

Effect of Cumulus Cells and Meiotic Stages on Survivability and Meiotic Competence in Vitrified Buffalo Oocytes

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Abstract.- Cryopreservation of oocytes is an open problem as a result of their structural sensitivity to the freezing process. Buffalo oocytes are sensitive to chilling injury, making their cryopreservation very difficult. To improve the efficacy of vitrification in buffalo oocytes, two experiments were conducted in this study, the first one was to evaluate the effect of existence of cumulus cells on the viability and maturation of vitrified oocytes. Oocytes with complete cumulus oocyte complexes (COCs), partial (COCs) and denuded were matured *in vitro* for 22h. The oocytes were vitrified in a mixture of 3M DMSO + 3M EG by the straw method. After warming the oocytes were cultured for further 2h. There was significant increase in morphologically normal, survivability and maturation rate of COCs and partial COCs than denuded oocytes. The second experiment was to evaluate the effect of meiotic stage on the viability and maturation of vitrified oocytes. Mature (22h) and immature (0h) oocytes were vitrified by the straw method. After warming, the oocytes were evaluated morphologically, and then cultured *in vitro* to complete the maturation period to 24h. Survivability of vitrified oocytes directly after warming was higher significantly ($P < 0.05$) in mature than immature oocytes. Also, the nuclear maturation rate was significantly higher ($p < 0.01$) in mature than immature oocytes. In conclusion, cumulus cells play vital role in oocytes protection during vitrification and mature oocytes able to survive vitrification more readily than immature oocytes.

Keyword: Buffalo, oocytes, meiotic stages, vitrification, cumulus cell.

INTRODUCTION

Oocyte cryopreservation is critically important in buffalo species because the major limitation to the diffusion of *in vitro* embryo production (IVEP) technology is the low number of oocytes recovered per ovary (Gasparrini, 2002). The difficulty, in obtaining acceptable rates of survival and functionality for oocytes after cryopreservation, is due to its size and unique morphologic characteristics (Le Gal and Massip, 1999). Buffalo oocytes are highly sensitive to chilling injuries because of their high intra cytoplasmic lipid content (Gasparrini *et al.*, 2007).

One of the factors that could affect oocyte quality following vitrification is the presence or absence of cumulus cells around the oocyte prior to cryopreservation. It have been reported that presence of cumulus cells is beneficial to the oocyte survival after cryopreservation (Imoedemhe and

Sigue, 1992, Li *et al.*, 2006); as it may minimize the release of cortical granules and prevent premature zona reaction, thereby cumulus cell improve the fertilization rates after cryopreservation (Vincent *et al.*, 1990). Cumulus cell removal prior to *in vitro* maturation or vitrification have shown to have a detrimental effect on oocyte morphology for both immature and mature vitrified buffalo (Gasparrini *et al.*, 2007; Attanasio *et al.*, 2010), equine (Tharasanit *et al.*, 2009), mouse (Suo *et al.*, 2009), bovine (Zhou *et al.*, 2010), goat (Purohit *et al.*, 2012) and human (Minasi *et al.*, 2008) oocytes.

Numerous studies have been conducted to determine optimal meiotic stage for oocyte vitrification, and results have been controversial. Many studies have been conducted to vitrify oocytes at different meiotic stages: GV (Cetin and Bastan, 2006; Vieira *et al.*, 2008; Abid *et al.*, 2011), GVBD (Barnes *et al.*, 1997; Mahmoud *et al.*, 2010a) and MII (Men *et al.*, 2002; Horvath and Seidel Jr., 2008). The optimal meiotic stage is still not clear because of various devices and solutions adopted by different research groups or due to membrane permeability (Richardson and Parks, 1992). So, the present study aimed to investigate the effect of

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cumulus cells and meiotic stage on viability and meiotic competence of buffalo oocytes.

MATERIALS AND METHODS

Chemicals

Chemicals for *in vitro* maturation including fetal calf serum and tissue culture medium (TCM 199) were obtained from Gibco BRL (Grand Island, New York, USA). Cysteamine, ethylene glycol (EG) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Company.

