

Post vitrification survivability of 2-cell stage IVP buffalo embryos: effect of concentration

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Received: 30 April 2008; Accepted: 24 August 2009

ABSTRACT

Good quality cumulus oocyte complex (COCs) aspirated from follicles >2 mm in abattoir buffalo ovaries were cultured in TCM-199 with 10% FCS, hormones, and antibiotics in 5% CO₂; 95% humidity at 38.5°C for 24–26 h for *in vitro* maturation (IVM). *In vitro* capacitation of bubaline spermatozoa was achieved using heparin. *In vitro* matured oocytes and *in vitro* capacitated spermatozoa were co-incubated in pre-equilibrated fertilization drops for *in-vitro* fertilization (IVF) and further cultured for cleavage and 2-cell *in-vitro* produced bubaline embryos were obtained, which were used for cryopreservation by vitrification. The 2-cell stage embryos were exposed to different mole concentrations (0–4 M) of dimethyl sulphoxide (DMSO) or 1, 2- propanediol (PROH) or the combination of DMSO and PROH in serially ascending concentrations grouped at 0.5 M interval along with 0.25 M sucrose with 2 min equilibration at each step. The embryos were vitrified and stored in liquid nitrogen for 7 or more days and then warmed and cryoprotectants were removed by placement of embryos in medium with serially descending concentrations of the cryoprotectants containing 0.5 M sucrose. The embryos were co-cultured with oviductal cells in TCM -199. The post vitrification developmental competence or cleaving ability was used for assessing suitability of the concentration of cryoprotectants. The results indicated that 1.5 M DMSO provided best cryoprotection (35.71%) to 2-cell stage buffalo embryos, whereas PROH performed better at 2.0 M (30.76%). When a combination of PROH and DMSO (1:1) was used the optimum concentration was found to be 1.0 M (33.33%).

Key words: Buffalo, Cryoprotectants, Embryos, Vitrification

Cryopreservation of embryos and oocytes has become a widely used method in commercial embryo transfer programme for many regions, as evident by the increasing number of cryopreserved embryos as well as their survival quality (Misra *et al.* 1992, Palasz and Mepletoft 1996, Shaw *et al.* 2000). Considerable differences of efficiency of embryos cryopreserved exist depending on the stage, species and origin (*in vivo* or *in vitro* produced). Factors that cause most of these differences are the amount of intra-cellular lipid droplets and different microtubular structure leading to chilling injury as well as the volume:surface ratio influencing the penetration of the cryoprotectants. Vitrification is the solidification of liquid by extreme increase in viscosity during very rapid cooling (Fahy *et al.* 1984).

Vitrification has been used to cryopreserve the oocytes

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or embryos with variable success of mice (Rall and Fahy 1985, Valdez *et al.* 1992, Chen *et al.* 2000, Lane and Gardner 2001, Edashige *et al.* 2003), rats (Nakagata 1989), goats (Begin *et al.* 2003), horses (MacLellan *et al.* 2002), rabbit (Cai *et al.* 2005), swine (Huang and Holtz 2002), cattle (Liebo and Loskutoff 1993, Hoshi *et al.* 1998, Vajta *et al.* 1998, Luna *et al.* 2001) and buffalo (Saxena and Maurya 1999, Dhali *et al.* 2000, Kumar 2000, Duran *et al.* 2004, Wani *et al.* 2004 a, b, Loganathasamy *et al.* 2006, Kumar *et al.* 2007).

Since a relatively high concentration of cryoprotective agent is required in vitrification technique, optimization of its level becomes essential and the information on cryopreservation of early buffalo embryos particularly by vitrification is lacking in literature except a few studies (Duran *et al.* 2004, Kumar *et al.* 2004, Kumar *et al.* 2007), present investigation was undertaken with the objective to establish a suitable concentration of dimethyl sulphoxide (DMSO), 1,2- propanediol (PROH) and their combination for vitrification and to study the effect of their concentration on post warmed developmental competence of *in vitro* produced 2-cell stage buffalo embryos.

