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Effect of type and concentration of cryoprotectant on post-thaw survivability of vitrified porcine follicular oocytes

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ABSTRACT

A total of 950 and 510 oocytes with two or more cumulus cell layers adhered to zona pellucida obtained by aspiration from follicles (2–8 mm dia) of 315 and 135 abattoir porcine ovaries, respectively, were utilized in the study to find the effect of cryoprotectant and concentration on post-thaw survivability of porcine follicular oocytes. Vitrified post-thaw oocytes with intact zona pellucida and vitelline membrane, normal spherical shape and dark and evenly granulated cytoplasm under a stereo-zoom microscope were considered as viable. Out of the 10 cryoprotectant treatments i.e. ethylene glycol (EG), propylene glycol (PG), dimethyl sulphoxide (DMSO), glycerol (GL), EG + PG, EG + DMSO, EG + GL, PG + DMSO, PG + GL and DMSO + GL used for vitrification of oocytes at a concentration of 35%, the post-thaw survivability rate was the highest in EG +DMSO followed by EG + PG group which had significantly higher mean post-thaw survived oocytes as compared to GL, PG + DMSO, PG + GL and DMSO + GL groups. Exposing the oocytes to 30, 35 and 40% of EG + DMSO and EG + PG yielded no significant difference in post-thaw survivability rate of vitrified oocytes, although the highest value was obtained with 35%. It was concluded that 35 to 40% EG + DMSO yielded efficient vitrification of porcine oocytes.

Key words: Concentration, Cryoprotectant, Porcine oocytes, Post-thaw survivability, Vitrification

Cryopreservation of oocytes rather than embryos provides greater flexibility in breeding programmes (Paynter and Fuller 2007), since cryopreservation of immature oocytes obtained from abattoir ovaries offers opportunity for planned *in vitro* maturation and *in vitro* fertilization in contrast to embryo cryopreservation. Vitrification of oocytes is rapid technique with radically increased cooling and warming rates that can obviate chilling injury and ice crystal formation associated with slow freezing. Although several vitrification protocols have been applied for porcine oocytes with varying survival rates, they are still associated with severely compromised embryo development. Several factors influence the survival rate of immature porcine oocytes following vitrification including type and concentration of cryoprotectant. Different cryoprotectants and their combinations with varying concentations effective for cryopreservation of mammalian oocytes were adopted for vitrification of oocytes in various species. However, the best cryoprotectant or combination of cryoprotectants and their concentrations are yet to be determined for vitrification of porcine follicular oocytes and hence the present study was taken up.

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MATERIALS AND METHODS

Porcine ovaries were collected from local abattoirs immediately after slaughter of the animal and transported to the laboratory within 1–2 h in a flask containing normal saline with antibiotic at normal environmental temperature (22–28°C). Oocytes were recovered from follicles (2–8 mm dia.) of the ovaries after washing by aspiration technique and examined on a sterile petri-dish under stereo-zoom microscope and good quality oocytes were selected for vitrification. The oocytes were first exposed to equilibration solution (cryoprotectant @ 15% v/v + sucrose 0.25 M in basic solution that contained 80 ml DPBS, 20 ml FBS and 5 mg Gentamicin, pH 7.2–7.4) and then to vitrification solution (VS) (cryoprotectant @ 35% (v/v) + sucrose 0.5 M in basic solution). Cryoprotectants i.e. ethylene glycol (EG), propylene glycol (PG), glycerol (GLY) and dimethyl sulphoxide (DMSO) singly and in combinations were used for vitrification of oocytes. For combination of cryoprotectants, the total concentration of cryoprotectants was 15% and 35% in equilibration and vitrification solutions respectively. Vitrification procedure was performed at room

temperature (24–25°C). Within 30 sec of exposure in VS, the oocytes with little quantity of VS were loaded in the French mini straw (0.25 ml), sealed and directly plunged into LN_2 tank. The thawing or warming of vitrified oocytes was done in a step-wise (1 min in 0.5 M sucrose followed by 2 min in each of 0.25 M and 0.125 sucrose in basic medium) manner at 37°C. The post-thaw vitrified oocytes were examined under stereo-zoom microscope to evaluate survivability based on morphology. Oocytes with intact zona pellucida and vitelline membrane, normal spherical shape, and a dark and evenly granulated cytoplasm were considered as viable. Oocytes with clear signs of membrane damage (brownish, faded cytoplasm) were considered as non-viable (Somfai *et al*. 2014). Two combinations of cryoprotectants that revealed higher rates of survivability of vitrified oocytes were further used separately for oocytes vitrification at total concentrations of 30, 35 and 40% in VS and survivability of vitrified oocytes was recorded as before. Statistical analysis of the data was done following SAS (version 4.04) software.

