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Meiotic stages during *in vitro* maturation regulating the post thaw survivability of vitrified buffalo oocytes

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ABSTRACT : The present study has been undertaken to assess the post thaw survivability of buffalo oocytes vitrified at different stages of *in vitro* maturation (IVM). Cumulus oocyte complexes (COCs) obtained from slaughter house ovaries were randomly divided into 6 different groups: control (non-vitrified oocytes were matured for 24 h in maturation medium (MM) consisted of TCM-199 supplemented with 10 per cent w/v fetal calf serum (FCS) at 38±1°C and 5 per cent $C0_2$ in a humidified atmosphere), 0 h (vitrified before the onset of maturation), 6,12,18 and 24 h groups (vitrified at 6, 12, 18 and 24 h, respectively, after the onset of maturation). Oocytes were exposed to vitrification solution (VS) consisted of 40 per cent w/v propylene glycol and 0.25 M trehalose in phosphate buffered saline (PBS) supplemented with 4 per cent w/v bovine serum albumin (BSA) for 3 min at 20-25°C. Oocytes in VS were loaded into 0.25 ml French mini straw with 1M sucrose solution separated by two airspace on either side of VS. The straws were sealed with hot forceps and plunged directly into liquid nitrogen (LN_2; -196°C). The straws were thawed after storage period of atleast 7 days by transferring them into a water bath at 37°C for 30 sec. The cryoprotectant was removed by exposing the oocytes to 1 M sucrose solution. Oocytes in 0, 6, 12, 18 and 24 h groups were further matured for additional 24, 18, 12, 6 and 0 h, respectively, to complete a total of 24 h maturation period.A total of 77, 64, 60, 51 and 62 post thawed morphologically normal oocytes in 0, 6, 12,18 and 24 groups, respectively, immediately after thawing, and 55 oocytes in control after 24 h maturation were stained with Trypan blue to assess the survivability. Survivability was significantly ($P<0.05$) higher in control (94.54%) than all five vitrification groups. Among vitrification groups, more survivability was observed in 24 h (61.29%) group as compared to 18 (56.86%), 12 (46.67%), 6 (40.62%) and 0 (37.66%) h groups. Oocytes vitrified at 24 after maturation survived significantly ($P<0.05$) better than oocytes vitrified before onset of maturation (0 h) and at 6 h after the onset of maturation. Though higher proportions of oocytes survived in 18 h group compared to 0,6 and 12 h groups, difference was significant ($P<0.05$) only with 0 h group, but not with 6 and 12 h groups. This study indicated that survivability of buffalo oocytes depends on the different developmental stages.

KEY WORDS : Vitrification, *In vitro* maturation, Post thaw survivability, Buffalo, Oocytes

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INTRODUCTION

Survivability assessment following freezing and thawing based on the morphological appearance of cumulus oocyte complexes led to inaccurate conclusion because of viable cumulus cells impaired observation of plasma membrane integrity. Even in nude oocytes, morphological appearance may not be judged properly. Therefore, use of vital stains such as Trypan blue (TB) and fluorescein diacetate (FDA) made the viability assessment of oocytes more accurate (Didion *et al.*, 1990). FDA has been used previously to assess the viability of cleavage stage cow (Church and Raines, 1980), mouse (Mohr and Trounson, 1980) and human (Trounson *et al.*, 1982) embryos. Didion *et al.* (1990) used FDA stain to assess the viability of cryopreserved GV stage pig oocytes. The metabolism of FDA depends on both esterase activity and an intact plasma membrane. Oocyte and cumulus cell viability are evidenced by intense fluorescence; non-fluorescence indicates non-viability. TB has

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also been used to detect the oocyte viability (Lenz *et al.,* 1983; Didion *et al.,* 1990) and it is based on plasma membrane integrity (Schrek, 1936). Oocyte and cumulus cell viability was evidenced by exclusion of TB. Didion *et al.* (1990) tested viability of cryopreserved pig oocyte using FDA and TB and reported that viability of oocytes was similar for both stains.TB is cheap and simple microscope is necessary whereas FDA is expensive and requires fluorescence microscope for assessment. Hence, the study was formulated using TB stain to assess the viability of vitrified oocyte and the cumulus cell.

MATERIAL AND METHODS

Materials :

Chemicals :

Sodium chloride (NaCl) and modified Dulbecco's phosphate buffered saline (mDPBS) used for ovary collection and washing were procured from Hi-Media, India. Tissue culture medium-199 (TCM-199), antibiotics, antimycotics and other chemicals used for preparation of maturation, fertilization and embryo development media were of embryo culture or tissue culture grade, procured from Sigma Chemicals Co., USA. Propylene glycol and trehalose used for preparation of vitrification solution were also procured from Sigma Chemicals Co., USA.

