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SURVIVAL OF VITRIFIED WATER BUFFALO CUMULUS OOCYTE COMPLEXES AND THEIR SUBSEQUENT DEVELOPMENT *IN VITRO*

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Summary

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The study aimed to determine the effects of different concentrations of glycerol and ethylene glycol and ultrarapid cryopreservation (vitrification) on survival and subsequent *in vitro* development of bubaline cumulus oocyte complexes (COCs) in order to recognize the optimum cryoprotectant. Survival and *in vitro* maturation, fertilization and cleavage of buffalo COCs was evaluated subsequent to their cryopreservation by vitrification. The vitrification solution (VS) consisted of Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5 M sucrose, 0.5% bovine serum albumin (BSA) and different molar (M) concentrations of the cryoprotectants glycerol (G) (4 M, 6 M, 8 M and 10 M), ethylene glycol (EG) (4 M, 6 M, 8 M and 10 M) and their combinations (2 M G + 2 M EG, 3 M G + 3 M EG, 4 M G + 4 M EG and 5 M G + 5 M EG). The COCs were pre-equilibrated in 50% of the VS for 3–5 min, then kept in VS for 1 min and loaded in pre-sterilized 0.25 mL semen straws. After 7–10 days of storage COCs were warmed (38 ^{o}C for 5 s) and evaluated for morphological damage. Morphologically normal COCs were cultured *in vitro* and evaluated for nuclear maturation (n=847), fertilization (n=621) and cleavage (n=1451) in two separate experiments. The survival of oocytes was 86.4% and 89.6% in experiment 1 and 2. The highest proportion of normal oocytes was seen in 6 M EG and the lowest – in 10 M G in both experiments. The *in vitro* maturation of oocytes at the end of experiment 1, and the *in vitro* fertilization and cleavage at the end of experiment 2, were significantly lower in all tested vitrification cryoprotectants compared to control. A dose-dependant increase in the proportion of oocytes matured, fertilized or cleaved was seen for both G and EG up to concentrations of 8 M. There was no specific benefit of combining G and EG on the subsequent *in vitro* maturation, fertilization and cleavage of oocytes. At equal concentrations EG proved to be a better cryoprotectant than G. It was concluded that there appeared to be a limit to the concentration of the cryoprotectant (8 M) beyond which the cryoprotectant exerted suboptimal effects and that there was no benefit of combining two similar cryoprotectants for cryopreservation of oocytes by vitrification.

Key words: buffalo, ethylene glycol, glycerol, oocytes,vitrification

INTRODUCTION

Current cryopreservation protocols have evolved from methods developed for freezing mouse, sheep and cattle embryos (Willadsen *et al.*, 1975; Whittingham, 1977; Willadsen *et al.*, 1978). The design of cryopreservation methods for living

cells and tissues, including embryos and eggs, usually disregard the biological complexity of such material and focus instead on the more immediate physical consequences of cooling and rewarming as potential causes of cellular damage. This may explain the difficulty and relatively poor success of oocyte cryopreservation (Cohen *et al.*, 1988; Carroll *et al.*, 1990; 1993; Gook *et al.*, 1993). According to the most widely accepted explanation, high solute concentrations, known as "solution effects" and intracellular ice are responsible for most, if not all, damage to cells during cooling and rewarming (Shaw *et al.*, 2000). While both factors often work simultaneously, intracellular ice is most likely to occur during rapid cooling and relatively slow rewarming, whereas solution effects are more pronounced with slow cooling (Mazur *et al.*, 1992). Each type of cell has its own optimal cooling rate. Cryoprotectant permeability also changes with changes in temperature (Mazur, 1977). As the cells are cooled slowly, ice crystals may be formed in the cytoplasm which may be lethal to the cells (Mazur, 1977). In contrast, when cells are cryopreserved by vitrification, they are cooled in such high concentrations of cryoprotectant solution and at such high cooling rates that intracellular ice crystals do not form (Porcu, 2001).

