



Effect of supplementation of vitrification solution on post-thaw survivability rate of porcine follicular oocytes

KRISHNA KALITA¹, B C DEKA², R K BISWAS³, P M BARUA⁴, P BORAH⁵, D J DUTTA⁶ and S K DAS⁷

Assam Agricultural University, Khanapara, Guwahati, Asom 781 022 India

Received: 1 December 2018; Accepted: 16 January 2019

Key words: Porcine oocytes, Post thaw survivability, Supplement, Vitrification, Vitrification solution

Oocytes cryopreservation provides greater flexibility in breeding programmes than embryo cryopreservation (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. In general, the combination of permeable cryoprotectants (CPAs) was known to be more effective for vitrification than individual CPA (Mahmoud *et al.* 2008, Somfai *et al.* 2013). Sucrose as a non-permeating agent was known to facilitate dehydration and vitrification which further reduced the toxicity of permeating cryoprotectant by decreasing its concentration. However, the best cryoprotectant or combination of cryoprotectants is yet to be determined in pig. It was suggested that addition of certain high molecular weight polymers like polyvinyl pyrrolidone (PVP) or ficol alone or in combination with sucrose had some beneficial effects on cryo-survivability of oocytes and they protected the cellular membrane and the zona pellucida from damage during cooling or warming procedures (Yang *et al.* 2003). In view of the beneficial effect of supplementation of vitrification solution with sucrose, PVP or their combination, present work was taken up to study the effect of supplementation of vitrification solution on post-thaw survivability rate of porcine follicular oocytes.

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. The oocytes were washed 3–4 times and classified into four grades (grade A, B, C and D) based on their gross morphology as integrity of cumulus cells (Jackowska *et al.* 2009). Only ‘A’ and ‘B’ grade oocytes were selected for vitrification.

The oocytes were exposed to 35% concentration vitrification solution (VS). Two types of VS, i.e. 17.5% EG + 17.5% PG and 17.5% EG + 17.5% DMSO were prepared in basic solution (BS) that contained 80 ml DPBS, 20 ml FBS and 5 mg gentamicin, pH 7.2–7.4. Both were again supplemented with three different types of supplements, i.e. sucrose (0.5 M), PVP (@50 mg/ml) and sucrose + PVP. Prior to vitrification, oocytes were first exposed to equilibration solution (cryoprotectant @ 15% v/v + sucrose 0.25 M in BS) for 5 min. Vitrification procedure was performed at room temperature (24–25°C). Within 30 sec. of exposure in vitrification solution (VS), the oocytes with little quantity of VS were loaded in the French mini straw (0.25 ml), sealed and directly plunged into LN₂ 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 solution) 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). Statistical analysis of the data was done using SAS (version 4.04) software.

The mean percentage of post-thaw survivability rate of oocytes following vitrification was 85.60±5.33, 43.94±5.40 and 85.03±4.14 in EG + PG and 86.99±3.07, 41.99±1.93

Present address: ¹Embryologist (embryologist.swagat.bongai@gmail.com), ⁷CMD (drsankarkumardas@rediffmail.com), Swagat Hospital and Research Center, Bongaigaon, Assam. ²Retired Professor and Head (bcdeka@gmail.com), ^{3,4}Professor (rkbiswascvsc@rediffmail.com, prithviraj.barua@gmail.com), Department of Animal Reproduction, Gynaecology and Obstetrics; ⁵Professor and Head (borahp@rediffmail.com), Department of Animal Biotechnology; ⁶Professor (duttjdj@hotmail.com), Department of Veterinary Physiology.

