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Research Article

STUDIES ON THE EFFECT OF VITRIFICATION TECHNIQUES AND ETHYLENE GLYCOL-DIMETHYL SULPHOXIDE-SUCROSE VITRIFICATION SOLUTION ON RECOVERY, MORPHOLOGY AND VIABILITY OF OOCYTES IN SHEEP

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ABSTRACT

The experiment was undertaken to study the effect of vitrification techniques and ethylene glycol-dimethyl sulphoxide-sucrose (EG + DMSO + S) solution on recovery, morphology and viability of cumulus oocyte complexes (COCs) in sheep. Ovaries of ewe were collected from municipal slaughter house from Jammu and oocytes were retrieved by aspiration, puncture and slicing. A total 1800 Good and Fair quality oocytes were vitrified by three techniques *viz.*, Conventional straw (CS), Open pulled straw (OPS) and Hemi straw (HS) (600 COCs each) at conc.10%, 15% and 20% (200 COCs each), respectively. Out of 600 COCs vitrified, the highest number recovered was in 15% of OPS and lowest in 20% of CS. Normal COCs at 10% and 15% conc. were significantly higher ($P<0.05$) than 20% within techniques. The normal COCs at 10%, 15% and 20% conc. in OPS and HS techniques were significantly higher ($P<0.05$) than corresponding values at same conc. in CS but value at 15% and 20% conc. in HS did not differ significantly with CS. The live COCs at 10% and 15% conc. were significantly higher ($P<0.05$) than 20% conc. within techniques. The live COCs at 10%,15% and 20% conc. of OPS were significantly higher ($P<0.05$) than corresponding values at same conc. in CS and HS but value in CS and HS did not differ significantly with each other. The recovery, morphology and viability of cumulus oocyte complexes (COCs) in sheep at 15 % conc. by OPS technique were better.

Keywords: Ethylene glycol-dimethyl sulphoxide-sucrose solution, Morphology, Oocytes, Recovery, Sheep

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Vitrification, a technique that involves a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling without formation of ice crystals (Niemann, 1991). Vitrification is a simple, faster, less expensive technology than slow freezing. Moreover, it was shown to be more effective than slow freezing for material more sensitive to chilling. Several researchers have used cryodevices to vitrify the oocytes of cow (Dalvit *et al.*, 2012) and sheep (Bhat *et al.*, 2013). Sheep oocytes have been vitrified using different cryodevices such as conventional straw, open pulled straw (OPS), hemistraw, solid surface vitrification (SSV), cryotop and cryoloop to increase rates of post-thaw survival and subsequent *in vitro* development (Rao *et al.*, 2012; Bhat *et al.*, 2013). Oocytes and embryos undergo considerable stress (cold shock and osmotic stress) during vitrification warming and may suffer considerable morphological and functional damage. Changes in temperature, several solution effects, or both are the cause of cellular damage and cell death during cryopreservation (Fuller, 2004). The present study was designed to study the effect of vitrification techniques and ethylene glycol-dimethyl sulphoxide-sucrose (EG+DMSO+S) vitrification solution on recovery, morphology and viability of oocytes in sheep.

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MATERIALS AND METHODS

Source of ovary and oocyte Retrieval: Sheep ovaries were brought from municipal slaughter house, Jammu in thermos containing physiological saline (0.9% w/v NaCl) and antibiotic (100 µg/ml streptomycin and 100 IU/ml penicillin) at 37 °C within an hour of slaughter. In the laboratory each ovary was removed from the surrounding tissue and overlying bursa. The ovaries were rinsed in physiological saline and 70% alcohol followed by three washings in Dulbecco Phosphate Buffered saline (DBPS) with antibiotics (100 µg/ml streptomycin and 100 IU/ml penicillin). Oocytes were collected by different methods (Aspiration, Puncture and Slicing) from the visible surface follicles with diameter *viz.*, small (<2 mm), medium (2-4 mm) and large (>4 mm) (Dar, 2014). The oocytes were pooled and graded under a stereozoom-microscope and only those having homogeneous cytoplasm surrounded by more than 3 layers of compact tcumulus cell were selected for vitrification. A total 1800 usable oocytes (Good and Fair quality) were vitrified using ethylene glycol-dimethyl sulphoxide-sucrose (EG + DMSO+S) vitrification solution by three different vitrification techniques *viz.*, Conventional straw (CS), Open pulled straw (OPS) and Hemi straw (HS) (600 COCs each) at conc. 10%, 15% and 20% (200 COCs each), respectively (Fig. 1). Statistical