Relatively few studies have focused on the cryopreservation of bubaline oocytes. These studies evaluated a single molar concentration of two cryoprotectants: dimethyl sulfoxide (DMSO) and ethylene glycol (EG) (Dhali *et al.*, 2000a; 2000b) or different combinations of EG, DMSO and 1,2-propanediol (PROH) (Wani *et al.*, 2004a) and studied the morphological survival and nuclear maturation of oocytes. The highest cleavage of buffalo oocytes was recorded at 7 M concentration of DMSO and other cryoprotectants (Wani *et al.*, 2004b). Successful vitrification can be achieved in two ways: i) increasing the speed of temperature conduction and ii) increasing the concentration of the cryoprotectant (Fahy *et al.*, 1984). It remains to be seen whether the morphological survival of oocytes can be further improved by increasing the concentration of the cryoprotectants or their combinations. Buffalo oocyte cryopreservation can be important because of the relatively lower oocyte yield per ovary in this species (Purohit *et al.*, 2003); however, the cryopreservation protocols need substantial improvement. The objective of the present study was to vitrify bubaline oocytes and determine their survival and subsequent development *in vitro*.

MATERIALS AND METHODS

Cumulus oocyte complexes (COCs) were collected by aspiration of surface follicles (2–8 mm) present on buffalo ovaries (n=1780) collected from a local abattoir. COCs with an evenly granulated cytoplasm and 3–4 or more layers of cumulus cells attached were selected for further work.

Oocyte cryopreservation

Freshly collected oocytes were cryopreserved by ultrarapid cooling as per methods described previously (Das, 1997) with some modifications. The vitrification solution (VS) comprised of Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5 M sucrose, 0.4% bovine serum albumin (BSA) and different molar (M) concentrations of the cryoprotectants glycerol (G) and ethylene glycol (EG) and their combinations in order to be tested as vitrification cryoprotectants. The concentrations tested were 4 M, 6 M, 8 M

and 10 M concentration of both G and EG and combinations of 2 M G + 2 M EG, 3 M G + 3 M EG, 4 M G + 4 M EG and 5 M G + 5 M EG. The oocytes were pre-equilibrated in 50% of the vitrification solution (prepared by dilution of VS in DPBS) for 3–5 min and then kept in VS for 1 min and loaded (4–5 oocytes per straw) in pre-sterilized 0.25 mL straws (IMV, France). The straws were heat-sealed and pre-cooled by keeping the straws over LN_2 vapour for 2 min at the height of about 5 cm from LN_2 level. The straws were then plunged in LN_2 and stored for 7–10 days.

Warming and evaluation

Frozen straws containing the oocytes were warmed in a water bath at 38 °C for 5 s. The contents of the straw were emptied in a 35 mm Petri dish and the oocytes were evaluated for morphological damage. The oocytes were considered abnormal when there was a change in shape, breakage of zona pellucida, uneven granulation or leakage of oocyte contents. The survival percentage was calculated as the proportion of oocytes seen to be normal against the total number vitrified. The cryoprotectant was removed by placing oocytes in 50% VS and then transferring to DPBS. The morphologically normal oocytes were matured and fertilized *in vitro* in two experiments to record their *in vitro* maturation, fertilization and cleavage. In experiment 1, morphologically normal oocytes (n=847) were matured *in vitro* (9 replicates with 5–10 oocytes per replicate) for 24 h and evaluated at the end of the experiment for maturation. Freshly collected oocytes were simultaneously matured *in vitro* and kept as control (n=95).

In experiment 2, oocytes were vitrified and those recovered in normal form were matured *in vitro* and then fertilized *in vitro* (n=2072). Of the fertilized oocytes, a part were evaluated for fertilization by fixing and staining (n=621) whereas the rest were evaluated for cleavage (n=1451) 48 h later. Freshly collected oocytes were also processed for *in vitro* maturation, fertilization and culture without freezing, evaluated for fertilization (n=72) and cleavage (n=158) and kept as control.

