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Types of damages on buffalo oocytes after vitrification with different concentration of ethylene glycol and dimethyl sulphoxide

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ABSTRACT

The objective of the study is to observe the oocytes damage after vitrification in combination of ethylene glycol (EG) and dimethyl sulphoxide (DMSO) at different concentration with 1 and 3 min equilibration time (ET). A total 826 cumulus oocytes complexes were obtained by follicular aspiration from 278 ovaries of buffaloes, slaughtered at local abattoir. The compact oocyte complexes (COC's) with evenly granulated ooplasm were vitrified using 4 different concentrations viz. 10, 15, 20, 25% of EG and DMSO with 1 and 3 min of ET. After 7 days of cryopreservation, the vitrified oocytes were thawed and these oocytes were evaluated for morphologically normal recovery, partial cumulus loss and different damages. The normal recovery was observed higher in 25% of EG and DMSO (3 min ET), partial cumulus loss found higher in 25% EG and DMSO (3 min ET) and damaged oocyte was observed higher in 15% EG and DMSO (1 min ET). Among all damages, the cracked zona was prevalent and observed significantly higher in 10% EG and DMSO (1 min ET)- 93.7% , shrinkage\ change in shape were found higher in 15% EG and DMSO (1 min ET)- 22.2%, and split in 2 halves were observed in 15% EG and DMSO (1 min ET)- 15.6%. The damage percent was significantly higher in 10% EG and DMSO (1 and 3 min ET) than other concentrations ($P< 0.05$). However, better results were obtained in 25% concentration compared to other concentrations of EG and DMSO.

Key words: Buffalo oocytes, Dimethyl sulphoxide, Ethylene glycol, Vitrification

India is the largest milk producing country in the world and the mainstay of source of milk is buffalo, contributing more than 50% of total milk production (Wani *et al.* 2004). Now it is very important to improve the quality and quantity of this species. But this is severally hampered by its own low reproductive efficiency, which can be overcome by the different reproductive biotechniques like embryo transfer technology, *In vitro* fertilization, embryonic stem cell production and cloning. For all these reproductive biotechniques, large quantity of buffalo oocytes will be required while the need of large quantity of oocytes will be fulfilled by the vitrification. Vitrification is the most promising technique used for the cryopreservation. The vitrification method is proposed with the aim of preventing loss due to cold injury and formation of ice crystals in suspension (Rall and Fahy 1985). In vitrification method oocytes or embryos plunged directly into liquid nitrogen (LN_2) due to which liquid phase turns into the solid phase without forming ice crystals, which is also known as glassy phase. To elude the problem of ice-crystal formation, the vitrification method plays a vital role due to its short time of proper rapid freezing and that is the main reason to minimize the damages to oocytes in vitrification method than other methods of cryopreservation like slow freezing.

Optimum vitrification may be obtained with high concentration of single cryoprotectant, but these concentrations are frequently toxic to the oocytes (Dhali *et al.* 2000a). Mixtures of cryoprotectants generally vitrify at lower concentrations and are likely to be less toxic.

The objective of the present study is to observe the oocytes damages after vitrification in combination of ethylene glycol (EG) and dimethyl sulphoxide (DMSO) with 1 and 3 min equilibration time (ET).

MATERIALS AND METHODS

Buffalo ovaries were collected from the local slaughterhouse and transported to the laboratory within 2 h in a thermos flask containing 0.9% normal saline (30–35°C)

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supplemented with gentamicin. Oocytes were collected from visible follicles of about 2–5 mm diameter with a sterile disposable 18-gauge needle and 10 ml plastic syringe. The oocytes were searched under a zoom stereomicroscope. Oocytes were washed 3 times in TL-Hepes and the number and quality of oocytes harvested were counted and classified according to the compactness of the cumulus cells (Pawshe and Totey 1993).