analysis of vitrified-thawed morphological normal oocyte and viability were done by chi-square test as per the method of Snedecor and Cochran (1989).

Vitrification of cumulus oocytes complex (COCs): Vitrification was carried out by successive immersions of COCs in solutions containing increasing concentrations of cryoprotectant in holding medium. Three different concentrations of EG + DMSO + S vitrification solutions (10%, 15% and 20%) were used in the present study. The COCs were initially equilibrated in vitrification solution I (VS-I) for 2-5 minutes followed by in vitrification solution II (VS-II) of different vitrification groups for 30 seconds before being loaded into Conventional straw (Rao *et al.*, 2012), Open pulled straw (Vajta *et al.*, 1998) and Hemi straw (Dar, 2014). Immediately after loading, cryo devices were plunged into liquid nitrogen (LN2) and stored for one week.

Thawing of vitrified COCs: Thawing of vitrified COCs was carried in two steps. COCs vitrified in different solutions at different concentrations were thawed in respective thawing solution I (TS-I) and thawing solution II (TS-II) at room temperature. Initially COCs were warmed in TS-I for 1 minute followed by into TS-II for 5 minutes. Thawed COCs were then washed twice in HM before being examined. For CS method, straws were exposed to air at room temperature until the frozen solution liquefied. Sealed ends were cut and the contents poured into 35 mm culture dish and COCs were transferred into TS-I followed by TS-II and then washings given in HM. For other method, straws were taken out of the LN2 and the open end immersed vertically in TS-I solution. The vitrification medium liquefied in 2-4 seconds and the COCs were released into the TS-I followed by TS-II and then washed into HM.

Post thaw morphological assessment and viability Evaluation: Post thaw evaluation for morphological damage was performed within 30 min of thawing (Garg and Purohit, 2007). Oocytes were considered abnormal when there was loss of cumulus cells, change in shape, breakage of zona pellucida, uneven granulation or leakage of oocyte contents. The morphologic survival per cent was calculated as the proportion of oocytes seen to be normal against the total number vitrified.

The viability of thawed oocytes was evaluated by the method of Ali *et al.* (2014). Some of normal COCs were taken for staining. One drop of trypan blue (0.4%) was mixed to a drop of holding media. To this solution, 4-5 oocytes were added and allowed to stain for five minutes, after which it was transferred to holding media and examined under phase contrast microscope. Both cumulus cells and ooplasm stain blue in case of dead oocytes while as live oocytes remained unstained.

RESULTS AND DISCUSSION

The recovery, morphology and viability of cumulus oocyte complexes after using Conventional straw (CS), Open pulled straw (OPS) and Hemi straw (HS) vitrification techniques and EG + DMSO + S vitrification solution is depicted in Table1.

Recovery: Out of the 600 COCs vitrified in each technique using EG + DMSO + S vitrification solution at conc. 10%, 15% and 20% (200 COCs each), the number of COCs recovered after thawing in CS, OPS and HS vitrification were 165, 169, 161; 171, 174, 165 and 165,168 and 164, respectively. The highest number of COCs recovered were in 15% of OPS (174). Easy handling of oocytes during vitrification-warming procedures with OPS method might be a factor in the present study for higher recovery rate of oocytes.