RESULTS AND DISCUSSION

The mean post-thaw survivability rates following vitrification of porcine follicular oocytes in Dulbecco's phosphate buffered saline containing 35% of different cryoprotectants either alone or in combination are presented in Table 1. The post-thaw survivability rate of oocytes differed significantly (P<0.01) between cryoprotectants and the highest rate was obtained in EG+DMSO followed closely by EG+PG, the difference being non-significant. Oocytes in EG+DMSO and EG+PG vitrification groups had significantly (P<0.05) higher mean post-thaw survivability as compared to GL, PG+DMSO, PG+GL and DMSO+GL vitrification groups, the rate of survival being the least in DMSO+GL. This could indicate lower toxicity of EG than PG, GL and DMSO. The lowest survivability of vitrified oocytes in DMSO+GL could be due to higher molecular weights of DMSO (78.13g/mol) and GL (92.094 g/mol), which might had impaired their permeability into and out of oocytes thus reducing the efficiency of vitrification. Lower efficacy of GL in vitrification was reflected in the mean survivability of oocytes which was recorded to be lower although not significantly as compared to that in EG, PG and DMSO. This was in agreement with the findings of Shahat and Hamman (2014) in buffalo oocytes. Lower post-thaw survivability rate obtained with GL as compared to EG was also reported by Yadav *et al.* (2008) in bubaline cumulus-oocytes complexes. EG+DMSO yielded significantly (P<0.05) higher post-thaw survived vitrified oocytes as compared to EG+GL, however, the difference between EG+PG and EG+GL was not significant. Significantly (P<0.05) higher post-thaw survivability of vitrified oocytes in EG+DMSO than in PG+DMSO was in conformity with the findings of Sharma *et al.* (2010) in buffalo oocytes. This could indicate efficacy of EG over PG in vitrification, which might be attributed to lower molecular weight of EG (62.07g/mol) which enabled it to permeate rapidly into and out of the oocytes as compared to PG (molwt: 76.09 g/mol). EG was reported to be an effective cryoprotectant for vitrification of oocytes (Somfai *et al*. 2006). The post-thaw survivability rates of follicular oocytes vitrified in EG+DMSO and EG+PG were apparently higher than that vitrified in EG, PG or DMSO alone. Similar observation was made by Mahmoud *et al.* (2008) for buffalo oocytes. It could be ascribed to reduction in the level of toxicity in oocytes exposed to cryoprotectant due to use of combinations of different permeable cryoprotectants at lower concentration thus obviating use of higher concentration of single cryoprotectant that was needed for avoiding ice crystal formation in oocytes but could be more toxic causing osmotic injury. Szurek and Eroglu (2011) also observed that combinations of different permeable cryoprotectants might be a possible means of improving oocytes cryopreservation protocol by reducing their specific toxicity. Somfai *et al.* (2013) reported higher post-thaw survivability of pig follicular oocytes vitrified in EG+PG as compared to EG alone. Thus maintenance of a balance between the toxicity and cryoprotective action of acryoprotectant was critical for the development of an efficient vitrification protocol. However, in the present study, some combinations of cryoprotectants had lower post-thaw survivability of vitrified oocytes when compared with single cryoprotectant. This might be due to inequality or disproportion in the level of permeability and toxicity of different cryoprotectants.

Table 1. Post thaw survivability rate of follicular oocytes vitrified using different cryoprotectants

Cryoprotectant	No. of oocytes vitrified	Survivability (%) (Mean±SE)
Ethylene glycol	110	75.59^{abc}±4.79 (41.67–91.67)
Propylene glycol	110	77.86^{abc}±5.77 (50.00–100.00)
Dimethyl sulphoxide	110	74.72^{abcd}±2.69 (60.00–87.50)
Glycerol	110	66.14^{cde}±3.50 (50.00-78.57)
Ethylene glycol + Propylene glycol	85	85.60^{ab}±5.33 (61.54-100.00)
Ethylene glycol + Dimethyl sulphoxide	85	86.99^{a}±3.07 (78.57–100.00)
Ethylene glycol + Glycerol	85	73.76^{bcde}±4.19 (57.14-84.61)
Propylene glycol + Dimethyl sulphoxide	85	57.57^{de}±5.81 (35.71-75.00)
Propylene glycol + Glycerol	85	66.14^{cde}±3.72 (50.00-81.82)
Dimethyl sulphoxide + Glycerol	85	56.23^{e}±7.32 (36.84-80.00)

Figures in the parentheses indicate range. Means bearing different superscripts differ significantly (P<0.05).

