were washed 6 to 8 times in maturation medium. The maturation medium comprising of TCM-199 containing FSH 5µg/ml, LH 5 µg /ml, estradiol 17ß 1 µg/ml, Sodium Pyruvate 0.25 mM, L- Glutamine 100 µg/ml, gentamycin 50 µg/ml, supplemented with 10% FBS and 0.4% BSA (W/V). Finally 10 to 15 oocytes were transferred into 50µl drop of maturation media under warm paraffin oil in a polystyrene culture dish (40X 10mm) previously incubated for 2h in a CO_2 incubator. Oocytes were cultured over a granulose cell monolayer along with granulose cells, for 27 h at 38.5°C in 5% CO_2.

The matured prepubertal goat oocytes after 27 h of *in vitro* maturation from group 1 (51) group 2 (77) and group 3 (101) were denuded with 0.1% hyaluronidase, fixed in 2.5% glutaraldehyde and stained with DAPI. The nuclear maturation was observed as per the method of Kharche *et al*. (2008).

In-vitro *fertilization of* in-vitro *matured oocytes:* The matured prepubertal goat oocytes after 27 h of *in vitro* maturation from group 1 (n=108), group 2 (n=106) and group 3 (n=101) were separated from cumulus and corona cells by treatment with 0.1% hyaluronidase. The denuded oocytes were washed 8–10 times in Fert. TALP medium, containing 20% EGS and 10 µg/ml heparin (pre-equilibrated in CO_2 incubator at 38.5 ± 1°C for 2 h). After washing 10–15 oocytes were placed in 50 µl drops of Fert-TALP medium. An aliquot of 50–µl capacitated sperm was added to each of the fertilization droplets (50 µl) having matured oocytes and then incubated in humidified atmosphere of 5% CO_2 in air at 38.5 ± 1°C for 18h.

In-vitro *embryo culture:* Following 18 h of sperm exposure, oocytes were examined under a stereozoom microscope (Nikon, Japan) and were freed from adhering sperm cells by repeated pipetting. The oocytes were washed 8–10 times in Embryo Development Media (EDM) and transferred to EDM drops in small culture dish covered with sterile mineral oil (equilibrated in CO_2 incubator). Ten to twelve pre cultured oviductal cells were washed in EDM and were added per drop having fertilized oocytes then cultured at 38.5 ± 1°C and 5% CO_2 in humidified air. Oocytes were observed for cleavage after 48h of insemination under inverted phase contrast microscope (Nikon, Japan) and resulting embryos were cultured for at least nine days.

Statistical analysis: The maturation rate of oocytes was calculated as a percentage. 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).

RESULTS AND DISCUSSION

The average recovery of good quality prepubertal goat oocytes for IVM was 1.85 per ovary by puncture method. The post thaw recovery of morphological normal immature prepubertal goat oocytes in vitrification, slow freezing and control groups were 53.73%, 80.0% and 100%, respectively (Table 1).

The oocytes recorded an 80.0% maturation rate based on the cumulus cell expansion after 27 h of culture at 38.5°C in 5% CO_2 of humidified air. It is evident from Table 2 that after 27 h of culture in maturation medium, 9.80% frozen oocytes of Group 1 reached metaphase II was significantly lower than control oocytes (29.70%). The maturation rate of 18.18% for Group 2 oocytes were found significantly higher than Group 1 oocytes but significantly lower than that for controls.

Most of the frozen–thawed oocytes that did not reached metaphase II during the maturation period did not resume meiosis. The cleavage rates of the *in vitro* matured oocytes in the different Groups were 2.0%, 5.80%, and 13.58% for vitrification, slow freezing and control group, respectively.