Morphology

Normal COCs: The proportion of morphologically normal COCs using EG+DMSO+S vitrification solution at 10% and 15% concentration were significantly higher ($P<0.05$) than 20% conc. within CS, OPS and HS vitrification techniques. Despite the critical importance of various cryoprotectants for avoiding ice crystal formation in oocytes, the higher concentrations of cryoprotectants used for vitrification may be toxic and can result in osmotic injury to the cell.

The proportion of normal COCs using EG + DMSO + S solution at 10%, 15% and 20% conc. in OPS and HS techniques were significantly higher ($P<0.05$) than corresponding values at same concentration in CS technique but value at 15% and 20% conc. of HS were non significant with CS technique. In the present study, the higher proportion of vitrified post-thawed oocytes obtained in the OPS and HS techniques may be because of the low volume of vitrification solution and higher cooling and warming rate which drastically reduced the chilling injury and decreased the toxic and osmotic damages (Liebermann and Tucker, 2002). Results of the present study indicate that using the hemistraw system has a positive effect on the survival after warming. Reason for this might be that cells loaded on the carrier developed a more effective viscous matrix for encapsulation in the hemistraw system, and also prevented crystallization during cooling and warming (Liebermann and Tucker, 2002). Vitrification of oocytes in 0.25 ml straws caused a delay in heat loss from the solutions, possibly leading to devitrification, i.e. intracellular recrystallization during

Table 1

Recovery, morphology and viability of cumulus oocyte complexes after vitrification using ethylene glycol-dimethyl sulphoxide-sucrose (EG+DMSO+S) solution

S.No.	Vitrification technique	EG+DMSO+S concentration	No. of COCs vitrified	No. of COCs Recovered	No. of normal COCs (%)	No. of damaged COCs (%)	Test of Significance	No. of live COCs (%)	No. of dead COCs (%)	Test of Significance
1	CS vitrification	10%	200	165	142 (86.06)	23 (13.94)	bA	135 (81.82)	30 (18.18)	bA
		15%	200	169	147 (86.98)	22 (13.02)	bA	137 (81.07)	32 (18.93)	bA
		20%	200	161	131 (81.37)	30 (18.63)	aA	120 (74.53)	41 (25.47)	aA
2	OP Svitrification	10%	200	171	152 (88.89)	19 (11.11)	bB	146 (85.38)	25 (14.62)	bB
		15%	200	174	156 (89.66)	18 (10.34)	bB	149 (85.63)	25 (14.37)	bB
		20%	200	165	138 (83.64)	27 (16.36)	aB	132 (80.00)	33 (20.00)	aB
3	HS vitrification	10%	200	165	144 (87.27)	21 (12.73)	bB	138 (83.64)	27 (16.36)	bA
		15%	200	168	148 (88.10)	20 (11.90)	bAB	140 (83.33)	28 (16.67)	bA
		20%	200	164	136 (82.93)	28 (17.07)	aAB	122 (74.39)	42 (25.61)	aA

Values with different superscripts within a column differ significantly at $P<0.05$
a, b -Within vitrification technique between different concentration
A, B-Within same concentration between different vitrification techniques
Figures in parentheses indicate percentage

warming which resulted in recovery of morphologically poor oocytes.

The present findings at 10% and 15% EG+DMSO+S in CS resembles with the previous studies of Dutta *et al.* (2013) in bovine who reported 88.37% morphologically normal vitrified-thawed oocytes with 15% EG+15% DMSO + 0.6M S vitrification solution using CS technique. However, the present study contradicts with the earlier reports of Ali *et al.* (2014), Dar (2014) and Quan *et al.* (2016) in goats who observed lower percentage of normal oocytes (59.00%, 60.63% and 64.42%, respectively). Dolakasaria *et al.* (2013) in cattle reported higher percentage (91.06%) of normal oocytes in EG + DMSO + S vitrification solution compared to the present findings.