In vitro maturation

Oocytes were matured *in vitro* in TCM-199 supplemented with 5 $\mu g.mL^{-1}$ FSH, 5 $\mu g.mL^{-1}$ LH and 1 ng/mL oestradiol, 25 mM Hepes, 0.25 mM pyruvate and antibiotics by keeping them in a CO_2 incubator with 5% CO_2 and 39.5 ^oC temperature as per previously described methods (Kumar & Purohit, 2004; Purohit *et al.*, 2005). After 24 h of *in vitro* culture, all oocytes in different groups in different replicates were fixed and stained as per Purohit *et al.* (2005) and evaluated. Briefly, the surrounding cumulus cells were removed by vortexing for 1 min. The cumulus-free oocytes were placed in the centre of an area delineated by two paraffin wax bars on a clean grease-free glass slide. They were compressed gently with a cover slip to hold and fixed by keeping them in acetic methanol (1:3, v/v) for 24 h. Oocytes were stained with 1% aceto-orcein (1% orcein in 45% glacial acetic acid). The nuclear status of oocytes was evaluated under a microscope and considered to be matured if they were at metaphase II stage (reduced number of chromatin, metaphase plate and extrusion of 1st polar body).

Sperm preparation and in vitro fertilization (IVF)

Frozen thawed buffalo bull semen was prepared for IVF using a discontinuous Percoll density gradient to separate live spermatozoa as previously described (Purohit *et al.*, 2005). Briefly, 4 mL of

90% isotonic Percoll was layered in a 15 mL centrifuge tube beneath 4 mL of 45% isotonic Percoll. The sperms were washed initially in TALP-BSA by centrifugation at 250×g for 4 min. The sperm pellet was re-suspended in 1 mL of the medium. The washed sperm pellet was layered on the top of Percoll gradient and centrifuged at 300×g for 35 min. The resultant pellet was removed from the bottom and washed twice in TALP-BSA by centrifugation.

The sperm pellet was re-suspended in TALP to give a final concentration of 3×10^9 spermatozoa per mL. They were incubated for 2–3 h in a CO_2 incubator. The matured cumulus oocyte complexes were transferred to another dish containing Fert-TALP medium (TALP supplemented with 30 $\mu g.mL^{-1}$ penicillamine, 15 mM hypotaurine mL^{-1}) under paraffin oil. This was inseminated with prepared sperms in a volume, so as to give a final concentration of 1×10^6 spermatozoa per mL. Following co-incubation for 20–24 h with sperm, approximately 30% of the oocytes from each treatment group were washed with fresh medium and vortexed for 1 min to separate the cumulus mass. They were prepared for fixing and staining in the same way as oocytes were fixed after *in vitro* maturation. The fertilization was evaluated as per previous method (Purohit *et al.*, 2005).

In vitro embryo culture

Following 20–24 h of sperm oocyte co-incubation, oocytes were washed 10–15 times in the IVM media and they were then cultured for another 48 h. The cleavage (division into two or more cells) was recorded.

Statistical analysis

The proportion of morphologically normal oocytes recovered between various groups was compared by the Chi-square test. The arcsin transformed data of the proportion of oocytes matured, fertilized or cleaved over various groups were compared by DNMR test (Purohit *et al.*, 2005).

RESULTS

Oocyte survival

In experiment 1 and 2 the survival of oocytes was 86.4% (1050/1214) and 89.6% (2072/2310) respectively. Some of the

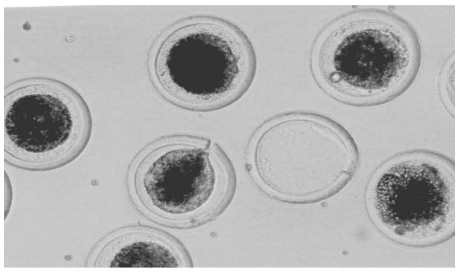

Fig. 1. Microphotograph showing morphologic damage (ruptured zona, leakage of oocyte contents, uneven granulation) in oocytes cryopreserved by vitrification in 4 M glycerol.