Fetal calf serum was procured from Sigma Chemicals Co., USA and heat inactivated at 56°C for 30 min and stored at -20°C. Blood sample was collected from the estrus buffalo. The serum was separated, heat inactivated at 56°C for 30 min and stored at-20°C. All the media were filtered through membrane filter (0.22 µm) and stock solutions were kept at 4°C for maximum of 1 month. The working solutions were prepared and pre-equilibrated 12 h before use.

Plasticware and glassware :

All the plasticware used for culture *viz.*, Petriplates, culture dishes, multiwell dishes, centrifuge tubes and culture bottles etc. were purchased from Nunc, Denmark.

All the glasswares' (Borosil, India) used for culture were washed, packed and sterilized at 180°C for 1 h. Other than glasswares, rest of culture accessories were autoclaved at 120°C and 15 psi for 30 min. All the plasticwares and glasswares were UV treated for 30 min before use.

Methods :

Collection of ovaries :

Buffalo ovaries were collected from local abattoir in sterile normal saline solution (NSS-0.89 per cent w/v) supplemented with antibiotics (Penicillin G-100 lU/ml and streptomycin-100 µg/ml) and antimycotic (Amphotericin B - 2.5 µg/ml) at 30-35°C in an isothermic container and transported to the laboratory within 2 h of slaughter.

The surrounding tissues were trimmed off and the ovaries were washed several times with sterile NSS. The ovaries were exposed to 70 per cent ethyl alcohol for 30 sec and finally washed in mDPBS.

Oocyte collection :

Oocytes from surface follicles (>3 mm) of buffalo ovaries were collected by aspirating the follicles with 18 gauge needle attached to 5 ml syringe in oocyte collection medium (OCM). Contents from syringe were poured into a 50 ml test tube having OCM. Oocytes were allowed for gravitational settlement for atleast 15-20 min. Supernatant was then discarded and the remaining fluid was poured into a Petridish containing OCM. Morphologically culturable oocytes *i.e.* those having compact, multilayered cumulus oocyte complexes (COCs) and evenly granulated cytoplasm were selected under stereo microscope (Bausch and Lamb, USA) and transferred to another Petridish containing OCM. Finally COCs were washed 5 times in oocyte washing medium and 5 times in maturation medium (MM).

In vitro maturation (IVM) :

The COCs were randomly divided into 6 different groups with approximately equal number of oocytes.

Group I (control) :

The COCs were matured in MM for 24 h at 38±1°C and 5 per cent $C0_3$ in humidified air.

Vitrification of oocytes :

Vitrification solution (VS) consisted of propylene glycol (40% w/v) and trehalose (0.25 mol l^{-1}) in phosphate buffered saline (PBS) supplemented with BSA (0.4% w/v).

Group II (vitrification before onset of maturation) :

The COCs immediately after collection (germinal vesicle stage) were exposed to VS at 20-25°C for 3 min. Then, the COCs in VS were loaded into 0.25 ml French mini straw (15-20 COCs in each straw) with sucrose (1 mol $l \sim^{1}$) solution separated by two air space on either side of VS. The straws were sealed with hot forceps and plunged directly into liquid nitrogen (LN_2).

The straws were thawed after storage period of 7 days by transferring them into a water bath at 37°C for 30 sec. The cryoprotectant was removed by exposing the COCs to sucrose (1 mol $^{-}$ l Finally, the COCs were matured in MM for 24 h at 38± 1°C and 5 per cent CO, in humidified air.

Group III (vitrification at 6 h maturation) :

The COCs were matured for 6 h and were vitrified, stored and thawed as in Group II. They were further matured for 18 h to complete 24 h maturation.

Group IV (vitrification at 12 h maturation) :

The COCs were matured for 12 h and were vitrified, stored

and thawed asin group II. They were further matured for 12 hto complete 24 h maturation.

Group V (vitrification at 18 h maturation) :

The COCs were matured for 18 h and were vitrified, stored and thawed as in group II. They were further matured for 6 h to complete 24 h maturation.

Group VI (vitrification at 24 h maturation) :

The COCs were matured for 24 h and were vitrified, stored and thawed asin group II.

Assessment of survivability :

COCs in nonvitrified (after 24 h maturation) and vitrified (immediately after thawing) groups were stained with 0.4% (w/v) Trypan blue (TB) and viability was determined by examining under inverted phase contrast microscope. Uptake of dye by COCs indicated nonviability and exclusion of dye by COCs was considered as viable. Viability was categorized into four groups as per the procedure described by Didion *et al.* (1990). They were :

- Viable oocyte and viable cumulus cells (VOC),
- Viable oocyte and non-viable cumulus cells (VO),
- Nonviable oocyte and viable cumulus cells (VC) and
- Nonviable oocyte and non-viable cumulus cells (NV).

