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Glycerol and ethylene glycol as cryoprotectants for vitrification of immature bovine oocytes

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There has been an increasingly rapid advance in the field biotechnology of reproductive biotechnology, especially for the cryopreservation of gametes (Dhali *et al*. 2000, Hurtt *et al.* 2000, Hadi *et al*. 2010, Hadi *et al*. 2011) and ovarian tissue (Al-aghbari *et al.* 2002, Zhang *et al.* 2009). Although the controlled freezing method is still widely used, its field application is limited by complex freezing procedures and the requirement for expensive freezing machines. Vitrification, which is a relatively recent approach, is defined as a glass-like solidification of solutions at low temperatures, without the formation of intracellular ice crystals (Prentice and Anzar 2011). Vitrification not only prevents ice crystals formation, but it quickly passes the temperature zone known to cause chilling injury (15º to –5ºC).

Vitrification may be successfully applied for cryopreservation of bovine oocytes (Diez *et al.* 2005, Hari *et al.* 2011, Bogliolo *et al.* 2012) and embryos (Abdalla *et al.* 2010, Hlavicova *et al.* 2010) at various developmental stages. However, the meiotic spindle in mature oocytes is sensitive to low temperature and in most of the cases it is disrupted during cryopreservation resulting in low survival rate after freezing of mature oocytes. Immature oocyte is the ideal state of freezing as there is no meiotic spindle present and the genetic material is confined within the nucleus (Massip 2003).

The type and concentration of cryoprotectants and the duration of time for which the oocytes are exposed to cryoprotectants are of critical importance. The duration of exposure to cryoprotectants depends primarily upon their permeability into the ocytes/ embryo. Ethylene glycol has the highest permeability due to its low molecular weight compared with glycerol, DMSO and propylene glycol (Dhali *et al.* 2000). Development of reliable method for cryopreservation of oocytes is therefore relevant for conservation of female animal genetic resources. Therefore, the present experiment has been undertaken to study the effect of glycerol (GLY), ethylene glycol (EG) and GLY+EG and their concentrations on vitrification of immature bovine oocyes and their subsequent development *in vitro*.

Oocyte collection: The cattle ovaries were collected from a local slaughter house in warmed (37ºC) normal saline solution (0.9%) containing penicillin G (100 IU/ ml) and streptomycin (0.125 mg/ ml) in a thermos flask and brought to the laboratory within 3 h of slaughter. The extraneous tissues were removed from the ovaries using a pair of scissors. The ovaries were then washed 3–4 times in physiological saline solution containing penicillin G (100 IU/ ml) and streptomycin (0.125 mg/ ml) prior to further processing. The cumulus-oocyte-complexes (COCs) from the visible follicles measuring 3–8 mm diameter were collected by aspiration and slicing technique in Medium-199 containing 25 mM HEPES, Earl's salt, L-glutamine and 2 mg/ ml sodium bicarbonate modified by addition of 4 mg/ ml bovine serum albumin (BSA, fraction V) and 50 µg/ ml gentamycin. The oocytes surrounded by minimum one layer of compact cumulus cells layer to the zona pellucida were subjected to vitrification and *in-vitro* maturation.

Vitrification and warming: Two numbers of vitrification solutions I (VS I) were prepared by using 3.5 M and 4 M concentrations of GLY, EG and GLY+EG in holding medium comprising Medium-199 with 20% fetal bovine serum (FBS). Again two numbers of vitrification solutions II (VS II) were prepared by using 7 M and 8 M concentrations of cryoprotectants and 0.5 M sucrose in holding medium comprising Medium-199 with 20% FBS. The immature oocytes were exposed to VS I for 1 minute followed by exposure to VS II solution for 30 sec. Oocytes

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were then loaded in the 0.25 ml straw and plunged in liquid nitrogen for vitrification. Vitrified straws were thawed in warm water at 37º C for a min and the content of the straw was transferred to a diluting medium containing TCM-199 with 0.5 M sucrose and subsequently diluted in step wise manner. Post thaw survivability rate was evaluated and processed for *in-vitro* maturation (IVM).

