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Research Paper

Post thaw in vitro maturation of vitrified immature oocytes in sheep using different cryoprotectant concentrations

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ABSTRACT

The present study was conducted to evaluate different cryoprotectant concentrations on post-thaw in vitro maturation rate of vitrified sheep oocytes.Vitrification was done in three vitrification solutions using different concentration of cryoprotectants (Ethylene Glycol + DMSO in equal ratio) i.e. (G1) 20%, (G2) 30%, (G3) 40% mixture of ethylene glycol and DMSO in equal ratio in open pulled straw. Post thaw IVM rate was evaluated based on cumulus cell expansion after 1 week of storage in LN2 and D2 cumulus cell expansion was considered as mature oocytes. In the present study, 152, 152 and 125 oocytes were recovered post thaw with 133,141,117 morphologically normal oocytes in G1 (20%), G2 (30%) and G3 (40%) respectively, which were then subjected to IVM. Significantly, higher percentage of in vitro maturation rate of post thaw oocytes based on D2 cumulus cell expansion was observed in G1 i.e. 20% (48.87%) followed by G2 i.e. 30% (32.6%) with lowest percentage in G3 i.e. 40% (26.4%) respectively. However, non-significantly higher percentage of oocytes was found with D1 expansion in 30% (11.34%) followed by 40% (8.5%) and 20% (5.26%) group. In conclusion, lower cryoprotectant concentrations (20%) of ethylene glycol and DMSO increases the maturation rate of vitrified immature oocytes in sheep possibly due to decreased toxic effect of cryoprotectants.

Key words: Cryoprotectants, immature oocyte, in-vitro maturation, sheep, vitrification

Oocytes cryopreservation in mammalian species has gained a rapid pace during the past couple of decades emphasizing its importance in various assisted reproductive technologies. Cryopreservation of oocytes is a challenging task because of its sensitive nature to chilling due to its size, shape, permeability, species, lipid content, that affect its survival and developmental competence after cryopreservation. Cryopreservation of immature oocytes allows immediate oocyte storage after follicular aspiration, eliminating the need for in vitro fertilization laboratories at the site of oocyte collection. Although recent approaches toward improving the technique have significantly increased the survival rates in some species, thorough investigation needs to be carried out to find a technique that could adjust to each species if required. In vitrification, highly concentrated solution of cryoprotectant solidifies during cooling without formation of ice crystals (Rall, 1987) and is currently being used as an emerging tool of choice for routine cryopreservation of oocytes and embryos (Baril *et al*., 2001).Vitrification of oocytes in OPS reduces the cryoprotectant volume and increases the freezing rate more than 10-folds when the straws are immersed in liquid nitrogen (Vajta *et al*., 1997, Lewis *et al*., 1999) thereby, inhibits the formation of ice crystals. In addition toxic and osmotic effects at warming are minimized by immersion of the capillary end containing the oocyte into a warming solution. There are few reports of vitrification of immature oocytes despite the convincing evidence regarding the superiority of vitrification for oocyte cryopreservation in several other species (Vajta and Kuwayama, 2006a; Vajta and Nagy, 2006b; Asgari *et al*., 2012). Therefore, the objective of this study was to evaluate the post thaw in vitro maturation rate of

abattoir derived sheep immature oocytes post vitrification using different concentrations of permeable cryoprotectants, ethylene glycol (EG) and dimethyl sulphoxide (DMSO).

MATERIALS AND METHODS

The study consisted of oocyte collection from ovaries of sheep slaughtered at nearby abattoirs, vitrification of immature oocytes using different concentrations of cryoprotectants and evaluation of post thaw in vitro maturation rate of oocytes.