The selected oocytes were processed for vitrification with 4 different concentrations of ethylene glycol (EG) and dimethyl sulphoxide (DMSO) i.e. 10%, 15%, 20% and 25%, respectively, and each supplemented with 0.5 M sucrose and 20% FBS (EDSF). The oocytes were exposed to equilibration solution (50% EDSF –10 in v/v DPBS) prior to final vitrification for 1 min. Oocytes were transferred to 5% and 7% solutions of EG and DMSO step-wise for 1 and 3 min, respectively. Finally, the oocytes were exposed to the 10% of final vitrification solution. After quick mixture with the final vitrification solution, oocytes were loaded into the 0.25 ml French straws with loading solutions containing 0.5 M sucrose in TCM–199 supplemented 20% FBS. The straws were sealed with the PVA powder and then plunged directly into LN_2 to store for at least 7 days. The same procedure was followed for remaining 7 groups. After 7 days, the straws were thawed in warm water at 28–30 °C for 20 sec and 4 steps dilutions were done in 0.5 M, 0.33 M, 0.17M and 0.00 M sucrose + holding medium in TCM-199, respectively, for 5 min each. Oocytes were washed 4 to 5 times in TL-Hepes medium. Vitrified-thawed oocytes morphology was evaluated by their post-thaw morphological appearance as normal or with any asymmetry, partial or complete cumulus loss, shrinkage and abnormal oocytes with ruptured zona pellucida under an inverted tissue culture microscope.

The statistical analysis of the present research work was done by factorial completely randomized design (FCRD) after Arcsin conversion for recovery and damaged oocytes analysis as prescribed by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Effect of vitrification on recovery of immature buffalo oocytes: In present experiment, good quality COC's were selected and vitrified by using combination of EG and DMSO at concentration of 10%, 15%, 20% and 25% with 1 and 3 min equilibration time. The total number of oocytes vitrified were 86, 115, 99, 92, 69, 82, 58 and 67 using above concentrations, respectively, however oocyte recovery was 49 (56.98%), 77 (66.95%), 83 (83.83%), 81 (88.04%), 50 (72.46%), 67 (81.70%), 50 (86.21%) and 67 (100%), respectively (Fig. 1) and data reflecting the effect of different concentrations of EG and DMSO on post-thaw oocyte recovery are summarized in Table 1.

The recovery of morphologically normal oocytes in present study are in resemblance with Dhali *et al.* (2000a) who reported the recovery rate of 88 to 98% using 3.5M and 4.5M EG concentration in combination with DMSO and glucose in buffalo. Also, Dhali *et al.* (2000b) reported that 89% to 96% recovery rate in the two-equilibration solutions and exposure times was 1 and 3 min in buffaloes.

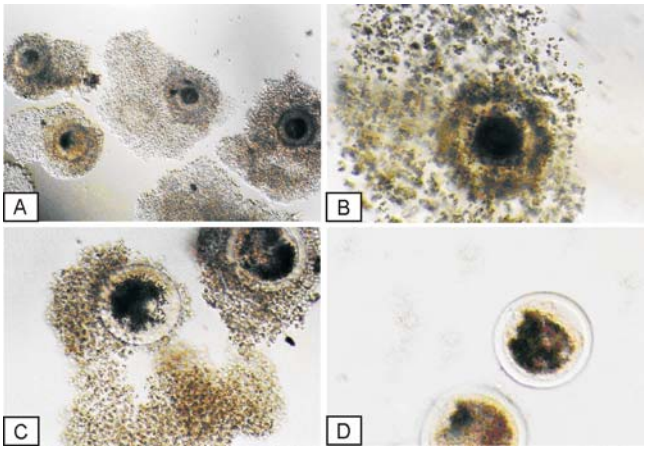

Fig. 1 (a-d). Morphological appearance of vitrified immature buffalo oocytes. **A.** Good quality oocytes. **B.** Expanded cumulus. **C.** Partial cumulus loss. **D.** Complete cumulus loss.

Table 1. Effect of different concentration, combination of cryoprotectant and equilibration time on recovery of immature buffalo oocytes after vitrification (r=4)