Oocyte recovery and selection

Buffalo ovaries were collected from abattoir within 2 h of slaughter. The ovaries were transported to the laboratory in physiological saline (0.9% NaCl) containing antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin) maintained at 30°C. Ovaries were washed three times in phosphate-buffered saline (PBS). Oocytes were aspirated from 2 to 5 mm follicles with a 20-gauge needle attached to a 5-ml syringe containing PBS with 0.3% bovine serum albumin (BSA), fraction V and antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin). Oocytes were screened using a stereo zoom microscope (Beco, Germany). The oocytes with intact layers of cumulus cells and homogenous cytoplasm were selected for the study (Warriach and Chohan, 2004).

In vitro maturation (IVM) of oocytes

Oocyte maturation was carried out as described by Mahmoud (2001). Briefly, the recovered oocytes were cultured in groups of 10 or 20 in 100 µl droplets of maturation medium (TCM-199 supplemented with 10% fetal calf serum, 50 µM cysteamine and 50 µg/ml gentamycin sulfate). The droplets were covered with mineral oil and pre-incubated for a minimum of 2 h in a humidified 5% CO₂ atmosphere at 38.5°C. The oocytes were placed into the droplets and incubated for 22 h in a humidified 5% CO₂ atmosphere at 38.5°C.

Vitrification and warming

Oocytes were exposed to two-step addition of cryoprotectants (Mahmoud *et al.*, 2010b). Briefly, oocytes were exposed to VS1 (1.5 M EG + 1.5 M

DMSO) for 45 seconds and to VS2 (3 M EG + 3 M DMSO) for 25 sec. The holding medium was TCM 199 containing 25 mM HEPES + 20% fetal calf serum. Oocytes (n=5-10) were immediately loaded in 0.25 ml straws in the middle column of VS2 and separated by air bubbles. The straws were sealed with straw plugs, pre-cooled by exposure to liquid nitrogen (LN2) vapor for at least 60 seconds and dipped vertically in LN2 and stored for two months. At thawing, oocytes were equilibrated for 5 minutes in 0.5M galactose solution (Horvath and Seidel, 2006) in TCM-199 for one-step dilution to remove the cryoprotectants. Oocytes were washed 4-5 times in fresh washing medium and cultured in IVM medium for 24 hours.

Morphological evaluation of vitrified warmed oocytes

The vitrified-warmed oocytes were examined under an inverted phase contrast microscope (Olympus; Tokyo, Japan). The criteria used for assessing morphology were followed as described earlier (Mahmoud *et al.*, 2010b). The morphologically normal oocytes have spherical and symmetrical shape with no signs of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content. Morphologically abnormal oocytes had a ruptured zona pellucida or ruptured vitelline membrane or had fragmented cytoplasm with signs of degeneration.

Evaluation of oocyte viability by trypan blue

Viability of oocytes was determined after thawing and two hours after incubation. Oocytes were stained with 0.4% trypan blue for 1 minute and examined under inverted phase contrast microscope. Uptake of dye by COCs indicated non-viable (blue color) and exclusion of dye by COCs indicated viable (colorless) oocytes (El-sokary *et al.*, 2013).

Evaluation of nuclear maturation of vitrified-warmed oocytes

At the end of the culture period, the stage of nuclear maturation of vitrified-warmed oocytes was studied as described by Tarkowski (1966). Briefly, cumulus cells were removed mechanically by vortexing. Each oocyte was transferred to 1%

hypotonic sodium citrate solution for 10 min and then placed on a slide with a minimal amount of hypotonic solution. Three drops of fixative (methanol: acetic acid, 3:1) were dropped onto the oocytes. Subsequently, the fixed oocytes were stained with 1% orcein. The stage of nuclear maturation was determined as described earlier (Mahmoud, 2004). Oocytes that reached telophase I or metaphase II stages were considered matured. Post thaw maturation rate (matured oocyte / survived oocyte) was calculated.

