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Immature buffalo (*Bubalus bubalis*) oocytes vitrification: Effect of ethylene glycol concentrations on survivability and nuclear maturation

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ABSTRACT

The objective of the present research work was to study the effect of 5, 6 and 7 M ethylene glycol (EG) concentrations during vitrification of immature buffalo oocytes. The COCs were retrieved by aspirating visible follicles on the ovaries collected from slaughterhouse and loaded into 0.25 ml French straws after exposing to the equilibration medium (50% VS v/v in DPBS) for 1 min and the final vitrification solutions of respective concentrations of EG. The straws were then directly plunged into the LN_2. After thawing in warm water (28–30°C), for equilibration COCs were kept for 4 step dilutions step-wise in 0.5, 0.33, 0.17 and 0.00 M sucrose holding medium for 5 min each. The percent morphologically normal oocytes recovered were significantly higher (75.00%) in 7 M concentration. For nuclear maturation, the COCs were placed in 50 µl droplets of maturation medium and cultured for 24 h in CO_2 incubator at 39°C temperature.

The proportion of maturation reached to metaphase-II was significantly higher in 7 M (47.42%) concentration whereas the highest nuclear degeneration was in 6 M (70.47%) concentration. From the results, it was revealed that 7 M concentration has beneficial effect on survivability and nuclear maturation of oocytes.

Key words: Buffalo, Ethylene glycol, *In vitro* maturation, Oocytes, Vitrification

Conventional method of cryopreservation of embryos and oocytes induces a significant damages leading to lower survivability and developmental rate (Wani *et al.* 2004a,b). Vitrification is an ultra-rapid freezing method by which a concentrated solution of cryoprotectant solidifies during cooling without ice-crystallization, i.e. glassy phase (Vajta 2000, Yavin and Arav 2007 and Cuello *et al.* 2008).

The successful vitrification depends on types or concentrations of the cryoprotectants used in vitrification solution. Change in concentration of cryoprotectants leads to toxic and hypertonic effect on oocytes during vitrification (Yavin and Arav 2007). The successful attempts were made for oocytes vitrification with different concentrations and combinations of cryoprotectants like ethylene glycol (EG), dimethyl sulfoxide (DMSO), propenidiol, poly-ethylene glycol (PG), and glycerol (Gly) in cattle (Vieira *et al.* 2002, Magnusson *et al.* 2008), sheep (Succu *et al.* 2007, Bogliolo *et al.* 2007), goat (Kharche *et al.* 2005, Sharma *et al.* 2006), pig (Shi *et al.* 2007, Huang *et al.* 2008), equines (Hurtt *et al.* 2000), cat (Merlo *et al.* 2008), rabbit (Saeed *et al.* 2000) and mouse (Gomes *et al.* 2008 and Ko *et al.* 2008). However, very less literature is available on vitrification of immature buffalo oocytes with the single use of ethylene glycol (Wani *et al.* 2004a and 2004b). The present research work was undertaken with the objectives to find out the suitable concentration of ethylene glycol (EG) for better survivability and nuclear maturation rate on vitrified-thawed immature buffalo oocytes.

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MATERIALS AND METHODS

Oocytes collection: Buffalo ovaries were collected from the local slaughterhouse and transported to the laboratory within 1 to 2h of slaughter in a thermos flask containing 0.9% warm normal saline (30–35°C) supplemented with gentamycin. Oocytes were retrieved from ovaries using aspiration method. Follicular content of visible small follicles of about 2–5 mm diameter were aspirated using a sterile disposable 18 gauge needle and 10 ml plastic syringe. The follicular contents were collected in a 50 ml centrifuge tube and allowed to stand for 10 min at 37°C in a water bath. The oocytes were searched under a zoom stereomicroscope at around 20× magnification. After collection, oocytes were washed 3 times in TL-Hepes media.

Vitrification of immature oocytes: The good quality COCs were taken and processed for vitrification with 3 different concentrations of ethylene glycol (EG) (5, 6 and 7 M, each