MATERIALS AND METHODS

Buffalo ovaries were brought from a local slaughter house within one hour of slaughter to the laboratory in Dulbecco's phosphate buffer saline (D'PBS) supplemented with antibiotics at 25–30°C. Ovaries were washed twice in 60% alcohol and then 30 times in DPBS. Small antral follicles (2–8 mm size) were aspirated with the help of glass syringe fitted with 18 gauge needle after blotting dry the ovaries. Cumulus oocyte complexes were isolated under stereo zoom microscope and washed five times in TL-HEPES medium and three times in oocyte maturation medium (TCM -199) containing serum (10% FCS) and hormones (OLH 10 IU, OFSH, 1 µg/ml and E₂ 1 µg/ml). COCs were then cultured in maturation medium for 24–26 h (10 oocytes in 100 µl droplet) under sterile paraffin oil at 38.5°C in an atmosphere of 5% CO₂ and 95% humidity. Strict aseptic precautions were taken during all procedures. The maturation was assessed by cumulus mass expansion and extrusion of first polar body after culture.

Frozen thawed bubaline semen was prepared for *in-vitro* insemination (Parrish *et al.* 1986). The spermatozoa were washed twice with sperm – TALP by centrifugation at 2000 rpm for 10 min each. The content of sperm pallet was then carefully layered under 1 ml sperm – TALP containing heparin (10 µg/ml). Sperm motility was assessed. The sperm concentration was assessed with a haemocytometer and adjusted to 20×10⁶ spermatozoa per ml.

The fertilization – TALP was prepared freshly (pH 7.4) and 100 µl droplets were prepared under sterile paraffin oil on 35 mm petriplates and equilibrated with 5% CO₂ in humidified air for at least 2 h before use. Matured oocytes were washed thrice in TL-HEPES and twice with fertilization – TALP. Cumulus cells were detached by repeated pipetting and vortexing. The matured oocytes and capacitated spermatozoa were co-incubated for 6 h at 38.5°C with 5% CO₂ in humidified environment. After 6 h of co-incubation, the oocytes were transferred to culture medium containing oviductal epithelial cells from parallel cell culture. The fertilization was assessed by extrusion of second polar body and followed by formation of zygote, i.e. cleavage.

Cryopreservation by vitrification: The 2-cell stage IVP buffalo embryos were exposed to holding medium containing DPBS with 0.4% BSA for 2 min and then exposed to different vitrifying solutions of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 M concentration of either DMSO or PROH or a combination of DMSO and PROH in equal volume and concentration (1:1) with 0.25 M sucrose in DPBS supplemented with 0.4% BSA. Experiments were conducted using one group of cryoprotectant mixture at different concentrations per experiment. The embryos were exposed for 2 min. at different vitrification solutions and then filled in middle column of 0.25 cc. French mini straw in vitrifying solution separated by air bubble from the other columns

containing the same solution. Each straw contained 1 embryo. The straws were kept in liquid nitrogen vapour for 30 sec and then plunged into liquid nitrogen and stored for at least 7 days.

The straws containing embryos were warmed at 34°C in water-bath and the cryoprotectants were removed in serially descending concentrations of same cryoprotectant along with 0.5 M sucrose and lastly in holding medium. The embryos were recovered and cultured for *in vitro* development over 5-days-old oviductal epithelial co-culture system in TCM-199 supplemented with antibiotics. The survivability and development of vitrified–warmed embryos was assessed by first and second cleavage on post warmed culture. The data were analyzed by simple CRD for statistical analysis using computer micro-32.

RESULTS AND DISCUSSION

Post vitrification survival rate of 2-cell stage IVP buffalo embryos: The recovery rate and subsequent development of 2-cell embryos vitrified with DMSO at different concentrations are presented in Table 1. The maximum recovery rate (77.77%) was observed at 1.5 M concentration which decreased on alteration of DMSO concentration to either side. The recovery rate of identifiable embryos did not differ significantly between concentration groups. In control (0.0 M) group 11 (61.11%) embryos out of 18 were recovered in identifiable condition but none of these could cleave on subsequent culture. On post thaw culture the highest rate of first cleavage (50.00%) was observed after 24–30 h of culture at 1.5 M DMSO concentration which was significantly higher ($P<0.05$) than its nearest value (35.29%) recovered at 2.0 M concentration of DMSO. No cleavage on post thaw culture was observed in embryos of control and 4.0 M DMSO groups. The highest rate (35.71%) of second cleavage was observed with 1.5 M DMSO group after 44–50 h of culture followed by 2.0 M DMSO group (27.27%). Although 67.48% embryos were recovered in identifiable form, only 26.36% embryos underwent first cleavage and 16.36% completed the second cleavage.