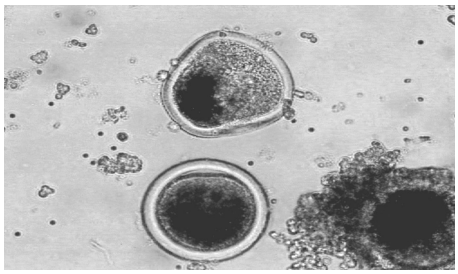

Fig. 2. Microphotograph showing change in shape of oocytes cryopreserved by vitrification in 6 M ethylene glycol.

Table 1. Survival of buffalo oocytes after vitrification in varying concentrations of glycerol, ethylene glycol and their combinations

Vitrification solution	Proportion of morphologically normal oocytes
4 M G	81.63 ^{acd}
6 M G	90.56 ^{cdfg}
8 M G	86.17 ^{ace}
10 M G	70.21 ^{a}
4 M EG	87.36 ^{abfg}
6 M EG	95.23 ^{eg}
8 M EG	94.0b ^{eg}
10 M EG	72.34 ^{d}
2 M G+2 M EG	83.65 ^{af}
3 M G+3 M EG	87.17 ^{ab}
4 M G+4 M EG	92.24 ^{beg}
5 M G+5 M EG	94.64 ^{g}

Values in same column with different superscripts differ significantly, X^2 test (P=0.05). Pooled data of two experiments.

abnormalities observed in the oocytes subsequent to warming of cryopreserved oocytes are shown in Fig. 1 & Fig. 2. The highest proportion of normal oocytes was seen in 6 M EG and the lowest – in 10 M G, in both experiment 1 and 2. Non-significant (P>0.05) differences were seen between the oocytes recovered in normal form for equal concentrations of both G and EG. Pooled data of survival of oocytes in the various concentrations of G and EG or their combination are shown in Table 1.

In vitro maturation

Oocyte maturation was significantly lower (p=0.05) in all vitrification cryoprotectants compared to control. The proportion of oocytes attaining M-II increased with increasing concentrations of both cryoprotectants up to 8 M, however, at 10 M concentrations of both G and EG, the nuclear maturation rates decreased significantly (P=0.05). Combination of both cryoprotectants did not further improve the nuclear maturation of oocytes compared to when either cryoprotectant (G or EG) was used alone, except for the combination of 5 M G and 5 M EG. EG was found to be a better cryoprotectant compared to G as evident by significantly higher (P=0.05) proportion of oocytes reaching M-II at equal concentration of EG (4 M, 6 M, 8 M and 10 M) as compared to G.

Fertilization and cleavage

In experiment 2, the proportion of oocytes fertilized or cleaved at the end of experiment was significantly higher for control oocytes as compared to vitrified oocytes (Table 2). A dose-dependant significant increase (P=0.05) in the proportion of oocytes fertilized or cleaved was seen up to 8 M concentration of both G and EG. At equal concentrations (4 M, 6 M, 8 M and 10 M), a significantly higher proportion of oocytes were fertilized, and cleaved in EG compared to G. A specific benefit of combining the two cryoprotectants (G and EG) on the fertilization or cleavage of vitrified oocytes was not seen and the combination yielded lower fertilization and cleavage rates compared to when either G or EG was used alone except at 10 M concentration (5 M G + 5 M EG) which tended to increase the fertilization and cleavage.