The present result at 10% and 15% EG+DMSO+S in OPS are in accordance with the studies of Ali *et al.* (2014) in goat who reported 86.20% morphological normal occytes after warming with EG+DMSO+S vitrification solution using OPS technique. Quan *et al.* (2016) in sheep observed 85.00% morphological normal vitrified post thawed oocytes using OPS in 20% EG+20% DMSO+0.6 MS which supports the observations of the present study at 20%

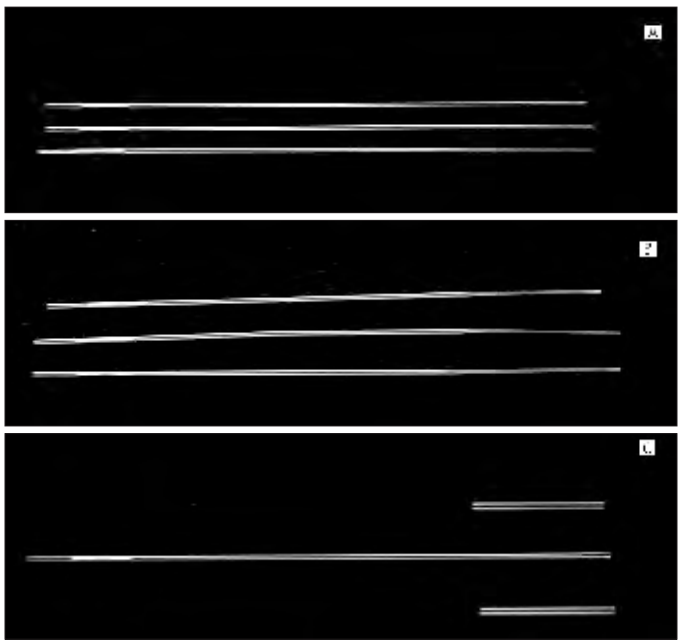

Fig. 1. **(A)** Conventional (French mini) straw, **(B)** Open pulled straw **(C)** Hemi straw

concentration. On the other hand, the present study contradicts with the findings of Bhat *et al.* (2013) in sheep

who observed lower percentage of morphologically normal oocytes (77.43%) with EG+DMSO+S. The present findings at 10% and 15% EG+DMSO+S in HS are favorably comparable with the results of Dar (2014) in goat who recorded 89.68% morphological normal oocytes after vitrification-thawing in EG+DMSO+S vitrification solution using HS method.

Viability

Live COCs: The proportion of live COCs using EG+DMSO+S vitrification solution at 10% and 15% concentration were significantly higher ($P<0.05$) than 20% conc. within CS, OPS and HS vitrification techniques. High concentrations of cryoprotectants are toxic and may cause osmotic injury to the oocytes (Liebennann and Tucker, 2002). A balance between maximizing cooling rate and minimizing the cryoprotectant concentrations is important to minimize the osmotic or chemical toxicity.

The proportion of live COCs using EG+DMSO+S vitrification solution at 10%, 15% and 20% conc. in OPS were significantly higher ($P<0.05$) than corresponding values at same conc. in CS and HS. The high cooling as well as warming rate in OPS compared to CS and HS technique might contributes largely for improvement of oocyte quality and higher viability of oocytes (Quan *et al.*, 2016). The present findings at 20% EG+DMSO+S in CS are in agreement with the study of El-Shalofy *et al.* (2017) in buffalo who obtained 73.60% post-thawed viabile oocytes with 20% EG+20% DMSO solution using CS technique. On the contrary, Ali *et al.* (2014) and Dar (2014) observed lower vitrified post-thawed viable oocytes, 63.78% and 66.3%, respectively with 20% EG+20% DMSO+0.6 MS using CS method in goats. The present results at 10% and 15% EG+DMSO+S in OPS are in close agreement with the study of Ali *et al.* (2014) and Dar (2014) in goats who recorded 88.57%, 90.40% vitrified post-thawed viable oocytes, respectively with EG+DMSO +S solution.