Chemicals and media: All the chemicals and media were purchased from Sigma-Aldrich (USA) and plastic ware from Nunc (Denmark) and Axiva Sichem Biotech (India). Media used in present study was supplemented with gentamicin (50µg/ml) prior to use**.**

Collection of ovaries and oocyte collection: Sheep ovaries were collected from slaughtered sheep in and around the vicinity of Veterinary Clinical complex (VCC), F.V.Sc & A.H, SKUAST-K, Srinagar, India. Ovaries were transported to the laboratory immediately after slaughter in a thermos containing DBPS with gentamicin (50µg/ml) at 37 °C. Ovaries were trimmed off from the surrounding tissue, rinsed in distilled water and washed in 70% ethanol followed by 2 washings in DBPS with gentamicin. Collection of oocytes was done in 35 mm petri dish containing collection media (DPBS+ 50 µg/ml Gentamicin + 0.3% BSA) from visible follicles of >2mm diameter by puncture technique. Oocytes were searched under stereo zoom microscope and good and fair oocytes were transferred to fresh warm collection media for further processing.

Vitrification of oocytes: Immature oocytes (good and fair based on cumulus cell layers and uniformity of cytoplasm; Fig-1) collected from ovaries were subjected to vitrification using different concentration of cryoprotectants (Ethylene glycol and DMSO) in open pulled straw and stored in LN2 using standard protocol. Usable oocytes were washed three times in holding medium (TCM-199+ 20% FBS + 50 µg/ml Gentamicin) and finally transferred to a drop of holding medium for 10-12 minutes for equilibration. Vitrification was done in three vitrification solutions using different concentration of cryoprotectants (Ethylene Glycol + DMSO in 1:1 ration) i.e. (G1) 20%, (G2) 30%, (G3) 40% mixture of Ethylene glycol and DMSO (v/v) in holding medium. Vitrification solution-I (VS-I) consisted of half the concentration of vitrification solution-II (VS-II). VS-II also was added with 0.5 M sucrose. After equilibration, oocytes were washed two times in VS-I and then transferred to a droplet of vitrification solution-I for 5 minutes. After 5 minutes in VS-I, oocytes were washed two times in VS-II, transferred to a droplet of vitrification solution-II for 30 seconds and then 3-5 oocytes were loaded in open pulled straw in a minimum volume (<1µl). Open pulled straw with oocytes was immediately plunged in LN2 and stored in LN2 in modified canisters with a lid to prevent outflow of straws especially during refilling with fresh liquid nitrogen.

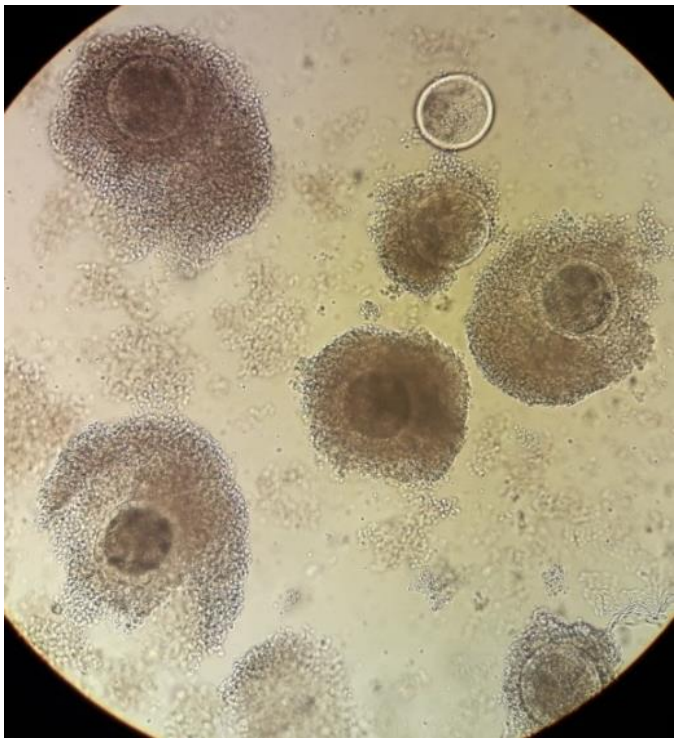

Fig-1: Immature oocytes before vitrification

Thawing and Evaluation: Warming of vitrified COCs was carried out in 2 steps. Straws were taken out of the LN2 and the capillary end of straw containing oocytes was immersed at an acute angle in WS-I solution containing HM + 0.5M sucrose. The vitrification medium liquefied in 2–4 s and the COCs were released into the WS-I and kept for 5 min and then transferred into WS-II (HM + 0.25M sucrose) and kept for 5 min. The oocytes were then released into HM for 5 min and then washed 3 times in TCM-199 plus 10% FBS before being

evaluated. The oocytes with normal shape and uniform cytoplasm, dense and regular layer of cumulus cells, intact cell membrane and zona pellucida after warming were considered as normal oocytes (Fig-2) and were used for IVM. All equilibration, dilution, as well as warming steps were performed at room temperature (approximately 25^0C).