The mean per cent post-thaw survivability rates of oocytes following vitrification using 30, 35 and 40% concentrations of EG+DMSO and EG+PG respectively are

Table 2. Post-thaw survivability rate of follicular oocytes vitrified using different concentrations of cryoprotectants

Concentration of cryoprotectant	Cryoprotectant			
	Ethylene glycol + Dimethyl sulphoxide		Ethylene glycol + Propylene glycol	
	No. of oocytes vitrified	Survivability (%) (Mean±SE)	No. of oocytes vitrified	Survivability (%) (Mean±SE)
30% (15% + 15%)	85	82.74±4.45 (68.75-100.00)	85	76.27±5.41 (63.16-93.75)
35% (17.5% + 17.5%)	85	86.99±3.07 (78.57-100.00)	85	85.60±5.33 (61.54-100.00)
40% (20% + 20%)	85	85.85±2.73 (77.78-94.44)	85	65.18±6.33 (50.00-80.00)

furnished in Table 2. The survivability rate of post-thaw vitrified oocytes was higher than that reported by Somfai *et al.* (2013) in pig, however, it did not vary significantly among the concentrations used which was in consonance with the findings of Mohmoud *et al.* (2013) who reported no significant difference between 6 M and 7 M concentrations of EG+DMSO in post-thaw survivability of vitrified buffalo oocytes. In the present investigation, the percentage of mean post-thaw survived oocytes was higher in 35% EG+DMSO (86.99±3.07) and EG+PG (85.60±5.33), however, it did not differ significantly from that using 30 and 40% concentrations in both the combinations. Present findings of level of post-thaw survivability were comparable with that reported by Shi *et al.* (2006) and Huang *et al.* (2006) in pig and Attanasio *et al.* (2010) in buffalo. Somfai *et al.* (2013) also observed that a combination of equal concentration of EG and PG as permeable cryoprotectant that had a total concentration of 35% improved the efficacy of vitrification protocol of immature porcine oocytes. In the present study, concentration of 35 and 40% of EG+DMSO and 35% EG + PG yielded higher post-thaw survivability following vitrification of follicular pig oocytes than utilizing 30% of the combination of the cryoprotectants. This indicated that higher concentrations of cryoprotectants inside cells became hyaloids material under ultra-cooling conditions and thereby prevented ice-crystal formation that were detrimental to cellular health. However, the toxic effects that might lead to oocyte damage emanating from using a higher concentration of cryoprotectant was evinced in the lower level of survivability of vitrified oocytes obtained by employing 40% total concentration of EG+PG in the present work. This could be due to inclusion of PG in the combination since use of 40% total concentration of EG+DMSO gave rise to much higher proportion of post-thaw survived vitrified oocytes. Somfai *et al.* (2015) also found that PG at high dose could be toxic. Szurek and Eroglu (2011) found that the toxicity of PG could be avoided by its combination with DMSO sharing equally for the total concentration. They further observed that toxicity of 1.5 M PG increased significantly as indicated by degeneration of vast majority of mouse oocytes with increase in exposure temperature and time while there was no significant increase in the toxic effect of 1.5 M DMSO and 1.5 EG under the same condition as revealed by viability of oocytes and fertilization and embryonic development. The mechanism of action of cryoprotectant (CPA) toxicity still remains poorly understood. Hydrophobic interactions between CPAs and proteins (Arakawa *et al.* 1990), the extent of hydrogen binding between CPAs and water molecules (Fahy *et al*. 2004) were proposed to explain cryoprotectant toxicity. Nevertheless, PG was implicated in inducing chemical toxicity (Huang *et al*. 2006). It could be concluded from the present study that the use of EG+DMSO as cryoprotectant at 35 to 40% concentration could bring about successful vitrification of porcine oocytes.

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