supplemented with 0.5 M sucrose and 20% FBS in TCM-199). The vitrification solutions (VS) were named as VS-1, VS-2 and VS-3 for each concentration of EG. At first, the oocytes were exposed to equilibration solution (50% VS-1 in v/v Dulbecco's phosphate buffered saline) prior to final vitrification for 1 min and then, transferred to 1.5 M and 3.5 M solutions of EG step-wise for 5 min and 3 min, respectively. Finally, the oocytes were exposed to the VS-1 and after quick mixture 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 poly-vynil alcohol powder and then plunged directly into LN_2 to store for 7 days. The time taken from the exposure of oocytes to the VS-1 and loading and plunging it into the LN_2, was 45–60 sec. The same procedure was followed for the VS-2 and VS-3 with 6 M and 7 M concentrations of EG, respectively. After 7 days, the straws were thawed in warm water at 28–30°C for 20 sec. After thawing, 4-step dilutions were done for removal of EG concentration effect by keeping oocytes step-wise in 0.5 M, 0.33 M, 0.17 M and 0.00 M sucrose holding medium in TCM-199, respectively, for 5 min each. Then oocytes were washed 4 to 5 times in TL-Hepes medium containing BSA. Vitrified-thawed oocytes morphology was evaluated by their morphological appearance as normal or with any asymmetry, partial or complete cumulus loss, shrinkage and oocytes with ruptured zona pellucida under an inverted microscope.

In vitro *maturation of vitrified-thawed immature oocytes:* Morphological normal oocytes from each group of vitrified-thawed oocytes were then transferred into 50 µl droplets (10–15 oocytes/droplet) of maturation medium (TCM-199 + 10% FBS + 5 µg/ml FSH + 1 µg/ml 17-α oestradiol) and cultured for 24 h in CO_2 incubator at 39°C temperature containing 95% humidity and 5% CO_2 in air. After 24 h of culture, the oocytes were examined for nuclear maturation. The oocytes were washed in TL-Hepes medium. The cumulus cells were removed by repeated pipetting using a fine bore pipette. The denuded oocytes were mounted on glass slides and covered with cover slip supported by vaseline: paraffin mixture and retained with rubber cement. The slides were then immersed in an ethanol: acetic acid (3: 1) solution for 24 h and stained with 1% aceto-orcein stain and examined under a phase contrast microscope at 100× and 200× magnification. Based on the changes occurring in their nuclear matrix, the oocytes were classified as germinal vesicle (GV), metaphase-I (MI), metaphase-II (MII) stage and degenerated oocytes.

Statistical analysis: The statistical analysis of the present work was done by completely randomized design (CRD) after Arcsin conversion (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

In the present study, low molecular weight cryoprotectant EG was used for cryopreservation of oocytes, which easily permeate through cell membrane and rapidly maintain concentration equilibrium across the cell membrane. In addition, the vitrification solution contained sucrose which increases intracellular osmotic pressure, dehydrating the oocytes and increasing the relative intracellular concentrations of cryoprotectants.

The high proportion of morphologically normal oocytes with compact cumulus cells (75.00%) were recovered after thawing using 7 M concentration of EG. However, this rate was significantly higher ($P<0.01$) than 5 M (47.70%) and 6 M (30.51%) concentrations of EG. The partial cumulus loss was significantly higher in 5 M (25.86%) concentration; however, there was no significant difference observed for 6 M (14.12%) and 7 M (11.81%) concentrations, respectively. The proportion of damaged oocytes was significantly lower ($P<0.01$) for the 7 M concentration than that for 5 M and 6 M concentrations of EG (Table 1).

Post-thaw recovery was better in the present study than that reported by Asada *et al.* (2002) in bovines, apparently due to the addition of the 20% FBS. Protein supplementation can reduce the amount of visible ice in the vitrification medium. Furthermore, it was proposed that large amounts of serum in the holding media can protect the membrane (Hou *et al.* 2005). In contrast to our study, Dhali *et al.* (2000a) reported the recovery rate of 88 to 98% in buffalo using 3.5 and 4.5 EG concentration in combination with DMSO and glucose. Also, Hou *et al.* (2005) observed 77 to 98% recovery of morphologically normal oocytes using EG with ficoll and DMSO in cattle. This may be due to the use of different cryoprotectants in combination, which reduces the concentration and exposure time dependant toxicity (Wani *et al.* 2004a).

The morphological abnormalities observed in vitrified-thawed oocytes include complete cumulus loss, shrinkage/change in shape, cracked zona and oocytes split into 2 halves. The complete cumulus loss was significantly higher in 7 M (68.42%) concentration and the shrinkage/change in shape of oocytes was significantly higher in 5 M (73.91%) concentration, respectively (Table 1, Fig. 1b). Cracked zona of oocytes was observed only in 6 M (5.10%) and 7 M (15.79%) concentrations (Figs 1d–g); however, the oocytes splitted into 2 halves were observed only in 6 M (4.08%) concentration of EG (Fig. 1h).