Concentration of EG and DMSO	Equilibration time (min)	Total no. of oocytes vitrified	Total no. of oocytes recovered (%)	No. of morphologically normal oocytes (%)	No. of partial cumulus loss oocytes (%)	No. of damaged oocytes (%)
10%	1	86	49(56.98)	28 ^{ab}(56.73±5.30)	02 ^{b}(4.42±2.60)	19 ^{a}(38.85±3.16)
	3	115	77(66.95)	40 ^{ab}(51.70±3.55)	04^{b}(5.20±2.05)	33 ^{a}(43.09±3.43)
15%	1	99	83(83.83)	40 ^{ab}(47.77±3.16)	07 ^{b}(8.63±1.47)	36 ^{a}(43.61±2.45)
	3	92	81(88.04)	53 ^{ab}(60.78±3.73)	02 ^{b}(2.70±1.56)	26 ^{a}(36.50±3.43)
20%	1	69	50(72.46)	28 ^{b}(55.72±2.22)	04 ^{a}(8.10±1.47)	18 ^{a}(36.17±1.96)
	3	82	67(81.70)	34^{b}(50.65±0.66)	05 ^{a}(7.36±1.10)	28 ^{a}(41.97±1.74)
25%	1	58	50(86.21)	30 ^{a}(61.36±7.54)	04 ^{a}(7.48±2.65)	16 ^{b}(31.15±5.28)
	3	67	67(100)	42 ^{a}(62.61±2.98)	08 ^{a}(11.86±2.1)	17 ^{a}(25.52±1.33)

Values with different superscripts within the same column differ significantly from each other (P<0.05).

The present findings resembles with the Bombatkar (2008), the recovery rate of morphologically normal oocytes significantly higher in 7M (75.00%) as compare to 5M (47.70%) and 6M (30.51%) concentration of ethylene glycol. Asada *et al.* (2002) reported that the survivability rate of oocytes ranged from 69 to 87% using EG and PVP in different concentrations in bovines. This is higher than the result of present experimental study using EG and DMSO. Thus, the delicate balance is important between the cryoprotectant concentrations and exposure time, which may enable the decreasing cryoprotectant toxicity and minimizing osmotic shock to improve recovery and survival rate of vitrified-thawed oocytes (Yavin and Arav 2007).

Effect of vitrification on different oocyte damages: Out of the total oocytes recovered, the higher percent of oocytes damaged found in 15% (1 min) concentration of EG and DMSO was 36 (47.77%) and the lower damage percent of oocyte observed in 25% (3 min) and 17 (25.37%). Data reflecting the observed types of damages on different concentrations are summarized in Table 2.

The cracking of zona in oocytes was observed higher in 10% (1 min) concentration 18 (93.75±6.25) and comparatively lower in 15% (1 min) concentration 30 (72.08%). The shrinkage/change in shape of oocytes observed significantly higher in 15% (1 min) concentration as 22.29%, whereas it was not observed in 10% (1 min) and 20% (1 min). The splitted oocytes into 2 halves were observed in all concentrations except in 10% (3 min) (Fig. 2). After thawing of vitrified oocytes different damages were observed i.e. cracked zona, split into 2 halves, and change in shape of oocytes in buffalo. Bombatkar (2008) observed that the proportion of damages was significantly higher in 6M (55.37%) than 5M (26.44%) and 7M (13.19%) concentrations. The cracked zona was observed only in 6M (5.10%) and 7M (15.79%) concentration with no significant difference. Dhali *et al.* (2000b) reported the proportion of cracked zona of oocytes as 9, 4, 5 and 7%, respectively, using the 3.5 and 4.5M EG and DMSO concentrations along with glucose (5.56 mM) for 2 different equilibration times.

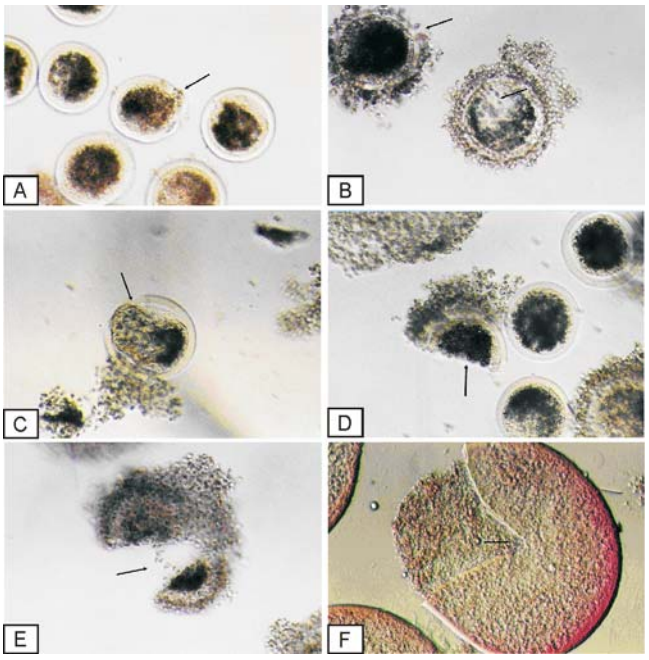

Fig. 2 (A-F). Damages after post thawing of vitrified buffalo oocytes. **A.** Change in shape of occyte with ooplasm (arrow). **B.** Degenerated oocytes with dense and shrunken ooplasm (arrow). **C.** Thinning of zona (arrow). **D.** Sliced zona (arrow). **E.** Split into two halves (arrow). **F.** Cracked zona observed after aceto- orecin stained after fixation (arrow).