When 2-cell stage IVP buffalo embryos were vitrified using PROH as cryoprotectant, the recovery rate of vitrified – warmed embryos was 64.83% and 25.42% embryos completed the first cleavage but only 15.25% could cleave twice on culture (Table 1). The recovery rate was maximum (72.22%) in 2.0 M PROH group which was significantly higher ($P<0.05$) than recovery rate observed with 3.5 M PROH group (50.00%). However, it did not differ significantly from all other PROH groups under study. The highest rate of post warmed first cleavage (38.64%) was observed in 2.0 M PROH group after 24–30 h of culture which did not differ significantly from 1.0, 1.5, 2.5 and 3.0 M PROH groups. No cleavage was observed in control, 3.5 and 4.0 M PROH groups.

Examination of 44–50 h of culture demonstrated that

Table 1. Post vitrification survivability of 2-cell stage buffalo embryos cryopreserved with DMSO/PROH/DMSO+PROH

Conc. (M)	DMSO			PROH			DMSO+PROH		
	Recovered/ vitrified (%)	Post vitrification development		Recovered/ vitrified (%)	Post vitrification development		Recovered/ vitrified (%)	Post vitrification development	
		1st cleavage (%)	IIInd cleavage (%)		1st cleavage (%)	IIInd cleavage (%)		1st cleavage (%)	IIInd cleavage (%)
0.0	11/18 (61.11 ^a)	00 (0)	00 (0)	3/5 (60.00 ^{ab})	00 (0)	00 (0)	03/05 (60.00 ^a)	00 (0)	00 (0)
0.5	15/22 (68.18 ^a)	03 (20.00 ^a)	02 (13.33 ^a)	5/8 (62.50 ^{ab})	00 (0)	00 (0)	07/10 (70.00 ^a)	03 (45.85 ^b)	02 (28.57 ^{ab})
1.0	14/20 (70.00 ^a)	04 (28.57 ^a)	03 (21.42 ^a)	7/10 (70.00 ^{ab})	02 (28.57 ^a)	01 (14.28 ^a)	12/16 (75.00 ^a)	06 (50.00 ^b)	04 (33.33 ^b)
1.5	14/18 (77.77 ^a)	07 (50.00 ^b)	05 (35.71 ^b)	11/18 (61.11 ^{ab})	04 (36.36 ^a)	02 (18.18 ^a)	12/16 (75.00 ^a)	05 (41.66 ^b)	03 (25.00 ^{ab})
2.0	11/16 (68.75 ^a)	05 (45.45 ^b)	03 (27.27 ^b)	13/18 (72.22 ^b)	05 (38.46 ^a)	04 (30.76 ^b)	11/18 (61.11 ^a)	03 (27.27 ^{ab})	02 (18.18 ^a)
2.5	14/20 (70.00 ^a)	06 (42.85 ^b)	03 (21.42 ^a)	8/12 (66.66 ^{ab})	03 (37.50 ^a)	02 (25.00 ^{ab})	11/15 (66.66 ^a)	02 (18.18 ^a)	00 (0)
3.0	10/16 (62.50 ^a)	02 (20.00 ^a)	01 (10.00 ^a)	5/8 (62.50 ^{ab})	01 (20.00 ^a)	00 (0)	07/10 (70.00 ^a)	00 (0)	00 (0)
3.5	12/18 (66.66 ^a)	02 (16.66 ^a)	01 (8.33 ^a)	3/6 (50.00 ^a)	00 (0)	00 (0)	07/11 (63.63 ^a)	00 (0)	00 (0)
4.0	09/15 (60.00 ^a)	00 (0)	00 (0)	4/6 (66.66 ^{ab})	00 (0)	00 (0)	06/10 (60.00 ^a)	00 (0)	00 (0)
Over	110/163 (67.48) ±5.64	29 (26.36) ±4.25	18 (16.36) ±4.16	59/91 (64.83) ±6.04	15 (25.42) ±7.63	09 (15.25) ±3.21	76/111 (68.46) ±6.63	18 (23.68) ±4.55	11 (14.47) ±4.14
	r=3/CD at 5% =18.03 CV=11.91	CD at 5% =13.59 CV=28.16	CD at 5% =13.32 CV=63.71	r=3/CD at 5% =19.33 CV=13.18	CD at 5% =24.42 CV=62.03	CD at 5% =10.29 CV=60.48	CD at 5% =21.21 CV=13.54	CD at 5% =14.56 CV=32.40	CD at 5% =13.24 CV=53.11

