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# Effect of vitrification techniques on post-thaw survivability and *in vitro* maturation of immature bovine oocytes

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ABSTRACT

The present study was conducted to evaluate the effectiveness of single-step and two-step vitrification techniques on post-thaw survivability and subsequent *in vitro* maturation of immature bovine oocytes. In two-step vitrification method, oocytes were first equilibrated in vitrification solution-I and then vitrification solution–II of 3.5M or 4.0M and 7.0M or 8.0M glycerol + ethylene glycol (GLY+EG) cryoprotectant respectively. In single-step vitrification technique oocytes were directly exposed to final vitrification solution (7 M or 8 M of GLY+EG) for 45, 60 and 75 sec to find out the suitable exposure time based on post-thaw survivability and subsequent development *in vitro*. In single step vitrification the per cent morphologically normal oocyte, cumulus cells expansion and polar body formation was found to be significantly highest in oocytes of least exposure (45 seconds) period for 8M of GLY+EG. The per cent recovery of morphologically normal oocytes was found to be higher in two steps (91.81±1.42 and 91.18±1.17) than single step vitrification technique (87.94±3.49 and 85.72±2.24 of 45 sec exposure time) for both 7M and 8M of GLY+EG. The rate of cumulus cells expansion and polar body formation was significantly higher in two-steps (81.34±2.65% and 76.54±3.60% and 56.93±1.52% and 51.76±2.87%) than single step vitrification technique (57.33±3.90% and 56.91±4.66% and 33.17±5.34% and 32.70±2.91%). From the study it was concluded that two-step vitrification technique was more effective on post-thaw survivability and subsequent *in vitro* maturation of immature bovine oocytes.

**Key words**: Bovine oocytes, *In vitro* maturation, Survivability, Vitrification

Development of a suitable protocol for vitrification of immature bovine oocyte is still one of the main objectives of the assisted reproductive technologies, although vitrification has been successfully applied for cryopreservation of bovine oocytes (Luna *et al*. 2001, Diez *et al.* 2005, Martins *et al.* 2005, Hadi *et al.* 2011). A few important variables that influence the survival rate of vitrified oocytes are the type and concentration of the cryoprotectant, the temperature of the vitrification solution at the time of cell exposure, and the duration of exposure to the final cryoprotectant before plunging in liquid nitrogen. Gradual equilibration seems to adjust permeability of plasma membrane, which may contribute to maintaining the oocytes and cumulus cells, and/ or may decrease rapid changes in osmotic pressure (Mahmoudi *et al.* 2012). Therefore, the present experiment was undertaken to study the effect of single-step and two-step vitrification techniques and three exposure times to cryoprotectant in single-step technique on vitrification of immature bovine oocytes.

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## MATERIALS AND METHODS

*Collection of ovary and oocyte:* The cattle ovaries were collected from a local slaughter house in warmed (37°C) 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 hours of slaughter (Hadi *et al.* 2011). The extraneous tissues were removed from the ovaries and washed 3–4 times in physiological saline solution containing penicillin G (100 IU/ml) and streptomycin (0.125mg/ml) prior to further processing. The cumulus-oocyte-complexes (COCs) from the visible follicles measuring 3–8mm diameter were collected by aspiration technique in Medium 199 containing 25 mM HEPES, Earl's salts, L-glutamine and 2 mg/ml sodium bicarbonate modified by addition of 4 mg/ml bovine serum albumin (BSA, fraction V) and 50µg/ml Gentamycin. The oocytes with three or more numbers of compact cumulus cells layer surrounding the zona pellucida were subjected to vitrification and *in vitro* maturation.

*Vitrification and warming:* A combination of glycerol and ethylene glycol (GLY + EG) was used for preparation of vitrification solution I (VS I: 3.5 M or 4.0 M cryoprotectant) and II (VS II: 7.0 M or 8.0 M cryoprotectant). The VS I solutions for 3.5M (1.75 M GLY+ 1.75 M EG) and 4.0M (2.0 M GLY+ 2.0 M EG) were

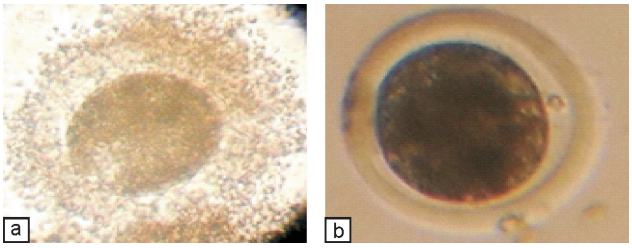


Fig.1. *In-vitro* matured oocyte: (a) Cumulus cell expansion; (b) Extrusion of polar body.

prepared in holding medium comprising Medium-199 with 20% fetal bovine serum (FBS). Similarly, 2 vitrification solutions of VS II for 7 M (3.5 M GLY+3.5 M EG) and 8 M (4.0 M GLY+4.0 M EG) concentrations were prepared with 0.5 M sucrose in holding medium.