During thawing, upon removal of the permeating cryoprotectant EG with hypertonic solutions containing sucrose as non-permeating solutes counteracts excess inflow of water and so oocytes remain shrunken (Kasai *et al.* 1980, Leibo 1980). However, the proportion of cracked zona observed was much lower and this may be due to the cryoprotectant combination permeability used during vitrification. DMSO used in combination having the lower permeating ability across the cell membrane than EG, that results into delay in equilibration, and recovery of normal morphology of oocytes (Mahmoudzadeh *et al.* 1993), which may resulted into more cracked zona percentage observed

Table 1. Effect of different concentrations of ethylene glycol on post-thaw recovery rate of oocytes (r = 4)

Ethylene Glycol Concentration	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 (%)			
					Complete cumulus loss	Shrinkage/ change in shape	Cracked zona	Split into two halves
5 M	200	174(87.00)	83 (47.70)[a]	45 (25.86)[a]	12 (26.08)[a]	34(73.91)[a]	0(0.00)	0 (0.00)
6 M	208	177(85.09)	54(30.51)[b]	25 (14.12)[b]	46 (48.93)[b]	43 (43.88)[b]	05 (5.10)	04 (4.08)
7 M	165	144(87.27)	108(75.00)[c]	17 (11.81)[b]	13 (68.42)[c]	03 (15.79)[c]	03 (15.79)	0 (0.00)

Values with different superscript within the same column differ significantly from each other (P<0.01)

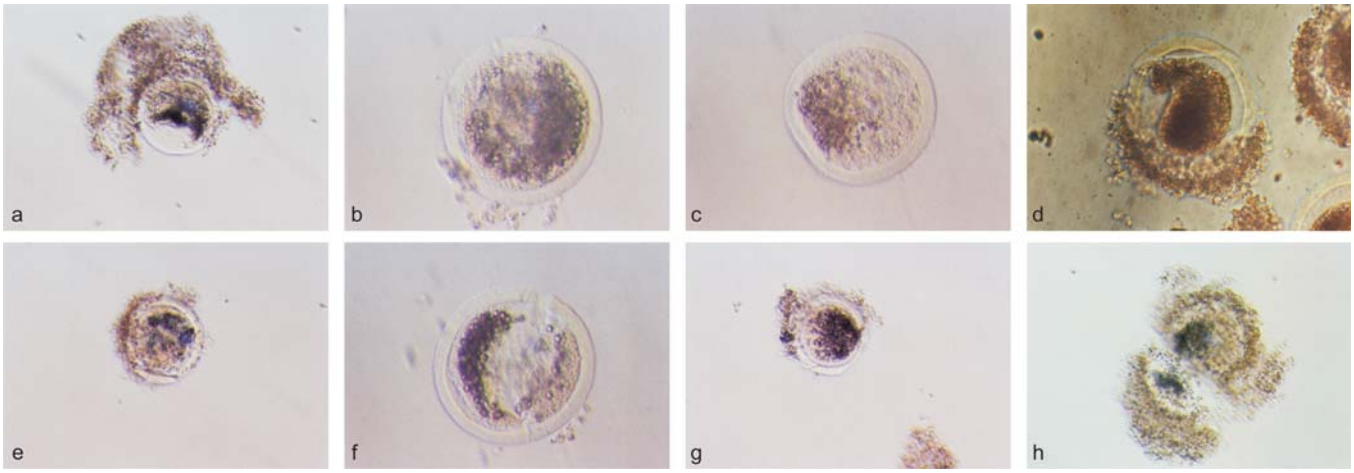

Fig. 1(a-h): Morphological abnormalities/damages observes in vitrified-thawed immature buffalo oocytes **(a)** partial cumulus loss with degenerated cytoplasm;**(b)** complete cumulus loss with normal cytoplasm; **(c)** shrinkage/change in shape of oocyte;**(d)** oocyte with cracked zona;**(e)** oocyte with slightly damaged zona;**(f)** oocyte with excessively cracked zona;**(g)** oocyte with exceessively damaged zona; **(h)** oocyte splitted into two halves.

than Dhali *et al.* (2000b) who reported 69% zona cracking. However, vitrification using straws, cracked zona can be minimized or prevented completely by cooling and warming to the phase transition temperature in a gas phase (Kasai 1997) and this may have resulted in lower per cent of damaged zona in present work (15.79%) compared with the earlier reports.

Buffalo oocytes are also more sensitive to cooling due to more cytoplasmic lipid droplets (Ledda *et al.* 2001, Kasai *et al*. 2002). During vitrification, change in temperature, solution effect or both are causes of cellular damage and death (Critser and Russel 2000). Also, less exposure to the cryoprotective agents leads to lower dehydration keeping intracellular water in cell which during cooling resulted into ice crystallization. Thus, formation, re-crystallization and/ or melting (Farrant *et al.* 1977) of intra and extracellular ice are related to these zona damages (Figs 1d–g).

