Morphological Survival and Subsequent *In Vitro* Maturation of Denuded and Cumulus Compact Bubaline Oocytes Cryopreserved by Ultra Rapid Cooling

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Abstract: Culturable grade oocytes (n=380) recovered by aspiration of surface follicles from buffalo ovaries (n=97) were either mechanically denuded (DN) or kept cumulus compact (CC) and were vitrified in Dulbecco's phosphate buffered saline + 0.4% sucrose, 0.4% bovine serum albumin and 6 M concentrations of either ethylene glycol (EG) or propylene glycol (PG). Oocytes were randomly allocated to four groups of vitrification (EGCC, EGDN, PGCC and PGDN) and cryostorage for 7-10 days in liquid nitrogen. They were then warmed to record morphological survival and morphologically normal oocytes were matured *in vitro* along with fresh oocytes (control) for 24 h in TCM-199 containing hormones (LH + FSH + estradiol) at 38.5 ^{0}C and 5% CO_2 in humidified air in a CO_2 incubator. The arcsine transformed data of the proportion of morphologic survival of oocytes and *in vitro* maturation of oocytes was compared by DNMR-test. The morphologically normal oocytes were significantly higher (P<0.05) for cumulus compact oocytes compared with denuded oocytes for both cryoprotectants EG and PG. The *in vitro* maturation was significantly higher (P<0.05) for non-vitrified oocytes (control) compared to vitrified oocytes. Significantly higher (P<0.05) proportion of cumulus compact oocytes matured *in vitro* compared to denuded oocytes for both cryoprotectants EG and PG. The differences between the cryoprotectants were non-significant. It was concluded that cryo-damage to the oocytes during vitrification can be minimized by the presence of cumulus cells with the oocyte, whereas the two cryoprotectants EG and PG are equally effective in preventing cryodamage to oocytes.

Keywords: Buffalo, cumulus cells, ethylene glycol, propylene glycol, oocytes, vitrification.

INTRODUCTION

Vitrification protocols have currently replaced slow rate cooling procedures for oocyte/embryo cryopreservation because many previous studies [1-7] have shown that oocytes evidence significant damage due to formation of ice crystals when cryopreserved employing slow cooling procedures.

Vitrification is defined as the solidification of a liquid as cooling occurs in such a way that ice crystals do not form [8] and instead the solution just become solid by increasing viscosity. This is achieved by increasing the viscosity of the solution as well as increasing the speed of cooling [8].

Bovine oocytes are much more difficult to be cryopreserved than cleavage stage embryo [9-13]. Their unique morphological and functional features, such as their size, their dynamics of sub-cellular organelles and active biochemical process along with the process of meiosis [14, 15] and the ability to develop ontogenetically upon the union with sperm during the fertilization, may account for their sensitive nature to cryopreservation.

Recovery of acceptable oocytes is low in the buffalo as compared to other livestock species. The low reproductive capacity of the buffalo as evidenced by the less number of follicles in the ovary [16] high percentage of atretic follicles, change in acrosomal proteins and membrane damage during freezing [17] is of great concern, and thus cryopreservation of oocytes could be of immense value in this species.

Relatively few studies have focused on the cryopreservation of bubaline oocytes [5-7, 18, 19]. These studies have focused on morphologic survival and subsequent development of cumulus compact buffalo oocytes cryopreserved using different molar concentrations of permeable cryoprotectants, but the effect of cumulus cell removal was not tested [20-23]. Buffalo oocyte cryopreservation can be important because of the relatively lower oocyte yield per ovary in this species [24] however, the cryopreservation protocols need substantial improvement.

MATERIAL AND METHODS

Collection of Oocytes

Oocytes from apparently non atretic surface follicles (5-8 mm) were aspirated using a 10 ml syringe with 18 gauge needle. The study was conducted during the breeding season (September to November, 2009).

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Buffalo ovaries were procured from local abattoir in sterile normal saline in an isothermic container with in 2h of slaughter. Oocytes with an intact cumulus and homogenous ooplasm were separated under a stereo microscope and washed 5-6 times in oocyte collection media followed by 3 washings in washing medium (TCM-199, buffered with HEPES 25mM+Pyruvate 0.25mM and antibiotics, pH 7.2-7.4) [25]. A part of the good quality oocytes were freed of the surrounding cumulus cells by placing them in 0.1% hyaluronidase (in TCM-199) for 10-15 min followed by repeat pipetting to be used as denuded (DN) oocytes [26].