Effect of presence of cumulus cells

Oocytes were classified based on the presence of cumulus cells into complete COCs, partial COCs and denuded oocytes. The oocytes were matured *in vitro* for 22h and then vitrified in a mixture of 3M DMSO + 3M E.G by the straw method. The oocytes were thawed and cultured for further 2hs. A group of mature oocytes was kept as a control without vitrification.

Effect of meiotic stage

Immature (0h) and mature (22h) oocytes were vitrified in a mixture of 3M DMSO + 3M E.G by the straw method. Both groups of vitrified oocytes were thawed and cultured *in vitro* to complete the maturation to 24h. A group of oocytes was kept as a control without vitrification.

Statistical analysis

Data were subjected to ANOVA using SPSS for Windows version 13.0, statistical software. Comparison of means was carried out by Duncan's Multiple Range Test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Effect of presence of cumulus cells

Three categories of oocytes were vitrified in a mixture of 3M DMSO + 3M E.G in 0.25 ml straw. After vitrifying, the oocytes were warmed and evaluated morphologically (Table I). The percentage of normal cumulus-enclosed oocytes was non-significantly differing with partially-enclosed oocytes, while there were significant ($P < 0.01$) difference between them and denuded oocytes.

Table II shows the survival rate of the three groups of oocytes. The survivability was non-significantly different between cumulus and partial oocytes. But, there was significant ($P < 0.05$) decrease in the viable oocytes (trypan blue-negative) in denuded oocytes than both cumulus and partial groups.

The maturation rate of vitrified oocytes with cumulus, partial and denuded is illustrated in Table III. The highest maturation rate was observed for cumulus-enclosed oocytes followed by partial-enclosed oocytes. But, a lower maturation rate was detected for denuded oocytes. There was a significant increase in the percentage of maturation rate of cumulus-enclosed oocytes than partial-enclosed ($P < 0.005$) and denuded ($P < 0.001$) oocytes.

Effect of meiotic stage

About 125 mature and 147 immature buffalo oocytes were vitrified in a mixture of 3M DMSO + 3M E.G by the straw method. Data in Table IV indicate that the percentage of morphologically normal vitrified oocytes directly after thawing was ($P < 0.05$) higher in mature than immature oocytes. The percentage of normal oocytes was significantly decreased in both vitrified groups than the control unvitrified one.

Oocytes were examined for viability 2h after thawing (Table V). The test was based on the presence or absence of blue color of trypan staining. The percentages of viable (colorless) oocytes and dead (blue) were calculated. There is non-significant difference between mature and immature oocytes but there was significant increase in survivability in the control group compared to both mature ($P < 0.01$) and immature ($P < 0.001$) groups.

Nuclear maturation rate in Table VI, represented by the percentage of oocytes reaching telophase I (TI) and metaphase II stages (MII), was higher ($p < 0.01$) increase in mature than immature oocytes. But, both groups were significantly lower than the control.

DISCUSSION

In the present study, the effects of cumulus cells on survival of vitrified buffalo matured oocytes

Table I.- Effect of presence of cumulus cells on morphology of vitrified buffalo oocytes after thawing (Mean \pm S. E).

Oocyte groups	No. vitrified oocytes	Morphological normal oocytes		Morphological abnormal oocytes	
		No.	%	No.	%
Cumulus	130	120	91.6 \pm 2.6 ^a	10	8.3 \pm 2.6 ^a
Partial	101	87	87.0 \pm 2.4 ^a	14	13.1 \pm 2.2 ^a
Denuded	99	78	78.0 \pm 1.8 ^b	21	21.9 \pm 1.8 ^b

^{a,b} Values with different superscripts within the same columns are differences (P < 0.01).

Table II.- Effect of presence of cumulus cells status on viability of vitrified buffalo oocytes after thawing (Mean \pm S.E).

Oocyte groups	No. vitrified oocytes	Viable oocytes		Non viable oocytes	
		No.	%	No.	%
Cumulus	117	96	82.2 \pm 1.9 ^a	21	17.7 \pm 1.9 ^a
Partial	92	76	82.0 \pm 1.5 ^a	16	17.9 \pm 1.5 ^a
Denuded	82	62	74.5 \pm 2.4 ^b	20	25.4 \pm 2.4 ^b

^{a,b} Values with different letters within the same columns are different (P < 0.05).