DISCUSSION

The results of the present study indicate that a high proportion of oocytes retain their normal morphology after a short exposure to high concentrations of different cryoprotectants except for 10 M glycerol at which a high proportion of oocytes turned out to be abnormal. These findings corroborate well with previous

Table 2. *In vitro* maturation, fertilization and cleavage of buffalo oocytes vitrified in different concentrations of glycerol (G), ethylene glycol (EG) and their combinations

Vitrification solution	*In vitro* maturation (%) [A]	*In vitro* fertilization (%) [B]	Cleavage (%)[C]
Control	65.26 [e]	54.16 [a]	31.64 [a]
4 M G	6.90 [a]	5.55 [d]	5.00 [e]
6 M G	21.17 [b]	19.23 [c]	15.00 [bc]
8 M G	25.00 [bc]	22.00 [c]	18.64 [b]
10 M G	5.45 [a]	4.16 [d]	0.89 [df]
4 M EG	22.66 [b]	19.23 [c]	9.91 [cd]
6 M EG	34.32 [cd]	27.08 [bc]	19.81 [b]
8 M EG	41.89 [d]	37.73 [b]	22.40 [b]
10 M EG	7.50 [a]	3.70 [d]	1.58 [h]
2 M G + 2 M EG	6.32 [a]	4.08 [b]	3.53 [eh]
3 M G + 3 M EG	9.52 [a]	7.54 [d]	5.60 [efg]
4 M G + 4 M EG	24.39 [bc]	20.34 [c]	10.23 [de]
5 M G + 5 M EG	28.73 [bc]	22.22 [c]	17.32 [b]

Values in same column with different superscripts differ significantly, DNMR-test (arcsin transformed data) (P=0.05). Data pooled from (A) 9 replicates, (B) 8 replicates and (C) 10 replicates.

findings of Dhali *et al.* (2000a) and Wani *et al.* (2004a) on buffalo oocytes. Studies by Vieira *et al.* (2001) and Mavrides & Morroll (2002) had also recorded survival/recovery rates of 65–95 % for bovine vitrified oocytes as in the present study. The damage to oocytes during cryopreservation may be because of the large lipid like material found in oocytes of many species, since lipid removal or lipid polarization reduces chill and cryo-injury (Otoi *et al.*, 1997).

The *in vitro* maturation, fertilization and cleavage of the vitrified immature buffalo oocytes were significantly lower compared to control in the present study. Wani *et al.* (2004b) recorded that less buffalo oocytes reached metaphase-II for oocytes cryopreserved in different concentrations of DMSO, EG, PROH and glycerol compared to fresh oocytes.

A dose-dependant increase in the proportion of oocytes that survived morphological damage and subsequently matured, fertilized and cleaved *in vitro* suggested that the optimum concentration of both cryoprotectants was 8 M. Many publications on the problems of mammalian oocyte cryopreservation contain information regarding the negative effects of low temperature including the cytoskeleton depolymerization effects of permeable cryoprotectants (Pickering & Johnson, 1987; Vincent *et al.*, 1989; Aigner *et al.*, 1992; Joly *et al.*, 1992; Yoon *et al.*, 2000). Such effects may be more pronounced with high concentrations of the two permeable cryoprotectants used in the present study. The freeze thaw process is known to induce an alteration in the physico-chemical properties of intracellular lipids (Isachenko *et al.*, 2001; Kim *et al.*, 2001) and such damages may render the oocyte incapable of retaining its developmental competence. Despite the protective effects of cryoprotectants during freezing they may

impose concentration, time and temperature dependant toxicity (Fahy *et al.*, 1990).