In vitro maturation (IVM): The morphologically normal oocytes were cultured in a drop of maturation medium (50 µl) consisting of TCM-199 + 10% FBS + FSH (1µg/ml) + EGF (20ng/ml) and Gentamicin (50µg/ml) overlayed with mineral oil for 24 h in a CO_2 incubator (5% CO2 and 95% relative humidity) at 38.5°C. The oocytes were washed twice in a drop of maturation medium before putting in a drop of final maturation medium. The oocytes vitrified in different cryoprotectant concentrations were matured in vitro separately. After 24 h of culture, oocytes were evaluated for maturation status under microscope (200X) based on the degree of cumulus cell expansion as described by Kobayashi *et al*., (1994) and categorized as Degree 0 (D0: No expansion of cumulus cells was recorded), Degree 1 (D1: Cumulus cells homogenously spread and clustered cells still present) and Degree 2 (D2: Cumulus cells homogenously spread and clustered cells no longer present) with D2 expansion (Fig-3) as mature oocytes. The total number of experiments (n) was 18, 19 and 16 in G1, G2 and G3 respectively.

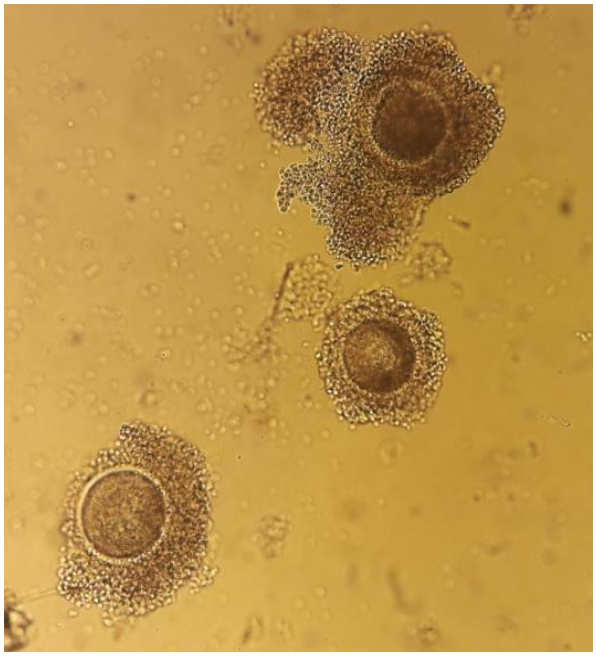

Fig-2: Morphologically Normal Immature oocytes Post thaw

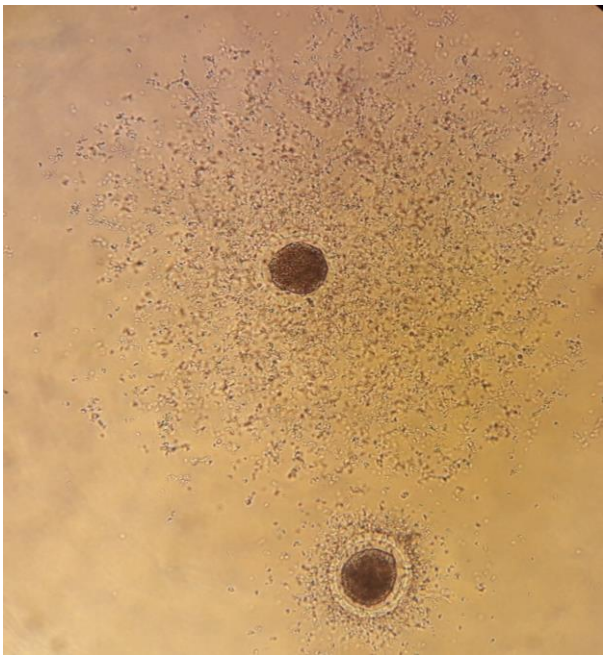

Fig-3: Mature oocytes with D2 Cumulus cell expansion

Statistical analysis: Data was analyzed by Chi square test with P value <**0.01** considered as significant.