However, the proportion of cracked zona observed in the present study was much higher. The higher proportion of cracked zona in the present study may be due to the cryoprotectant permeability used during vitrification. DMSO has lower permeating ability across the cell membrane than EG, which may results into delay in equilibration and recovery of normal morphology of oocytes (Mahmoudzadeh *et al.* 1993), which might have resulted into more cracked zona percentage. However, fracture or cracked zona is to be caused by non-uniform changes in the volume of liquid and solid phases of the medium during rapid phase change (Rall and Meyer 1989). In the present experimental work, percent

Table 2. Effect of different concentration of EG and DMSO on oocyte damage after vitrification with 1 and 3 min equilibration time (r=4)

Conc. of EG and DMSO	Equilibration time (min)	Total no. of oocytes recovered	Types of damages in oocytes no. (%)			
			Cracked zona	Shrinkage	Split into 2 halves	Empty zona
10%	1 min	49[a](56.98)	18 [a](93.7±6.2)	0 [a](0±0)	1 [a](6.2±6.2)	0 [a](0±0)
	3 min	77 [a](66.95)	30 [a](90.2±5.7)	2 [a](6.9±4.1)	0 [a](0±0)	1 [a](2.7±2.7)
15%	1 min	83 [a](83.83)	30 [a](72.0±7.5)	3 [a](22.2±15.0)	2 [a](15.6±11.8)	1 [a](2.5±2.5)
	3 min	81 [a](88.04)	22 [a](87.3±7.4)	2 [a](6.3±3.7)	2 [a](6.3±3.7)	0 [a](0±0)
20%	1 min	50 [a](72.46)	16 [a](90.8±5.3)	0 [a](0±0)	2 [a](9.1±5.3)	0 [a](0±0)
	3 min	67 [a](81.70)	24 [a](85.7±10.1)	2 [a](7.1±4.1)	1 [a](3.5±3.5)	1 [a](3.5±3.5)
25%	1 min	50 [a](86.21)	14 [a](82.4±8.5)	1 [a](6.2±6.2)	1 [a](8.3±8.3)	0 [a](0±0)
	3 min	67 [a](100)	14 [a](80.4±7.0)	1 [a](6.2±6.2)	1 [a](5.0±5.0)	1 [a](8.3±8.3)

Values with different superscripts within the same column differ significantly from each other (P<0.05).

of cracked zona was higher (93.375%) compared with the earlier reports (69%) by Dhali *et al.* (2000b).

Bombatkar (2008) observed shrinkage was significantly higher in 5M (73.91%) compared to 6M (43.88%) and 7M (15.79%). Whereas, splitting in 2 halves were seen in 6M (4.08%) concentration. Upon removal of the cryoprotectant during thawing with hypertonic solutions, containing sucrose as non-permeating solutes counteracts excess inflow of water and due to which oocytes remain shrunken (Kasai *et al.* 1980). This may be the reason for shrinkage/change in shape of oocytes.

During vitrification, change in temperature and effect of cryoprotectant solution or both causes cellular damage and death of oocytes (Fuller *et al.* 2004). However, buffalo oocytes content more cytoplasmic lipid droplets, which make them more sensitive to chilling injury (Kasai *et al.* 2002). In addition, cooling process resulted into the intracellular and extra-cellular ice crystallization, during which oocytes might be crushed resulting in various zona damages. These zona damages are related to formation, re-crystallization or melting of intra and extra-cellular ice (Mazur *et al.* 1972).

From the present study it is concluded that the recovery of the morphologically normal oocytes as well as lower percentage of damage, after vitrification was higher in 25% concentration of EG and DMSO in 3 min of equilibration time. The cracked zona was the most prevalent damage found in 10% concentration of EG and DMSO in 1 and 3 min of equilibration time than other concentrations.

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