The findings of better performance of ethylene glycol as a vitrification cryoprotectant, compared to glycerol and the optimum concentration of both cryoprotectants being 8 M during the present study suggested that there was a limit to the concentration of the cryoprotectant to be used beyond which it may exert sub-optimal effects. This is in part similar to previous findings of Wani *et al.* (2004b). These workers observed that the highest IVM of buffalo oocytes was observed for oocytes vitrified in 7 M solution of all cryoprotectants and that EG was better compared to glycerol. Ethylene glycol has been found to be an effective cryoprotectant for the vitrification of mouse (Miyake *et al.*, 1993), cattle (Delval *et al.*, 1996; Saha *et al.*, 1996) and buffalo (Dhali *et al.*, 2000a; 2000b, Wani *et al.*, 2004b) oocytes, since it offers advantages over other cryoprotectants in terms of higher permeation into oocytes/embryos for vitrification, and, faster removal during dilution, as its molecular weight is lower than that of glycerol (Dhali *et al.*, 2000a). Ethylene glycol has been found to be less toxic than glycerol and propylene glycol to mouse embryos (Kasai *et al.*, 1990) and the post-vitrification survival of bovine embryos has been found to be much higher in ethylene glycol than in either a combination of DMSO, PG and polyethylene glycol or a combination of glycerol and PG (Mahmoudzadeh *et al.*, 1993).

A combination of both cryoprotectants did not improve the nuclear maturation, fertilization and cleavage rates of vitrified oocytes compared to when either was used alone. Although many workers (Critser *et al.*, 1997, Vajta *et al.*, 1998, Paynter *et al.*, 1999) had recorded marginal benefit of combining more than one cryoprotectant on *in vitro* maturation of bovine oocytes, Wani *et al.* (2004b) however, had found no benefit of combining DMSO with either EG or propylene glycol on the nuclear maturation of vitrified buffalo oocytes. The overall composition of the solution appears to be important (Shaw *et al.*, 2000) as oocyte survival is modified by other components including sugars, macro-molecules or polymers (Carroll *et al.*, 1993; Miyake *et al.*, 1993; O'Neil *et al.*, 1997). It was concluded that there appeared to be a limit to the concentration of the cryoprotectants G and EG (8 M) beyond which they exerted sub-optimal effects and that there was no benefit of combining these two permeable cryoprotectants for oocyte vitrification.

REFERENCES

Aigner, S., J. Van der Elst, E. Siebzehnrubl, L. Wildt, N. Lang & A. C. Van Steirteghem,1992. The influence of slow and ultra rapid freezing on the organization of the meiotic spindle of the mouse oocytes. *Human Reproduction,* **7,** 857–867.

Carroll, J. D., G. Whittingham, M. J. Wood, E. Telfer & R. G. Gosden, 1990. Extra ovarian production of mature viable mouse oocytes from frozen primary follicles. *Journal of Reproduction and Fertility,* **90**, 312–327.

Carroll, J., M. J. Wood & D. G. Whittingham, 1993. Normal fertilization and development of frozen thawed oocytes: Protective action of certain macro-molecules. *Biology of Reproduction,* **48**, 606–612.

Cohen, J., K. L. Inage, S. R. Wiker & G. Sright, 1988. Duration of storage of cryopreserved human embryos. *Journal of In Vitro Fertilization and Embryo Transfer,* **5**, 301–303.

Critser, J. K., Y. Agca & K. T. Gunasena, 1997. The cryobiology of mammalian oo-

cytes. In: *Reproductive Tissue Banking,* eds. A. M. Karow & J. K. Critser, Academic Press, San Diego, pp. 329–357.

Das, S. K., 1997. Vitrification procedure for cryopreservation of oocytes. In: *In Vitro Fertilization, Embryo Transfer and Associated Techniques in Farm Animals*. NDRI, Karnal, India, pp. 69–72.

Delval, A., F. J. Ectrors, K. Touati, J. F. Beckers & F. Ectoss, 1996. Vitrification of bovine embryos produced *in vitro*: Survival, hatching and pregnancy rates. *Theriogenology,* **45**, 178 (Abstract).

Dhali, A., R. S. Manik, S. K. Das, S. K. Singla & P. Palta, 2000a. Vitrification of buffalo (*Bubalus bubalis*) oocytes. *Theriogenology,* **53**, 1295–1303.