Values with unidentical superscripts in columns differ significantly (P<0.05).

30.76% of recovered embryos in 2.0 M PROH had undergone the second cleavage, while in 3.0 M group second cleavage failed to occur. There was no significant difference in the rates of second cleavage in 1.0, 1.5 and 2.5 M PROH groups. However, 2.5 M PROH group also differed non-significantly from 2.0 M group.

Vitrification of 2-cell embryos using equal volumes of DMSO and PROH as cryoprotectant resulted in overall recovery rate of 68.46% with 23.68% embryos developing to 4-cell stage and 14.47% to 8-cell stage (Table 1). The highest rate of recovery of embryos in identifiable form was observed with 1.0 M and 1.5 M groups (75.00%) which were not significantly different from other corresponding concentration groups. Culture of recovered embryos for 24–30 h failed to initiate any development in control, 3.0, 3.5 and 4.0 M DMSO+PROH groups. The highest rate of the first cleavage (50.00%) post warmed culture was recorded with embryos of 1.0 M group which was not significantly different from cleavage rates observed for 0.5, 1.5 and 2.0 M groups. The first post thaw cleavage rates recorded with 2.0 and 2.5 M groups also differed non-significantly. The highest

rate of second cleavage (33.33%) was recorded with 1.0 M which did not differ significantly from 28.57% rate recorded with 0.5 M group and 25.00% rate recorded at 1.5 M but differed significantly (P<0.05) from 2.0 M DMSO+PROH group (18.18%). However, in 2.5 M none of the 4-cell embryos developed after cleavage from 10 recovered embryos could resume cleavage further.

The recovery rates observed in the present investigation is similar to the embryo survival rate (69–74%) reported by earlier workers and Valdez *et al.* (1990) when rapid cooling of the bovine/mouse embryos was done using 2.0 M glycerol, 2.0 M DMSO, 2.0 M ethylene glycol, 2.0 M ethytriethylol or 3.0 M methanol as cryoprotectant. Our findings also lends support to the findings of Mohr and Freeman (1984) who also cryopreserved the 2-cell embryos. Almost similar recovery rates (71.76%) were also reported by Mishra (1992) with 2-cell mouse embryos cryopreserved with ethylene glycol+DMSO and these findings also corroborate our earlier reports (Kumar *et al.* 2004, Kumar *et al.* 2007) of pronucleate buffalo embryos. More than 85.0% survival rate was reported by Crister *et al.* (1988) when mouse embryos were

equilibrated for 20 min with 1.5 M DMSO alone and slow cooling was done. The difference may be attributed to the difference in the species as well as difference in cooling rates used, however, the better result at 1.5 M DMSO supports our findings that DMSO produced better results at 1.5 M concentration. No much report of vitrification of IVP early embryos in buffaloes is available in the literature.

Hence it can be concluded from the present study that DMSO (1.5 M) alone proved a better cryoprotectant for 2-cell stage IVP buffalo embryos than PROH (2.0M) or a combination of PROH and DMSO (1.0 M) the other acceptable cryoprotectants used for cryopreservation of early buffalo embryos by vitrification.

ACKNOWLEDGEMENTS

Senior author is grateful to Council of Scientific and Industrial Research for providing financial assistance during the course of investigation.

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