Table 1. Effect of cryopreservation methods on post thaw recovery of immatured prepubertal goat oocytes

Groups	No of oocytes frozen	Morphologically abnormal post-thawed oocytes		Morphologically normal post-thawed oocytes (%)
		Cracked zona pellucida	Shrinked cytoplasm	
Group 1 (vitrification)	281	40	84	151 (53.73)
Group 2 (slow freezing)	290	14	44	232 (80.00)
Group 3 (control)	263	-	-	263 (100.00)

Table 2. Nuclear status of *in vitro* matured prepubertal goat oocytes subjected to different cryopreservation technique

Groups	Morphological normal Matured Oocytes (n)	Nuclear status of oocytes			Maturation (%)
		GV (%)	GVBD (%)	MII	
Group 1 (vitrification)	51	15(29.41)	20(39.21)	5	9.80[a]
Group 2 (slow freezing)	77	13(16.88)	30(38.96)	14	18.18[a]
Group 3 (control)	101	15(14.85)	28(27.72)	30	29.70[b]

The values bearing different superscript in the column were significantly different (P<0.05).

Significant differences (P<0.05) were recorded between treatments for these variables. The influence of different methods of cryopreservation on cleavage rate of *in vitro* matured goat oocytes is summarized in Table 3.

Advances in the cryopreservation of preimplantation embryos have made this technique a useful adjunct to IVF, allowing the storage of embryos for future use in embryo transfer programs. Cryopreserving embryos enables better embryo selection; therefore, oocyte cryopreservation can be seen as an integral part of assisted reproductive technologies and Caprine oocytes were matured *in vitro* followed by vitrification, thawing and for IVF as well following 27 h of *in vitro* maturation (Taru Sharma *et al*. 2006). It is also proposed that at the time of vitrification, blastocyst maturity may be a key determinant of outcome measures. As embryos from prepubertal females show a lower *in vitro* re-expansion rate in sheep (Leoni *et al*. 2006) and goat (Leoni *et al*. 2009) after vitrification and warming compared to those derived from their adult counterparts. Similarly, in our study, we obtained 53.73% normal oocytes after thawing with intact zona pellucida. These results can be compared to the reports of Kharche *et al*. (2005) and Taru Sharma *et al*. (2006) in caprine. Caprine oocytes (94.3%) were recovered by Taru Sharma *et al*. (2006) following post-thaw, 16.1% showed abnormalities and 83.0% oocytes were found to be normal when vitrified in propylene glycol and trehalose. In Post-thaw vitrified oocytes evidence of abnormalities have been reported in 18.6% of the oocytes (Kharche *et al*. 2005), suggesting the lysis of the plasma membrane. Possible reasons for lysis may be the freezing procedure used and the steps involved therein, as well as the osmotic shock. The incomplete development of both nuclear (Ptak *et al*. 2006) and cytoplasmic (Anguita *et al*. 2008, Leoni *et al*. 2006) oocyte compartments might be responsible for the reduced cryotolerance of embryos from prepubertal females.

Table 3. Effect of cryopreservation on cleavage rate of immature prepubertal goat oocytes

Groups	Morphological normal oocytes	Cleavage (n)	Cleavage (%)
Group 1 (Vitrification)	100	2	2.00 [a]
Group 2 (Slow freezing)	155	9	5.80[a]
Group 3 (Control)	162	22	13.58[b]

The values bearing different superscript in the column were significantly different (P<0.05).

Oocytes undergo changes in nuclear status that involves exit from diplotene stage of the first meiotic prophase to the metaphase II stage. Resumption of meiosis and subsequent sequential configuration are species specific (Kharche *et al*. 2006, Le Gall *et al*. 1992). In the present study also, most of the vitrified oocytes that did not reach metaphase II did not resume meiosis (29.41%). Some oocytes did not progress to maturation (39.21%) before metaphase I stage, 9.80% of the vitrified thawed GV stage oocytes were able to mature *in vitro*. This rate of maturation was lower than that reported for immature goat oocytes (Le Gal 1996, Kharche *et al*. 2005) bovine immature oocytes (Lim *et al*. 1992) and equine immature oocytes (Tharasanit *et al*. 2006). Post thaw vitrified oocytes showed evidence of abnormalities in the oocytes suggesting the lysis of plasma membrane. Le Gal (1996) also reported this block in the oocyte development to be due to an aberrant protein synthesis after freezing.