Cryopreservation of Oocytes

Oocytes were cryopreserved (soon after collection and evaluation) by vitrification in presterilized conventional 0.25 cc semen straws (IMV, France). Collected denuded (DN) and cumulus compact (CC) oocytes were cryopreserved by vitrification separately. They were vitrified in two vitrification solutions (VS) comprising of either 6M ethylene glycol (EG) or propylene glycol (PG) with 0.5 M sucrose and 0.4% BSA prepared in DPBS. The 50% vitrification solution was prepared by dilution of vitrification solution in DPBS [18, 22, 27]. Thus there were four groups of vitrification ethylene glycol cumulus compact (EGCC), ethylene glycol denuded (EGDN), Propylene glycol cumulus compact (PGCC) and propylene glycol denuded (PGDN) oocytes.

Vitrification of Oocytes in Conventional Straw

The oocytes to be used for cryopreservation were pre- equilibrated in 50% of the vitrification solution for 3-5 min and then loaded in 0.25cc mini straw in the middle column of vitrifying solution separated by air bubbles from about 40µl of the same medium on each side. After loading 5-8 oocytes in each straw, the straw was heat sealed and pre-cooled by keeping the straw over LN_2 vapor for 2 min, at the height of about 5 cm from the LN_2 level. The straws was then plunged in LN_2 for storage and kept for at least 7 days, after which they were taken out, held in the air for 5s and then plunged into water at 37 °C for 10 s. The straw was removed from the water and wiped dry. It was cut with scissors and the content containing oocytes were expelled into a drop of DPBS with 0.5 M sucrose in a petri dish [23, 28]. The oocytes were diluted in stepwise, sucrose solutions and then oocytes were evaluated for morphological survival and subsequent *in vitro* maturation.

Morphological Evaluation and Culture

The oocytes were defined as having morphologically survived if the cell possessed an intact zona pellucida and plasma membrane and showed a homogeneous cytoplasm. They were counted and recovered. The morphologically normal oocytes from conventional straw were further subjected to *in vitro* maturation as described previously [25]. However, the medium used for *in vitro* maturation was slightly different which is described.

***In Vitro* Maturation**

Control and vitrified warmed oocytes recovered in morphologically normal form in all groups were separately cultured in TCM-199 supplemented with 5 µg/ml FSH, 5 µg/ml LH and 1 µg/ml oestradiol and 25 mM Hepes, 0.25 mM pyruvate and antibiotics in 50-100 µl maturation media for 24 h at 38.5±1°C and 5% CO_2 in humidified air in a CO_2 incubator [29]. Freshly collected oocytes were also matured *in vitro* to be kept as control.

Evaluation of Oocyte Maturation

The cytoplasmic maturation of oocytes was evaluated as per Kumar and Purohit [29]. After 24 h of maturation, all oocytes from different groups were collected for staining. The surrounding cumulus cells were removed by vortexing for 1 min. in TCM-199 with hyaluronidase (0.3%). The oocytes were placed in the centre of an area delineated by two paraffin wax bars on a clean grease free glass slide. The denuded oocytes were compressed gently with a cover slip to hold and were fixed for 24h in acetic acid methanol. [1:3(v:v)] and stained with 1% giemsa stain for evaluation of the nuclear status. The different stages of maturation were classified as, (i) Germinal vesicle stage (GV) (ii) Metaphase-I (M-I) (iii) Ana-telophase-I (AT-I) (iv) Metaphase-II (M-II) [25].

The data of each replicate were recorded separately for two end points, morphologic survival (number of replicates=8) and nuclear maturation (number of replicates=6). The arcsine transformed data of the proportion of oocytes that were retrieved in morphologically normal from over the various groups was compared by DNMR test. Arcsine transformation of the proportion of the proportion of oocytes matured *in vitro* over the various treatment was done and the replicate data compared by Duncan's New Multiple Range test (DNMR-test).