Table III.- Effect of presence of cumulus cells on nuclear maturation rate of vitrified buffalo oocytes (Mean \pm S. E).

Oocytes quality	No. oocytes examined	Matured oocytes (MII + TI)	
		No.	(%) (% \pm S. E)
Cumulus	72	54	(73.7 \pm 2.6) ^a
Partial	73	46	(64.8 \pm 2.4) ^b
Denuded	65	39	(53.0 \pm 1.8) ^c

^{a,b,c} Values with different superscript letters in the same column differ (P < 0.05- P < 0.01- P < 0.001) respectively.

were studied and evidenced that survival rate was the same for partial and cumulus oocytes. Both partial and cumulus enclosed oocytes were better than denuded oocytes in survivability and maturation. It was reported that cumulus cell removal prior to *in vitro* maturation or vitrification has a detrimental effect on oocyte morphology for both immature and mature vitrified buffalo (Gasparrini *et al.*, 2007), equine (Tharasanit *et al.*, 2009), mouse (Suo *et al.*, 2009) and bovine (Modina *et al.*, 2004) oocytes. In contrast, Zhang *et al.* (2009) reported no difference in the development of vitrified ovine oocytes enclosed with or without cumulus cells cumulus cells and glycoproteins slow cryoprotectant penetration that may lead to unequal intracellular distribution of the cryoprotectant and

inadequate cell protection (Hyttel *et al.*, 2000). Bogliolo *et al.* (2007) reported that bovine and ovine matured oocytes without cumulus cells had a higher survival rate after vitrification. Moreover, the rates of embryo development to the 8-cell stage in cumulus cells free oocytes were significantly higher than those of cumulus-intact oocytes.

The functions of cumulus cells are different in immature (GV) and mature (MII) oocytes. Zhou *et al.* (2010) studied the effects of cumulus cells on bovine oocyte vitrification at both the GV and the MII stage; they reported that the survival; cleavage and blastocyst rates of cumulus-enclosed vitrified bovine oocytes of GV were significantly higher than those of partially-denuded vitrified and control oocytes. While, for MII stage bovine oocytes, no significant differences were detected between cumulus-enclosed vitrified oocytes and partially denuded vitrified oocytes in survival, cleavage or blastocyst rates. Modina *et al.* (2004) reported that immature bovine oocytes can be vitrified after cumulus cell removal, and that they successfully mature and develop up to the blastocyst stage after *in vitro* fertilization. Moreover, It has been reported that the absence of cumulus cells could provoke a possible shortcoming in protein synthesis and could reflect the levels of molecules involved in the regulation of meiotic and mitotic cell cycles (Combelles *et al.*, 2005).

Table IV.- Morphological evaluation of vitrified mature and immature buffalo oocytes directly after thawing (Mean ± S. E).

Oocyte groups	No. vitrified oocytes	Viable oocytes		Non viable oocytes	
		No.	%	No.	%
Mature	125	105	85.3±3.2 ^a	20	14.5±3.1 ^a
Immature	147	113	76.8±0.9 ^b	34	23.1±0.9 ^b
Control (unvitrified)	110	109	99.1±0.8 ^c	1	0.8±0.8 ^c

^{a, b, c} Values within column without common superscripts differ (p<0.05- P<0.0001).

Table V.- Viability evaluation of mature and immature vitrified buffalo oocytes by trypan blue 2h after thawing (Mean±SE).

Oocyte groups	No. vitrified oocytes	Viable oocytes with live or dead cumulus.		Dead oocytes with live or dead cumulus	
		No.	%	No.	%
Mature	106	76	73.2±3.7 ^a	30	28.0±3.4 ^a
Immature	99	66	66.6±2.8 ^a	33	33.9±2.7 ^a
Control (unvitrified)	102	91	88.9±2.2 ^b	11	11.0±2.2 ^b

^{a, b} Values with different superscript letters in the same column differ (p<0.01 - P< 0.0001).

Table VI.- Nuclear maturation rate of mature and immature vitrified buffalo oocytes after staining with orcein stain (Mean ± S. E).