Morphological evaluation of the slow freezed cumulus enclosed oocytes after thawing revealed that 80.00% of the

oocytes had a normal morphological appearance with intact zona pellucida and expanded cytoplasm. These results suggested a reduction in the maturation rate due to the cumulative effect of the cryoprotectant and freezing process (Kharche *et al*. 2005). Moreover, nuclear maturation is affected by many factors including cooling, crystallization of the medium, warming potential toxicity of the cryoprotectant, osmotic stock generated by the cryoprotectant exposure and removal (Le Gal 1996, Kharche *et al*. 2005). The maturation rate observed in the present study is similar to the results reported by Le Gal (1996) and Gautam *et al*. (2008) who reported 23.2% of the slow cooled thawed immatured oocytes to mature *in vitro* up to metaphase II stage and lower than that reported for immature goat oocytes (Agarwal 1998) might be due to the cumulative effect of the cryoprotectant and freezing process, the major damage occurred during cryopreservation (Kharche *et al*. 2005). Therefore, the *in vitro* maturation process is supposed to be completed when the highest percentage of M-II oocytes is observed (Kharche *et al*. 2006).

In our study the cleavage rates of frozen thawed oocytes from vitrification group were significantly lower (2.0%) than control group (13.58%). The cleavage rate observed in present study was lower than the results reported by Arav *et al*. (1993), Vieira *et al*. (2002), Wani *et al*. (2004), Kharche *et al*. (2005), Tharasanit *et al*. (2006) and Magnusson *et al*. (2008). The low cleavage rate of the vitrified oocytes might be due to complex sub-cellular structure within which many of the sub-cellular components are particularly temperature sensitive, osmotically and ionically sensitive, also cooling affects spindle fiber integrity and cortical granules (Gh. Mahmoud *et al*. 2010). Depolymerization of the spindle fiber lead to zona hardening. The Zonae of dead immature oocytes are penetrated at lower rates than those of *in vitro* matured oocytes (Gh. Mahmoud *et al*. 2010). Also the permeability to cryoprotectant and water in mature oocytes is greater than those in immature stage (Agca *et al*. 1998) and the immature oocytes have reduced developmental capacity after thawing/ warming as compared to control. Magnusson *et al*. (2008) indicated that the tolerance of oocytes at different meiotic stage to cryopreservation is affected by the type of cryoprotectant and may be one of the reasons for the differing results obtained in our study.

In the present work fewer oocytes cleaved after slow cooling (5.80%) compared to control which is lower to the results of Le Gal (1996), who obtained 89.2% morphological normal oocytes after slow cooling. We used propanediol as cryoprotectant, since propanediol previously shown to have an enhanced ability to decrease intracellular ice formation (Renard *et al*. 1984) and rapidly permeate goat oocytes. Frozen oocytes matured after thawing exhibited a lower fertilization rate than control (13.58%). Similarly Le Gal (1996) also observed a lower cleavage rate (19.2%) in frozen oocytes than control (56.2%) or denuded oocytes (50.1%). Agrawal (1998) obtained 94.80% morphological normal caprine oocytes after 2-step freezing method and recovered 5/96 oocytes exhibited damage in zona pellucida and ooplasm. He found more adverse effect on GV-stage oocyte's maturation after long storage (more than one year) in LN_2 container. Gautam *et al*. (2008); Saragusty and Arav (2011) also concluded that the type of the cryoprotectant as well as its concentration were important factors affecting the post thaw survival of immature oocytes subjected to slow freezing.

In conclusion, low cleavage rate and nuclear status of *in vitro* matured prepubertal goat oocytes was affected by different cryopreservation techniques (vitrification and slow freezing).

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