In two-step vitrification technique, the immature oocytes were exposed for 1 min in VS I before being transferred to VS II and kept for 30 sec. Oocytes were loaded in 0.25 ml straw and plunged in liquid nitrogen for vitrification. In single-step vitrification technique the oocytes were directly placed from holding medium to VS II solution for 45, 60 or 75 sec before plunging the straws loaded with oocytes into liquid nitrogen.

Vitrified straws were thawed in warm water at 37°C for 1 min. The content of the straw along with oocytes was transferred subsequently into four diluting medium at 1 min interval into each which contained TCM-199 with 0.5M, 0.25M, 0.125M and 0.0625M of sucrose.

*In vitro maturation (IVM):* Post-thawed normal oocytes were subjected to IVM. Culture medium for IVM was prepared using medium-199 supplemented with 10% FBS, 10% follicular fluid, 50 µg/ml gentamicin, 50 µM/ml cysteamine, 1µg/ml oestradiol 17ß, 5µg/ ml p-FSH and 0.8 mM sodium pyruvate. The oocytes were incubated in droplets of IVM culture medium, covered with sterile mineral oil (50 µl/ 10–12 oocytes) and maintained at 38.5°C in 5% $CO_2$ in humidified air.

After 24 hours of maturation, oocytes were denuded using 0.1% (W/V) hyaluronidase in Medium 199 by vortexing. The denuded oocytes in groups of 1–2 were mounted on micro-slides, under a cover slip and then fixed in aceto-ethanol mixture (1:3, v/v) for 24 h. Fixed oocytes were then transferred to absolute alcohol solution for 5 min and stained with aceto-orcein stain (1% orcein in 45% glacial acetic acid). The maturation rate was evaluated based on cumulus cell expansion and polar body formation (Fig. 1).

*Statistical analysis:* The statistical analysis was done by using SAS enterprise guide 4.3.

## RESULTS AND DISCUSSION

The recovery of morphologically normal oocytes in single-step vitrification technique for 60 and 75 sec of exposure to 7 M and 8 M GLY+EG was significantly ($P<0.05$) lower than that in control two-step technique (Table 1). Previous studies on vitrified mouse oocytes (Farsani *et al.* 2010, Mahmoudi *et al.* 2012) had recorded lower rate of post-warming morphologically normal oocytes in single-step vitrification technique than in step-wise vitrification controls.

Bautista *et al*. (1998) recorded higher survival rate of mouse oocytes in two-step technique than in single-step technique (91.2 and 88.7%, respectively).This might be due to step-wise addition of cryoprotectant which reduced its toxic effects.

In the present study, the recovery rate of morphologically normal oocytes in single-step vitrification using 7 M and 8 M concentration of GLY+EG although did not differ significantly between exposure times, it was apparently higher for 45 sec exposure time. This might be due to the possibility of increased biochemical toxicity that occurs due to increase in exposure time since there is a parallel relationship between biochemical toxicity, exposure time and temperature (Hadi *et al*. 2010).

The cumulus cell expansion (Table 2) in single-step vitrification technique using 7 M and 8 M concentrations of GLY+EG was significantly lower ($P<0.05$) than that in two-step vitrification (control). Similarly, lower percentage of M-II was reported after IVM of vitrified mouse oocytes in single-step vitrification than in two-step vitrification by earlier workers (Farsani *et al*. 2007 and Mahmoudi *et al.* 2008). The significantly ($P<0.01$) higher percentage of cumulus cell expansion observed in two-step vitrification technique might be due to the fact that two-step addition of cryoprotectant might reduce the toxic effects of cryoprotectant. Gradual equilibration seems to adjust permeability of plasma membrane, which might contribute