Statistical analysis :

Statistical analysis was carried out by standard method described by Snedecor and Cochran (1987).

RESULTS AND DISCUSSION

A total of 55, 77, 64, 60, 51 and 62 COCs were stained with 0.4 w/v Trypan blue dye to assess the survivability in control, 0, 6, 12, 18 and 24 h groups, respectively (Table 1). Exclusion of dye indicated viable and uptake indicated non-viable cells. Viability was categorized into 4 groups *viz.*, (1) viable oocyte and viable cumulus cells (VOC), (2) viable oocyte and nonviable cumulus cells (VO), (3) non-viable oocyte and viable cumulus cells (VC) and (4) non-viable oocyte and nonviable cumulus cells(NV). Only viable oocyte and cumulus cells (VOC) were considered as survivors.

Survivability was significantly ($P<0.05$) higher in control (94.54%) than all vitrification groups. Among vitrification groups, more oocytes survived in 24 h (61.29%) group as compared to 18 (56.86%), 12 (46.67%) 6 (40.62%) and 0 (37.66%) h groups. Oocytes vitrified at 24 h after maturation survived significantly ($P<0.05$) better than oocytes vitrified before the onset of maturation (0 h) and at 6 h after maturation. Though higher proportions of oocyte survived in 18 h group compared to 0, 6 and 12 h groups, it differed significantly ($P<0.05$) with 0 h group, but not with 6 and 12 h groups.

Post thaw survivability of buffalo oocytes in this study vitrified at 0, 6, 12, 18 and 24 h groups was 37.66, 40-62,46.67, 56.86 and 61.29 per cent, respectively. Survivability of vitrified oocytes was lower than non-vitrified oocytes (control-94.54 %). Among the different vitrification groups, lower survivability in immature and maturing oocytes may be due to lower membrane permeability. Permeability of plasma membrane to cryoprotectant changed according to the meiotic stages of oocytes (Richardson and Parks, 1992). Immature oocytes are less permeable to water and cryoprotectant than matured oocytes (Agca *et al.*, 1997; 1998). Low permeability (Lim *et al.*, 1992; Hochi *et al.*, 1998) and low stability (Hong *et al.*, 1999) of plasma membrane were responsible for lower survival and greater freezing injuries. Also, low permeability rendered the immature oocytes susceptible to osmotic damage upon addition of cryoprotectant (Ruffling *et al.*, 1993; Arav *et al.*, 1994; Le Gal *et al.*, 1994; Le Gal and Massip, 1999). Vitrification of immature oocytes resulted in more damage to plasma membrane itself (Lim *et at.*, 1992; Fuku *et al.*, 1995; Arav *et al.*, 1996). Addition of antifreeze proteins had protective effect on plasma membrane against thermal effects of oocytes and embryos (Rubinsky *et al.*, 1992; Arav *et al.*, 1994; Arav *et al.*, 2000). Later, Edashige *et al.* (2003) reported that artificial expression of Aquaporin-3 on the plasma membrane of immature mouse oocytes facilitated water and cryoprotectant permeability and thereby improved the survival of oocytes after vitrification.

Table 1 : Viable and non-viable status of buffalo cumulus oocyte complexes (COCs) vitrified at different stage of maturation *in vitro*

Group	Number of oocytes examined	Viable oocyte & viable cumulus cells	Viable oocyte & non-viable cumulus cells	Non-viable oocyte & viable cumulus cells	Non-viable oocyte & non-viable cumulus cells
Control	55	52^{a} (94.54)	1^{a} (1.82)	1^{a} (1.82)	1^{a} (1.82)
0 h	77	29^{b} (37.66)	17^{b} (22.08)	7^{ab} (9.09)	24^{b} (31.17)
6 h	64	26^{bd} (40.62)	10^{b} (15.62)	9^{b} (14.06)	19^{bd} (29.70)
12h	60	28^{bde} (46.67)	9^{b} (15.00)	6^{ab} (10.00)	17^{bd} (28.33)
18 h	51	29^{cde} (56.86)	6^{ab} (11.76)	7^{b} (13.73)	9^{bd} (17.65)
24 h	62	38^{ce} (61.29)	8^{ab} (12.90)	7^{ab} (11.29)	9^{cd} (14.52)

In parenthesis, percentage is shown.
Values with different superscripts in a column differ significantly ($P<0.05$).

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