RESULTS

Oocyte Recovery

A total of 411 oocytes were recovered by aspiration of surface follicles over buffalo ovaries (n=97). From the oocytes recovered only 380 were of culturable grade and used in experiments during the present study. The average number of culturable grade oocytes per ovary was thus 3.91 per ovary. Out of the total 380 culturable grade oocytes recovered 51 oocytes were used as fresh oocytes for *in vitro* maturation with cryopreservation and kept as control, whereas, 329 oocytes were used for vitrification and subsequent *in vitro* maturation of morphologically normal oocytes. From 329 oocytes used for vitrification 180 oocytes were denuded of cumulus cells, whereas 149 cumulus compact oocytes were vitrified for study of subsequent morphologic survival. A total of 167 (72 cumulus compact and 95 denuded) and 162 (77 cumulus compact and 85 denuded) oocytes were vitrified using two different cryoprotectants ethylene glycol (EG) and propylene glycol (PG).

Morphological Survival of Vitrified Oocytes

The oocyte recovery was not significantly different between any of the groups compared. Significantly higher (P<0.05) proportion of cumulus compact oocytes were recovered in morphologically normal form, compared to denuded oocytes for both EG and PG cryoprotectants respectively. The differences between the two cryoprotectants were however non significant both for cumulus compact and denuded oocytes respectively. Hence, cumulus cell showed a protective effect in preventing buffalo oocytes from cryodamage whereas; the cryoprotectant exerted a similar effect. The overall proportion of oocytes that were seen to been morphologically abnormal was 13.11 (40/305) percent. Abnormalities included change in shape and uneven granulation, rupture of zona with leakage of oocyte contents and abnormal shrinkage of vitellus. The denuded oocyte groups (EGDN and PGDN) evidenced significantly higher (P>0.05) proportion of abnormal oocytes compared to the cumulus compact oocyte group (EGCC and PGCC) respectively.

In Vitro Maturation of Vitrified Normal Oocytes

The proportion of oocytes matured was 68.62%, 42.85%, 28.76%, 48.52% and 13.14% in control, EGCC, EGDN, PGCC, and PGDN groups respectively (Table **2**). Significantly higher (P<0.05) proportion of oocytes were seen at M-I stage in EGCC and EGDN group compared to control and PGDN group respectively. The PGCC and PGDN group evidenced non-significantly higher proportion of oocytes at M-I stage compared to the control group. The EGDN group showed highest proportion at the M-I stage at the end of experiment.

The proportion of oocytes at GV stage was similar for control, EGCC and PGCC groups. Significantly higher (P<0.05) proportion of oocytes were seen at GV stage in the denuded (EGDN and PGDN) group compared to cumulus compact groups (EGCC and PGCC) respectively. However, comparison of EGCC

Table 1: Morphologic Survival of Cumulus Compact (CC) and Denuded (DN) Buffalo Oocytes Subsequent to their Vitrification in Either Ethylene Glycol (EG) or Propylene Glycol (PG).

Cryopreservation group	Number of Oocytes Vitrified	Nuber of Oocytes recovered	Morphologically	
			Normal oocytes	Abnormal oocytes
EGCC	72	69 (95.83%)[a]	63 (91.03%)[a]	6 (8.69%)[a]
EGDN	95	88 (92.63%)[a]	73 (82.95%)[b]	15 (17.04%)[a]
PGCC	77	72 (93.50%)[a]	68 (94.00%)[a]	4 (5.55%)[a]
PGDN	85	76 (89.41%)[a]	61 (80.26%)[b]	15 (19.73%)[b]
Total	329	305 (92.70%)	265 (86.88%)	40 (13.11%)

Number of replicates in each treatment =8.
Figures in parenthesis represent percent.
Proportion (in bracket) with different superscripted letter in the same column are significantly (P<0.05) different.
(DNMR test on arcsine transformed data).

Table 2: Nuclear Status of Cumulus Compact (CC) and Denuded (DN) Morphologically Normal Buffalo Oocytes *In Vitro* Subsequent to Vitrification in Ethylene Glycol (EG) or Propylene Glycol (PG) and Warming