Table 1. Recovery of morphologically normal oocytes after vitrification of immature bovine oocytes using 7M and 8M cryoprotectant (Glycerol+Ethylene glycol) by single-step and two-step vitrification techniques

| Single step (Exposure time) | Number of oocytes | | | | Recovery of morphologically normal oocytes | | | |
|---|---|---|---|---|---|---|---|---|
| | vitrified | | Recovered | | Number | | %(mean±SE) | |
| | 7 M | 8 M | 7 M | 8 M | 7 M | 8 M | 7 M | 8 M |
| 45 sec | 95 | 76 | 82 | 68 | 74 | 59 | 87.94 $^{ab}$ ±3.49 | 85.72$^{ab}$ ±2.24 |
| 60 sec | 90 | 89 | 79 | 73 | 65 | 60 | 80.89 $^{b}$ ±2.78 | 80.32 $^{bc}$ ±3.49 |
| 75 sec | 105 | 79 | 89 | 61 | 72 | 46 | 80.57 $^{b}$ ±3.23 | 75.83 $^{c}$ ±1.44 |
| Two step | 88 | 92 | 82 | 86 | 75 | 78 | 91.81 $^{a}$ ±1.42 | 91.18 $^{a}$ ±1.17 |

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

Table 2. Cumulus cell expansion and polar body formation after *in vitro* maturation (IVM) of immature bovine oocytes vitrified by single-step and two-step vitrification techniques using different exposure time at 7M and 8M concentration of cryoprotectant (glycerol+ethylene glycol)

| Single step (Exposure time) | Number of oocytes in IVM | | Number of oocytes | | | |
|---|---|---|---|---|---|---|
| | | | Cumulus cell expansion (%) mean±SE | | Polar body (%) mean±SE | |
| | 7 M | 8 M | 7 M | 8 M | 7 M | 8 M |
| 45 sec | 74 | 59 | 57.33 $^{b}$ ±3.90 | 56.91 $^{b}$ ±4.66 | 33.17 $^{b}$ ±5.34 | 32.70 $^{b}$ ±2.91 |
| 60 sec | 65 | 60 | 54.55 $^{b}$ ±1.70 | 49.32 $^{b}$ ±1.71 | 28.97 $^{b}$ ±2.99 | 25.92 $^{bc}$ ±2.65 |
| 75 sec | 72 | 46 | 42.39 $^{c}$ ±1.59 | 38.88 $^{c}$ ±1.2 | 24.44 $^{b}$ ±1.35 | 18.92 $^{c}$ ±2.58 |
| Two-step | 75 | 80 | 81.34 $^{a}$ ±2.65 | 76.54 $^{a}$ ±3.60 | 56.93 $^{a}$ ±1.52 | 51.76 $^{a}$ ±2.87 |

*Means with different superscripts in a column differ significantly ($P < 0.01$).

to maintaining the oocytes and cumulus cell and/ or might decrease rapid changes in osmotic pressure (Mahmoudi *et al*. 2012). The percentage of oocytes showing cumulus cell expansion in single-step vitrification technique using 7 M and 8 M concentration of GLY+EG was found to be significantly higher ($P<0.05$) for 45 and 60 sec than for 75 sec of exposure time.

The rate of polar body formation (Table 2) in single-step vitrification technique using 7 M and 8 M concentration of GLY+EG was significantly ($P<0.05$) lower than that in two-step vitrification (control). Similar results were obtained by previous workers (Farsani *et al*. 2010, Mahmoudi *et al*. 2012) after IVM of single-step vitrified mouse oocytes. Yang *et al*. (2003) obtained lower maturation rate in single-step vitrified oocytes than that in non-vitrified control after IVM of immature bovine oocytes. The results of the present study revealed that polar body formation after IVM of single-step vitrified oocytes did not differ significantly between exposure times in case of 7 M GLY+EG but differed significantly in case of 8 M GLY+EG. It was further observed that percentage of oocytes showing polar body extrusion decreased gradually as the exposure time in vitrification solution increased. The decrease in polar body formation rates with increase in equilibration time (exposure time) might be due to the aberrant protein synthesis after freezing (Moawad *et al*. 2012). The decline of developmental ability could be due to the toxic effects of cryoprotectant and osmotic injury.

The present study concluded that two-step technique was superior to single-step technique for vitrification of bovine follicular oocytes and there was decrease in post-thaw survivability and developmental competence of vitrified oocytes when exposure time to final vitrification solution was gradually increased in single-step vitrification technique.

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