Oocytes quality	No. oocytes examined	Matured oocytes (MII + TI)	
		No.	(%) (% ± S. E)
Cumulus	67	32	(69.8±2.6) ^a
Partial	65	36	(55.6±1.7) ^b
Denuded	83	66	(79.7±1.7) ^c

^{a, b, c} Different superscripts within a column denote statistical differences (P< 0.01 - P< 0.0001).

With respect to the effect of meiotic stage on viability and maturation rate of vitrified buffalo oocytes, in the present study, buffalo oocytes were vitrified at different stages of maturation to assess the post-thaw morphology, survivability and nuclear status. Post-thaw survivability of buffalo oocytes was higher in mature oocytes than GV stage or immature oocytes. The difference may be due to membrane permeability. Permeability of plasma membrane to cryoprotectant changes according to the meiotic stage of the oocytes (Richardson and Parks, 1992). Immature oocytes are less permeable to water and cryoprotectant than mature oocytes (Agca *et al.*, 1998) and low permeability (Hochi *et*

al., 1998) of plasma membrane has been considered responsible for lower survival and a higher incidence of damage to immature oocytes. Chilling injury is higher in immature than mature oocytes, probably due to low membrane stability and susceptibility of the cytoskeleton (Hong *et al.*, 1999) and differences in the hydraulic conductivity (Agca *et al.*, 2000). The increased percentage of morphologically normal oocytes vitrified at 24 h than 0 h may support the fact that MII stage buffalo (El-shahat and Hammam, 2005; Sharma and Loganathasamy, 2007; Mahmoud *et al.*, 2010a), human (Boiso *et al.*, 2002), bovine (Hurtt *et al.*, 2000 and Albarracin *et al.*, 2005), goat (Purohit *et al.*, 2012), porcine (Rojas *et al.*, 2004) and equine (Hurtt *et al.*, 2000) oocytes evidenced a higher capacity to recover cryopreservation damages than GV stage oocytes. In contrast, some workers suggested that GV stage is more resistant to cryodamage due to their smaller size, lack of cortical granules and a longer period to recover from cryoinjury (Shaw *et al.*, 1999). In addition, Al-Hasani *et al.* (2007) and Cao (2009) found that GV of human oocytes are more resistant to cryoinjury because they have less depolymerization of the microtubules and are less prone to aneuploidy than mature oocytes.

There is a controversy in literature regarding the suitable nuclear stage for oocyte vitrification. In some reports, stage of nuclear maturation of oocytes had no effect on survival following cryopreservation (Le Gal and Massip, 1999; Hurtt *et al.*, 2000) and on cleavage or blastocyst development (Prentice, 2010). On the other hand, cryopreservation of an intermediate stage oocyte, such as GVBD, has been thought to avoid some of the cryoinjuries associated with cryopreservation of GV or MII oocytes (Fuku *et al.*, 1995a; Albarracin *et al.*, 2005). Variations in maturation rate in immature buffalo oocytes from one study to another may be due to differences in types, concentrations, use of single or combinations of cryoprotectants, and exposure time (Sharma and Loganathasamy, 2007).

The cytoskeleton in first meiotic division of immature oocytes is particularly vulnerable to cryodamage, whereas matured oocytes display a more flexible cytoskeleton. This may explain why matured oocytes are less subject to cryodamage (Allworth and Albertini, 1993). The numerically lower maturation rate of vitrified matured oocytes compared to non-vitrified oocytes may be due to chromosomal aberrations that would have taken place during vitrification owing to alteration in the meiotic spindle (Carroll *et al.*, 1993). The meiotic spindle holding the chromosomes at the metaphase plate depolymerizes when the temperature is lowered. Although spindles are repolymerized once the temperature returns to normal, chromosomes may not realign correctly on the newly formed spindle (Shaw *et al.*, 2000).

CONCLUSION

Cumulus cell presence is very important for survival of vitrified buffalo oocytes. The MII stage of *in vitro* matured buffalo oocytes is able to survive vitrification and has better nuclear maturation than the GV stage.

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