Cryo group	Total no. of oocytes matured	Nuclear status			
		GV	M-I	AT-I	M-II
Control	51	5 (9.80)[a]	4 (7.84)[a]	7 (13.72)[a]	35 (68.62)[c]
EGCC	63	8 (12.69)[a]	13 (20.63)[bc]	15 (23.80)[a]	27 (42.85)[b]
EGDN	73	19 (26.02)[bc]	20 (27.39)[c]	13 (17.80)[a]	21 (28.76)[a]
PGCC	68	10 (14.70)[ab]	7 (10.29)[ab]	18 (16.47)[a]	33 (48.52)[b]
PGDN	61	20 (32.78)[c]	6 (9.83)[a]	16 (26.22)[a]	19 (13.14)[a]
Total	316	62 (19.62)	50 (15.82)[a]	69 (21.83)	135 (42.72)

GV=Germinal vesicle stage; M-I= Metaphase-I; AT-I= Ana-telophase-I; M-II= Metaphase-II.
Number of replicates in each group = 6.
Figures in parenthesis represent percent.
Proportions in bracket with different superscripted letter in the same column are significantly different (P<0.05).
(DNMR test on arcsine transformed data).

and PGCC and EGDN and PGDN showed non significant differences.

DISCUSSION

Vitrification procedures for cryopreservation of buffalo oocytes have tested different concentration and combination of permeating low and high molecular weight cryoprotectants [18, 21, 23, 30]. More recently, two methods of vitrification were tested; open pulled straw (OPS) and conventional method (CON) [22]. A consensus on the optimum concentration of cryoprotectants like ethylene glycol, propylene glycol, DMSO and glycerol appear to be somewhere between 5-7 molar concentration. An important feature of buffalo oocyte retrieval and *in vitro* development appears to be a poor oocyte yield and the presence of a large number of cumulus deprived oocytes [25]. The present study thus examined whether mechanical deprivation of the cumulus cells from the buffalo oocytes would render them useless for cryopreservation and subsequent development *in vitro* or only reduce this ability. Since, the cryopreservation of buffalo oocytes has immense, potential, supplementary utilization of denuded oocytes would be an added advantage.

During the present study the proportion of post vitrification morphologically normal oocytes recovered after warming was significantly higher in cumulus compact oocytes frozen either using ethylene glycol or propylene glycol. This reflect that the cumulus cells attached to the oocytes partly offer some protection from cryodamage due to vitrification and the cryoprotectant affects the oocyte morphology only with a narrow margin. The recovery rate of oocytes was however, similar in all the groups.

The cumulus cell removal prior to *in vitro* maturation or vitrification have shown a detrimental effect on oocyte morphology for both immature and mature equine [31], mouse [32] and bovine [26] vitrified oocytes. The cumulus cell removal is known to increase the MPF activity and accelerate the transition to the metaphase stage and the redistribution of cortical granules [33]. The presence of cumulus cells affects many processes of oocyte growth and Le Gal [34] found a higher blastocyst rate for oocytes denuded at the start of vitrification compared to those denuded later. Smith and associates [35] had stressed that many structural components of oocytes have cell to cell communication and essential functional roles in development. Thus, the removal of oocytes before immature and mature oocytes before cryopreservation must affect their functional capability.

During the present study the *in vitro* maturation of buffalo oocytes was significantly lower for vitrified oocytes compared to non vitrified control. The proportion of fresh oocytes (control) that matured in the present study is similar to previous findings on vitrified and non vitrified buffalo oocytes [19, 20, 22, 23]. These

studies recorded that less number of oocytes reached metaphase-II for the oocytes cryopreserved by conventional straw method in different concentration of DMSO, EG, PROH and glycerol compared to fresh oocytes.

Both ethylene glycol and propylene glycol proved equally good cryoprotectants as evident by nearly similar *in vitro* maturation rate in vitrified oocytes. Ethylene glycol has been found to be an effective cryoprotectant for the vitrification of mouse [36], cattle [37, 38] and buffalo [18, 19, 22, 23, 39] oocytes as it offers advantage because of its higher permeation into oocytes and faster removal during dilution owing to its low molecular weight [18]. Likewise, propylene glycol has been shown to be an effective cryoprotectant for goat [40, 41] bovine [42], sheep [43] and buffalo [18] oocyte vitrification. The concentration of the cryoprotectant used was based on previous studies conducted [18, 19] and since its performance is similar to these and other studies the present study confirm that concentration of cryoprotectant used was optimal.

It was concluded that cryo-damage to the oocytes during vitrification can be minimized by the presence of cumulus cells with the oocyte, whereas the two cryoprotectants EG and PG are equally effective in preventing cryodamage to oocytes.

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