In-vitro maturation: Post thaw morphologically normal immature bovine oocytes (533) were vitrified by using 2 molecular concentrations (7 M or 8 M) of cryoprotectants, glycerol (GLY), ethylene glycol (EG) and their combination (GLY+EG) and then used for *in-vitro* maturation. Another 200 immature bovine oocytes were used for *in-vitro* maturation without vitrification and these served as control. Culture media for IVM was prepared using Medium-199 supplemented with 10% FBS, 10% follicular fluid, 50 µg/ml gentamycin, 50 µM/ml cysteamine, 1µg/ml oestradiol 17ß, 5 µg/ml p-FSH and 0.8 mM sodium pyruvate. The oocytes were incubated in IVM culture media droplet (50 µl/ 10–12 oocytes), covered with sterile mineral oil and maintained at 38.5ºC in 5% CO_2 in humidified air. After 24 h of maturation, oocytes were denuded using 0.1% (W/V) hyaluronidase in hepes buffered Medium-199 by vortexing. The denuded oocytes in groups of 1–2 were mounted on micro-slides, in between two parallel lines of Vaseline bars and covered with cover slip by gently pressing the cover slip over the Vaseline bars until the oocytes were fixed between the slide and cover slip. Then the cover slip was fixed with the micro slide using 2 side bars of melted wax and Vaseline (1:1, w/w) which became hard on cooling. The micro slide was then fixed in aceto-ethanol mixture (1:3, v/v) at 5ºC for 24 h. Fixed oocytes were then transferred to absolute alcohol solution for 5 min. Then the micro slide was taken out from absolute alcohol and stained with aceto-orcein stain (1% orcein in 45% glacial acetic acid).The statistical analysis was done by using SAS enterprise guide 4.3.

The rate of recovery of morphologically normal oocytes is presented in Table 1. Present result is comparable with that reported by earlier researchers (Matsumoto *et al.* 2001, Hadi *et al.* 2010, Dutta *et al.* 2013). Purohit *et al.* (2012) and El-sokary *et al.* (2013) reported similar findings for buffalo oocytes.

In the present study, analysis of variance revealed that the recovery rate of morphologically normal oocytes after vitrification of immature bovine oocytes by using 7 M and 8 M concentrations of GLY, EG and GLY+EG did not differ significantly between cryoprotectants and between concentrations of cryprotectants. However, although not significant, the recovery rate was slightly better for 7 M concentration in both GLY and EG and for combination of GLY+EG. These findings corroborate with previous findings of Garg and Purohit (2007) and Yadav *et al.* (2008). Lower rate of morphologically normal oocytes after vitrification might be due to the toxic effects of cryoprotectants and osmotic injury.

The mean percentage of oocytes showing cumulus cells expansion and polar body formation after *in-vitro* maturation (IVM) of oocytes vitrified by using 7 M and 8 M concentrations of GLY, EG and GLY+EG cryoprotectants is presented in Table 2. Results obtained were found to be

Table 1. Recovery of morphologically normal immature bovine oocytes (%) after vitrification using different concentrations of different cryoprotectants

Cryoprotectants	Concentration	Number of oocytes		Recovery of morphologically normal oocytes	
		Vitrified	Recovered	Number	%
GLY	7 M	90	76	68	89.84±1.81
	8 M	110	94	83	88.21±1.61
EG	7 M	98	86	78	88.33±6.38
	8 M	88	81	70	86.46±3.10
GLY+EG	7 M	88	82	75	91.81±1.42
	8 M	92	86	76	91.18±1.17

Table 2. Cumulus cells expansion (%) and Polar body formation (%) after *in-vitro* maturation (IVM) of immature bovine oocytes vitrified using different concentrations of different cryoprotectants

Cryoprotectant	Concentration	No. of oocytes used for IVM	Oocytes (mean±SE)	
			% Cumulus cells expansion	% Polar body
GLY	7 M	68	71.92^{b}±7.08	47.08^{c}±2.10
	8 M	83	70.08^{b}±3.76	46.78^{c}±2.37
EG	7 M	78	77.00^{ab}±4.16	52.29^{abc}±1.70
	8 M	70	75.35^{ab}±2.47	49.90^{bc}±1.14
GLY+EG	7 M	75	81.34^{ab}±2.65	56.93^{ab}±1.52
	8 M	80	76.54^{ab}±3.60	51.76^{abc}±2.87
Control(Non-vitrified)		200	86.73^{a}±1.41	59.22^{a}±2.69

*Means with different superscripts in a column differ significantly (P< 0.05).