Table 1. Post-thaw survivability rate of follicular oocytes vitrified using different supplements in vitrification solution containing different cryoprotectants

Additive	Cryoprotectant			
	Ethylene Glycol + Propylene Glycol		Ethylene Glycol + Dimethyl Sulphoxide	
	No. of oocytes vitrified	Survivability (%) (Mean±SE)	No. of oocytes vitrified	Survivability (%) (Mean±SE)
Sucrose	85	85.60 ^a ±5.33 (61.54–100.00)	85	86.99 ^a ±3.07 (78.57–100.00)
Polyvinyl pyrrolidone	85	43.94 ^b ±5.40 (25.00–58.33)	85	41.99 ^b ±1.93 (36.84–46.15)
Sucrose + Polyvinyl pyrrolidone	85	85.03 ^a ±4.14 (75.00–100.00)	85	90.79 ^a ±1.66 (85.00–95.00)

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

and 90.79±1.66 in EG + DMSO containing vitrification solutions supplemented with sucrose, PVP and sucrose + PVP respectively (Table 1).

In the present study, supplementation with sucrose + PVP combination and sucrose alone resulted in significantly ($P<0.05$) higher mean post-thaw survivability rate of vitrified oocytes (Table 1) both for EG + PG and EG + DMSO as compared to the supplementation with PVP alone. The higher post-thaw survivability of oocytes obtained with sucrose + PVP supplementation was comparable with that reported by Yang *et al.* (2003) in bovine oocytes and Kim *et al.* (2008) in mouse embryo.

In our study, addition of PVP alone to the CPA showed significantly lower post-thaw survivability. Wang *et al.* (2014) also reported that addition of PVP at 20% or higher concentration of CPA (EG) did not increase survivability of mouse oocytes.

Sucrose, a disaccharide sugar, which consists of large molecules exerts its beneficial effect by contributing towards control of osmolarity in the vitrification and warming protocols (Arav *et al.* 1993). Also, sugars are known to stabilize and protect cell membrane under dehydrated conditions (Crowe *et al.* 1984). Higher survivability of vitrified oocytes in the present study using sucrose as an additive in vitrification solutions could be attributed to these factors. Beneficial effects of addition of sugars like sucrose or trehalose in vitrification solution were also recorded by other workers in bovine (Yang *et al.* 2003) and porcine (Shi *et al.* 2006, Huang *et al.* 2008, Somfai *et al.* 2015) oocytes.

Polyvinyl pyrrolidone (PVP) is the soluble homopolymer of N-vinyl-2 pyrrolidone. PVP tends to increase viscosity of the freezing solution and also form interaction through hydrogen bonding water, thereby decreasing the propensity for ice crystal formation. The efficacy of supplementation of PVP in the freezing solution was evidenced by successful production of the first live birth following cryopreservation of 8-cell mouse embryos (Whittingham 1971). However, addition of PVP did not bring about significant cryoprotective function in presence of CPA having 20% or higher concentration (Wang *et al.* 2014). Although non-significant, the highest post-thaw survivability rate of oocytes recorded with the addition of PVP in combination with sucrose in EG + DMSO obtained in the present study

suggested synergistic action of PVP with sucrose in protecting the oocytes during the process of vitrification and warming. PVP was reported to be used in conjugation with other CPA to freeze bovine oocytes (Checura and Seidel 2007). However, the exact mechanism of PVP in preventing the damage of oocytes during the process of vitrification and warming is still unclear. It can be concluded that addition of sucrose alone or in combination with PVP in vitrification solution significantly improved survivability of vitrified porcine follicular oocytes. Further studies are warranted on the dosage of PVP and selection of effective cryoprotectant with optimum vitrification and warming methods for efficient use of PVP in vitrification solution as supplement.

SUMMARY

Oocytes (510) with two or more cumulus cell layers adhered to zona pellucida obtained by aspiration from follicles (2–8 mm dia) of 127 abattoir porcine ovaries were utilized to study the effect of supplementation of 0.5 M sucrose (Suc), 50 mg/ml polyvinyl pyrrolidone (PVP) and Suc + PVP into two different vitrification solutions, i.e. 35% concentration of both ethylene glycol (EG)+ propylene glycol (PG) and EG + dimethyl sulphoxide (DMSO). 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 three supplements treatments, supplementation with sucrose + PVP combination and sucrose alone resulted in significantly higher mean post-thaw survivability rate of vitrified oocytes both for EG + PG and EG + DMSO as compared to the supplementation with PVP alone. It was concluded that addition of sucrose alone or in combination with PVP in vitrification solution, significantly improved survivability of vitrified porcine follicular oocytes.