Dhali, A., R. S. Manik, S. K. Das, S. K. Singla & P. Palta, 2000b. Post-vitrification survival and *in vitro* maturation rate of buffalo (*Bubalus bubalis*) oocytes: Effect of ethylene glycol concentration and exposure time. *Animal Reproduction Science,* **63**, 159–165.

Fahy, G. M., D. R. MacFarlane, D. A. Angell & H. T. Meryman, 1984. Vitrification as an approach to cryopreservation. *Cryobiology,* **21**, 407–426.

Fahy, G. M., T. H. Lilley, H. Linsdell, M. S. Douglas & H. T. Meryman, 1990. Cryoprotectant toxicity and cryoprotectant toxicity reduction in search of molecular mechanism, *Cryobiology,* **27**, 247–268.

Gook, D. A., S. M. Osborn, & W. I. H. Johnston, 1993. Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. *Human Reproduction,* **8**, 1101–1109.

Isachenko, V., H. W. Michelman, J. L. Alabart, I. Vazquez, E. Isachenko, N. Bezugly & F. Nawroth, 2001. Lipolysis and ultrastructural changes of intracellular lipid vesicles after cooling of bovine and porcine GV oocytes. *Anatomia, Histologia, Embryologia,* **30**, 333–338.

Joly, C., O. Bchini, H. Boulekbache, J. Testart & B. Maro, 1992. Effect of 1,2-propanediol on the cytoskeletal organization of the mouse oocytes. *Human Reproduction,* **7**, 374–378.

Kasai, M., J. H. Komi, K. Takakamo, H. Tsudera, T. Sakurai & T. Machida, 1990. A simple method for mouse embryos cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *Journal of Reproduction and Fertility,* **89**, 91–97.

Kim, J. Y., M. Kinoshita, M. Ohnishi & Y. Fukui, 2001. Lipid and fatty acid analysis of fresh and frozen-thawed immature and *in vitro* matured bovine oocytes. *Reproduction,* **122**, 131–138.

Kumar, D. & G. N. Purohit, 2004. Effect of epidermal and insulin-like growth factor-1 on cumulus expansion, nuclear maturation and fertilization of buffalo cumulus oocyte complexes in simple serum free media DMEM and Ham's F-10. *Veterinarski Arhiv,* **74**, 13–25.

Mahmoudzadeh, A. R, A. Van Soom, I. Van Vlaenderen & A. De Kruif, 1993. A comparative study of the effect of one-step addition of different vitrification solutions on *in vitro* survival of vitrified bovine embryos. *Theriogenology,* **39**, 1291–1302.

Mavrides, A. & D. Morroll, 2002. Cryopreservation of bovine oocytes: Is cryoloop vitrification the future to preserving the female gamete? *Reproduction, Nutrition and Development,* **42**, 73–80.

Mazur, P., U. Schneider & A. P. Mahowald, 1992. Characteristics and kinetics of subzero chilling injury in Drosophila embryos. *Cryobiology,* **29,** 39–68.

Mazur, P., 1977. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. *Cryobiology,* **14**, 251–272.

Miyake, T., M. Kasai, S. F. Zhu, T. Sakurai & T. Machida, 1993. Vitrification of mouse oocytes and embryos at various stages of development in an ethylene glycol based solution by simple methods. *Theriogenology,* **40**, 121–134.

O'Neil, L., S. J. Paynter, B. J. Fuller & R. W. Shaw, 1997. Vitrification of mature mouse oocytes: Improved results following addition of polyethylene glycol to a dimethylsulfoxide solution. *Cryobiology,* **34**, 295–301.

Otoi, T., K. Yamamoto, S. Tachikawa, M. Murakami, Y. Kikkawa & T. Suzuki. 1997. Cryopreservation of mature bovine oocytes following centrifugation treatment. *Cryobiology,* **34**, 36–41.

Paynter, S. J., A. Cooper, B. J. Fuller & R. W. Shaw, 1999. Cryopreservation of bovine ovarian tissue: Structural normality of follicles after thawing and culture *in vitro*. *Cryobiology,* **38**, 301–309.