corroborated with that of Rao *et al.* (2012) and Dutta *et al.* (2013). However, Moawad *et al.* (2012) recorded lower rate of cumulus cells expansion (41.30%) in bovine oocytes. The rate of cumulus cells expansion was significantly (P< 0.05) higher in non-vitrified control group than in GLY vitrification group and apparently higher than EG and GLY+EG groups. The lower rate of cumulus cells expansion of vitrified *in-vitro* matured oocytes might be due to damage and interruption that occurred to the cumulus cells during vitrification (Moawad *et al.* 2012). Exposure of oocytes to the cryoprotectants enhanced disorganization of actin filaments within transzonal processes through which cumulus cells establish physical contact with the zona pellucida of oocytes. Vitrification causes alteration of the secondary structure of the zona pellucida proteins as well as the carbohydrate residues (Bogliolo *et al.* 2012).

Our findings also revealed that the mean per cent cumulus cells expansion after *in-vitro* maturation of immature oocytes vitrified using GLY, EG and GLY+EG was apparently higher for 7 M than 8 M concentration of cryoprotectant.

Critical difference test revealed that IVM rate of vitrified oocytes in respect of polar body formation did not differ significantly between 7 M and 8 M concentrations. The mean incidence of polar body formation for 7 M and 8 M GLY was significantly (P< 0.05) lower than that for non-vitrified control and for 7 M GLY+EG. The incidence of polar body formation in IVM of oocytes did not differ significantly among control, 7 M and 8 M GLY+EG and 7 M EG and among 8 M GLY+EG, 7 M and 8 M EG and 7 M and 8 M GLY. The mean incidence of polar body formation after vitrification with 7 M GLY+EG was significantly (P< 0.05) higher than that in GLY group and nonsignificant than that with EG group. The findings reported by Hadi *et al.* (2010), Hadi *et al.* (2011) and Dutta *et al.* (2013) were similar to that of the present study. This might be due to the low viscosity, low toxicity and good vitrifying capacity of the mixture of glycerol and ethylene glycol cryoprotectants. A good vitrifying capacity can be achieved in the mixture of cryoprotectants with no major alteration of ultrastructure in bovine follicular oocytes because a mixture of cryoprotectants could decrease individual specific toxicities (Cocchia *et al.* 2010, Bhat *et al.* 2013).

The rate of polar body formation in non-vitrified control was apparently higher than that in 7 M and 8 M GLY+EG and 7 M EG vitrified groups and significantly (P< 0.05) higher than that in 7 M and 8 M GLY and 8 M EG vitrified groups. This might be due to the fact that when oocytes were exposed to vitrification solutions the developmental ability of vitrified oocytes get reduced because of toxic effects of cryoprotectants and osmotic injury. Degeneration of bovine follicular oocytes may also occur during vitrification as well as during warming of oocytes, where damage mainly occurs in oolema, zona pellucida and ooplasm (Djuwita *et al.* 2005). The freeze thaw process is also known to induce an alteration in the physio-chemical properties in the intra-cellular lipids and such damages might render the oocyte incapable of retaining its developmental competence.

In conclusion, based on rate of maturation of oocytes, combination of glycerol and ethylene glycol was superior to glycerol and ethylene glycol for vitrification of bovine immature oocytes. The present study also revealed that both 7 M and 8 M concentrations of cryoprotectants were equally effective for vitrification of bovine immature oocytes.

SUMMARY

In the present study A and B catergory oocytes were collected by aspiration and slicing techniques from cattle ovaries obtained from abattoir within 3 h of slaughter. Oocytes (566) were vitrified by two-step vitrification technique for studying the effects of 2 concentrations (7M and 8M) of glycerol (GLY), ethylene glycol (EG) and GLY+EG on *in-vitro* maturation of vitrified oocytes. Two hundred oocytes were used for *in-vitro* maturation without vitrification that served as control. Recovery of morphologically normal oocytes did not differ significantly between types of cryoprotectants and between concentrations of cryoprotectants. The rate of cumulus cells expansion did not differ significantly between types of cryoprotectants and their concentrations but was significantly higher in non-vitrified control group (86.73±4.41) than in 7M and 8M GLY groups (71.92±7.08 and 70.08±3.76 respectively). The mean % occurrence of polar body in GLY, EG and GLY+EG was 47.08±2.10, 52.29±1.70 and 56.93±1.52 and 46.78±2.37, 49.90±1.14 and 51.76±2.87 in 7M and 8M concentration of cryoprotectants in comparison to 59.22±2.69 in control group.

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