REFERENCES

- Arav A, Shehu D and Mattioli M. 1993. Osmotic and cytotoxic study of vitrification of immature bovine oocytes. *Journal of Reproduction and Fertility* **99**: 353–58.
- Checura C M and Seidel G E. 2007. Effect of macromolecules in solutions for vitrification of mature bovine oocytes. *Theriogenology* **67**(5): 919–30.

- Crowe L M, Mouradian R, Crowe J H, Jackson S A and Womersley C. 1984. Effect of carbohydrates on membrane stability at low water activities. *Biochimica et Biophysica Acta* **769**: 141–50.
- Huang J, Li Q, Zhao R, Li W, Han Z, Chen X, Xiao B, Wu S, Jiang Z and Hu J. 2008. Effect of sugars on maturation rate of vitrified thawed immature porcine oocytes. *Animal Reproduction Science* **106**: 25–35.
- Jackowska M, Kampisty B, Antosik P, Bukowska D, Budna J, Lianeri M, Rosinka E, Wozna M, Jagodzinski P P and Jaskowski J M. 2009. The morphology of porcine oocytes associated with zona pellucida glycoprotein transcript contents. *Reproductive Biology* **9**: 79–85.
- Kim C G, Yong H, Lee G and Cho J. 2008. Effect of the polyvinyl pyrrolidone concentration of cryoprotectant on mouse embryo development and production of pups: 7.5% of PVP is beneficial for *in vitro* and *in vivo* development of frozen-thawed mouse embryos. *Journal of Reproduction and Development* **54**(4): 250–53.
- Mahmoud KGh M, Scholkamy T H, Ahmed Y F, Seidel G E and Nawito M F. 2008. Effect of different combinations of cryoprotectants on *in vitro* maturation of immature buffalo (*Bubalus bubalis*) oocytes vitrified by straw and open-pulled straw methods. *Reproduction in Domestic Animals* **45**(4): 565–71.
- Paynter S J and Fuller B J. 2007. Cryopreservation of mammalian oocytes methods. *Molecular Biology* **368**: 313–24.
- Shi W Q, Zhu S E, Zhang D, Wang W H, Tang G L, Hou Y P and Tian S J. 2006. Improved development by Taxol pre-treatment after vitrification of *in vitro* matured porcine oocytes. *Reproduction* **131**: 795–804.
- Somfai T, Kikuchi K, Kaneko H, Naguchi J and Yoshika K. 2013. Cryopreservation of female germplasm in pigs. *Society of Reproduction and Fertility Supplement* **68**: 47–60.
- Somfai T, Thi Men N, Noguchi J, Kaneko H, Kashiwazaki N and Kikuchi K. 2015. Optimization of cryoprotectant treatment for the vitrification of immature cumulus-enclosed porcine oocytes: comparison of sugars, combinations of permeating cryoprotectants and equilibration regimens. *Journal of Reproduction and Development* **6**(6): 571–79.
- Somfai T, Yoshioka K, Tanihara F, Kaneko H, Noguchi J, Kashiwazaki N, Nagai T and Kikuchi K. 2014. Generation of live piglets from cryopreserved oocytes for the first time using a defined system for *in vitro* embryo production. *PLoS ONE* **9**(5): e97731.
- Wang Y, OkitsuO, Zhao X M, Sun Y, Di W and Chian R C. 2014. The effect of minimal concentration of ethylene glycol (EG) combined with polyvinyl pyrrolidone (PVP) on mouse oocytes survival and subsequent embryonic development following vitrification. *Journal of Assisted Reproduction and Genetics* **31**: 55–63.
- Whittingham D. 1971. Survival of mouse embryos after freezing and thawing. *Nature* **233**: 125–26.
- Yang B Ch, Im G S, Chang W K, Lee Y K, Oh S J, Jin D I, Im K S and Lee CK. 2003. Survival and *in vitro* development of immature bovine oocytes cryopreserved by vitrification. *Asian Australasian Journal of Animal Science* **16**(1): 23–28.