Pickering, S. J & M. H. Johnson, 1987. The influence of cooling on the organization of the meiotic spindle of the mouse oocytes. *Human Reproduction,* **2**, 207–216.

Porcu, E., 2001. Oocyte freezing. *Seminal Reproductive Medicine,* **19**, 221–230.

Purohit, G. N, G. P. Duggal, D. Dadarwal, D. Kumar, R. C. Yadav & S. Vyas, 2003. Reproductive biotechnologies for improvement of buffalo: The current status. *Asian Australasian Journal of Animal Science,* **16**, 1071–1086.

Purohit, G. N, M. S. Brady & S. S. Sharma, 2005. Influence of epidermal growth factor and insulin-like growth factor-1 on nuclear maturation and fertilization of buffalo cumulus oocyte complexes in serum free media and their subsequent development *in vitro*. *Animal Reproduction Science,* **87,** 229–239.

Saha, S., A. Boediono, E. Sumantri, M. Murakani, Y. Kikkawa & T. Suzuki, 1996. Vitrification of bovine *in vitro* matured and pronuclear oocytes with different vitrification solutions. *Theriogenology,* **45**, 179 (Abstract).

Shaw, G., A. Oranratnachai & A. O. Trounson, 2000. Fundamental cryobiology of mammalian oocyte and ovarian tissue. *Theriogenology,* **53**, 59–72.

Vajta, G., P. Holm, M. Kuwayama, P. J. Booth, H. Jacobsen, T. Greve & H. Callesen, 1998. Open pulled straws (OPS) vitrification: A new way to reduce cryoinjuries of bovine oocytes and embryos. *Molecular Reproduction and Development,* **51**, 53–58.

Vieira, D. G. I., R. S. Fontes, A. B. Dias, C. S. Paes, P. R. Adona, M. C. C. Bussiere, C. L. V. Leal & L .P. L Pinheiro, 2001. *In vitro* vitrification of mature bovine oocytes. XIV Congresso Brasileiro de Reproducao Animal. Belo Horizonte, Brazil, 1–5 August, 2001. *Revista Brasileira de Reproducao Animal,* **25**, 416–417.

Vincent, C., V. Garnier, Y. Heyman & J. P. Renard, 1989. Solvent effects on cytoskeletal organization and *in vivo* survival after freezing of rabbit oocytes. *Journal of Reproduction and Fertility,* **87**, 809–820.

Wani, N. A, A. K. Misra & S. N. Maurya, 2004a. Maturation rates of vitrified thawed immature buffalo (*Bubalus bubalis*) oocytes: Effect of different types of cryoprotectants. *Animal Reproduction Science,* **84**, 327–335.

Wani, N. A., S. N. Maurya, A. K. Misra, V. B. Saxena & B. D. Lakhchaura, 2004b. Effect of cryoprotectants and their concentration on *in vitro* development of vitrified warmed immature oocytes in buffalo (*Bubalis bubalis*). *Theriogenology,* **61**, 831–842.

Whittingham, D. G., 1977. Fertilization *in vitro* and development to term of unfertilized mouse oocytes previously stored at –196 °C. *Journal of Reproduction and Fertility,* **49**, 89–94.

Willadsen, S. M, C. Polge, L. E. Rowson & R. M. Moor. 1975. Deep freezing of sheep embryos. *Journal of Reproduction and Fertility,* **46**, 151–154.

Willadsen, S. M, C. Polge & L. F. Rowson. 1978. The viability of deep frozen cow embryos. *Journal of Reproduction and Fertility,* **52**, 391–393.

Yoon, T. K., H. M. Chung, J. M. Lim, S. Y. Han, J. J. Ko & K. Y. Cha, 2000. Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimu-

lated *in vitro* fertilization-embryo transfer programme. *Fertility and Sterility,* **74**, 180–181.

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