The complete cumulus loss was the most prevalent damage observed in the present study, whereas the maturation of oocytes and its development is directly related to the intactness of cumulus cells. Cumulus cells are one of the factors responsible for the nuclear and cytoplasmic maturation (Trounson 1992) by providing nutritional and hormonal support via 'gap junctions' (Down *et al.* 1988). Due to high metabolic activity of cumulus cells *in vitro*, it secretes protein and steroids during maturation (Eppig 1977) which is pre-requisite for both cytoplasmic and nuclear maturation (Pawshe and Totey 1993). However, vitrification of the COCs with EG leads to expansion of cumulus cells probably due to damage of gap junctions between cumulus cells and oocytes (Hurtt *et al.* 1999), which may result into lower maturation rate compared to control group due to lack of stimulatory signals and low nutrition and this may also be reason for incomplete or poor expansion of cumulus cells during *in vitro* maturation in this study than the control group.

The percent oocytes that reached to M-II stage was significantly higher in 7 M concentration (47.42%) than 5 M (23.42%) and 6 M (12.38%), however, significantly lower than the control group (77.77%) (Fig. 2). There was no significant difference observed in 5 M and 6 M concentrations of EG with respect to the M-II maturation stage. The data reflecting the effect of different concentrations of EG on *in vitro* maturation oocytes are summarized in Table 2.

The degeneration rate was significantly higher in 6 M

Table 2. Effect of different concentrations of ethylene glycol on nuclear maturation of vitrified-thawed immature buffalo oocytes (r = 4)

Ethylene glycol concentration	Total no. of oocytes used for maturation	Stages of maturationno. (%)			
		GV	M-I	M-II	Degenerated oocytes
Control (non-vitrified)	81	05(6.17)^{ab}	05(6.17)^{a}	63(77.77)^{a}	08 (9.87)^{a}
5 M	111	16(14.41)^{c}	16(14.41)^{a}	26 (23.42)^{b}	53 (47.74)^{b}
6 M	105	07 (6.66)^{bc}	11(10.47)^{a}	13 (12.38)^{b}	74 (70.47)^{c}
7 M	97	02 (2.06)^{a}	03 (3.09)^{a}	46 (47.42) ^{c}	46 (47.42)^{b}

Values with different superscript within the same column differ significantly from each other (P<0.01); GV,germinal vesicle; M-I,metaphase-I; M-II,metaphase-II.

concentration (70.47%) than 5 M (47.74%) and 7 M (47.42%) concentration and control group (9.87%), however, no significant difference was observed between 5 M and 7 M EG concentration groups. During maturation, the transient of intracellular calcium (Ca_i) results into the cell activation initiating cortical granule and plasmalemma fusion releasing a proteolytic enzyme that prevents polyspermy by targeting sperm binding proteins (Kline and Kline 1992,Tahara *et al.* 1996). This process is referred as 'zona hardening'. However, cryopreservation of oocytes resulted into zona hardening by initiating its own course of development before *in vitro* maturation and fertilization (Larman *et al.* 2006) thereby compromising maturation rate and developmental procedures. During the maturation oocytes undergoes changes in nuclear status from GV stage to M-II with extrusion of first polar body (Magnusson *et al.* 2008) with reorganization of organelles and cortical granules leading to cumulus expansion. Also the meiotic spindles are crucial for the events following fertilization as completion of meiosis, second polar body formation and migration of the pronuclei (Zhou *et al.* 2002), but disorganization of meiotic spindles could result in chromosomal dispersion, failure of normal fertilization, and termination of development (Chen *et al.* 2003). However, during vitrification organization and migration of cortical granules and organelles get disturbed as well as disappearance of spindle during cooling and reassembling during warming compromised the development (Diez *et al.* 2005). Thus, proper balance between cryoprotectant concentration and combination, oocyte dehydration, and temperature during cooling and warming is necessary, which are related with the osmotic shock, and intracellular as well as extracellular chilling injuries to the oocytes.

In summary, experimental results of vitrification using 7 M concentration of EG in the present study demonstrated higher proportion of morphologically normal oocytes recovery, lower shrinkages and oocyte's splitting into 2 halves. It also showed higher nuclear maturation rate as compared with 5 M and 6 M EG concentrations. Therefore, it is concluded that 7 M EG concentration has overall beneficial effect on survivability and *in vitro* maturation of vitrified-thawed immature buffalo oocytes. However, further experiments are needed to establish suitable protocol for vitrification of immature buffalo oocytes.

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