The present results at 10% and 15% EG+DMSO+S of HS are in close agreement with the study of Liebermann and Tucker (2002) in buffalo who observed 85.40% survival of oocytes after vitrification-thawing with EG+DMSO+S using HS vitrification. The present results are contrary with the findings of Dar (2014) in goat who recorded higher percentage (92.86%) of survivability of oocytes with 20% EG+20% DMSO+0.6 MS vitrification solution. It can be concluded that recovery, morphology and viability of cumulus oocyte complexes (COCs) at 15% concentration of ethylene glycol-dimethyl sulphoxide-sucrose by open pulled straw technique is better than Conventional straw and Hemi straw techniques at other different concentration in sheep.

REFERENCES

Ali, R., Razzaque, W., Sharma, U., Pandey, A. and Hussain, K. (2014). Survivability of caprine oocytes vitrified in conventional and open pulled straws. *Turk. J. Vet. Anim. Sci.* **38**: 707-711.

Bhat, M.H., Yaqoob, S.H., Khan, F.A., Waheed, S.M., Sharma, V., Vajta, G., Ganai, N.A. and Shah, R.A. (2013). Open pulled straw vitrification of *in vitro* matured sheep oocytes using different cryoprotectants. *Small Rumin. Res.* **122(1-3)**: 136-140.

Dalvit, G., Gutnisky, C., Alvarez, G. and Cetica, P. (2012). Vitrification of bovine oocytes and embryos. *Cryobiology.* **65**: 341–342.

Dar, R.A. (2014). Studies on follicular oocyte recovery and freezing techniques in goat (*Capra hircus*). Ph.D. thesis submitted to SKUAST-J, J&K, India.

Dolakasaria, D., Dutta, D.J., Dev, H. and Raj, H. (2013). Developmental competence of post-thaw vitrified bovine oocytes. *Int. J. Adv. Res.* **1(9)**: 825-830.

Dutta, D.J., Dev, H. and Raj, H. (2013). *In vitro* blastocyst development of post-thaw vitrified bovine oocytes. *Vet. World.* **6**: 730-733.

El-Shalofy, A.S., Moawad, A.R., Darwish, G.M., Ismail, S.T., Badawy, A.B.A. and Badr, M.R. (2017). Effect of different vitrification solutions and cryodevices on viability and subsequent development of buffalo oocytes vitrified at the germinal vesicle (GV) stage. *Cryobiology.* **74**: 86-92.

Fuller, B.J. (2004). Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryo Letters.* **25**: 375-388.

Garg, N. and Purohit, G.N. (2007). Effect of different cryoprotectant concentrations for ultrarapid freezing of immature goat follicular oocytes on their subsequent maturation and fertilization in vitro. *Anim. Reprod.* **4(3-4)**: 113-118.

Liebermann, J. and Tucker, M.J. (2002). Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. *Reproduction.* **124**: 483-489.

Niemann, H. (1991). Cryopreservation from livestock: current status and research needs. *Theriogenology.* **35**: 109-124

Quan, G.B., Wu, G.Q., Wang, Y.J., Ma, Y., Lv, C.R. and Hong, Q.H. (2016). Meiotic maturation and developmental capability of ovine oocytes at germinal vesicle stage following vitrification using different cryodevices. *Cryobiology.* **72(1)**: 33-40.

Rao, B.S., Mahesh, Y.U., Charan, K.V., Suman, K., Sekhar, K. and Shivaji, S. (2012). Effect of vitrification on meiotic maturation and expression of genes in immature goat cumulus oocyte complexes. *Cryobiology.* **64(3)**: 176-84.

Snedecor, G.W. and Cochran, W.G. (1989). Statistical Methods, (8th Edn.), Iowa University Press, Ames, Iowa, USA.

Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., Greve, T. and Callesen, H. (1998). Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol. Reprod. Dev.* **51(1)**: 53-58.