RESULTS AND DISCUSSION

In the present study, 152, 152 and 125 oocytes were recovered post thaw with cumulative loss of 5.59%, 11.1% and 11.3% of the oocytes in G1 (20%), G2 (30%) and G3 (40%) vitrification solutions respectively. Out of post thawed oocytes 133,141,117 oocytes were morphologically normal and were subjected to IVM. The present study (Table-1) revealed significantly higher percentage of in vitro maturation rate of post thaw oocytes based on D2 cumulus cell expansion in G1 i.e. 20% (48.87%) followed by G2 i.e. 30% (32.6%) with lowest percentage in G3 i.e. 40% (26.4%) respectively. However, non-significantly higher percentage of oocytes was found with D1 expansion in 30% (11.34%) followed by 40% (8.5%) and 20% (5.26%) cryoprotectant concentrations of ethylene glycol and DMSO.

In the present study the reduction in maturation rate of vitrified immature oocytes could be due to many causes including toxic effect of cryoprotectants, ultrastructural damage to the oocytes, and deleterious effects on chromosomes and other cytoplasmic structures as mentioned by Dobrinsky (1996). Cryopreservation outcome can be affected by cryoprotectant solution, time and temperature of exposure, cooling rate, thawing protocols and cytoskeleton stabilizers (Hochi *et al.,* 2001; Magnusson *et al.,* 2008).

Table-1: In vitro maturation rate (D2) post thaw vitrified immature oocytes in different cryoprotectant concentrations

Rx	No. of Experiments	Oocytes vitrified	Oocytes kept for IVM	D1 Expansion	D2 Expansion
20%	18	161	133	7 [a] (5.26%)	65[a] (48.87%)
30%	19	171	141	16[a] (11.34%)	46[b] (32.6%)
40%	16	141	117	10[a] (8.5%)	31[c] (26.4%)

Values within the same column with different superscripts differ significantly at P value <0.01

The present study revealed significantly higher in vitro maturation rate of post thaw oocytes in 20% (48.87%) followed by 30% (32.6%) with lowest percentage in 40% (26.4%) cryoprotectant concentrations. Amidi *et al.* (2018) reported survival rate of 85.5% and a fertilization rate of 29.2 in vitrified mouse oocytes using 10%EG, 10% DMSO, and 0.5 M sucrose in the base medium. Turathum *et al.* (2010) reported maturation rate of 3.9% only in vitrified-thawed canine immature oocytes using 20% ethylene glycol and 20% dimethyl sulphoxide as vitrification solution. Garg and Purohit (2007) reported that nuclear maturation and fertilization rate of vitrified oocytes decreased significantly ($P < 0.05$) at higher (10 M) concentration of cryoprotectants (Glycerol and Ethylene glycol). Martins *et al.* (2005) reported that high concentration (40%) of EG in addition to a long equilibration time (5 or 15 min) was detrimental to oocyte maturation after vitrification of immature bovine oocytes. Mahmoud *et al*. (2010) indicated that the nuclear maturation rate was higher (41.5%) in 3M EG + DMSO than the control i.e; 6 M ethylene glycol (EG) as control vitrification treatment. However, in contrast to our findings, Wani and Lakhchaura (2004) reported highest maturation rate of vitrified immature oocytes in buffaloes in 7M cryoprotectant concentration of DMSO and Ethylene glycol compared to 3, 4, 4.5.and 6 M cryoprotectant concentration of DMSO and Ethylene glycol which may be due to species difference. Similarly, Moawad *et al.* (2018) revealed that the best quality of vitrified-warmed camel oocytes demonstrated by higher percentage (58.95%) of maturation rate was achieved in 40% EG and DMSO. The results indicated that increase in the maturation rate of vitrified immature oocytes was observed with lower cryoprotectant concentrations (20%) of ethylene glycol and DMSO compared to 40% concentration of ethylene glycol and DMSO in sheep.

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