VETERINARY SCIENCE RESEARCH JOURNAL
Volume 5 | Issue 1&2 | Apr. & Oct., 2014 | 33-37
DOI : 10.15740/HAS/VSRJ/5.1and2/33-37
e ISSN-2230-942X | Visit us - *www.researchjournal.co.in*

RESEARCH ARTICLE

Effect of meiotic stages during *in vitro* maturation on the post thaw recovery of buffalo oocytes

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Members of the Research Forum

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Abstract : The present study has been undertaken to assess the post thaw recovery of buffalo oocytes vitrified at different stages of *in vitro* maturation (IVM). Cumulus oocyte complexes (COCs) obtained from slaughterhouse ovaries were randomly divided into 6 different groups: control (non-vitrified oocytes were matured for 24 h in maturation medium (MM) consists of TCM-199 supplemented with 10 per cent w/v fetal calf serum (FCS) at 38±1^{0}C and 5 per cent CO_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) consists 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^{0}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^{0}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 sum of 495, 432, 457, 416 and 420 oocytes were vitrified in 0, 6, 12, 18 and 24 h groups, respectively. After thawing, 444 (89.70%), 384 (88.89%), 418(91.47%), 381 (91-59%) and 387 (92.14%) oocytes were recovered in 0, 6, 12, 18 and 24 h groups, respectively. It is evident that no significant difference was observed under different vitrification groups.

Key words : Vitrification, *In vitro* maturation, Post thaw recovery, Buffalo oocytes

How to cite this paper : Loganathasamy, K. and Sharma, G. Tara (2014). Effect of meiotic stages during *in vitro* maturation on the post thaw recovery of buffalo oocytes. *Vet. Sci. Res. J.*, **5**(1&2) : 33-37.

Paper History : Received : 06.12.2013; Revised : 10.09.2014; Accepted : 21.09.2014

INTRODUCTION

In vitro embryo production (IVEP) plays a pivotal role in propagation of superior germplasm in livestock and provides basic research tool for further investigations like embryo splitting, sexing, cloning and production of transgenic animals. IVEP uses the oocytes obtained from live animals by ultrasound guided transvaginal ovum pick-up (OPU) as well as from ovaries collected at abattoir.The continuous availability of viable, developmentally competent oocytes has been critical to recent progress in IVEP because of the relatively short fertile life span of mammalian oocytes (Parks and Ruffling, 1992). Hence, the storage of unfertilized oocytes would generate a readily available source, which allows the experiments to be carried out at convenient time (Moor and Crosby, 1985) and could therefore be of practical importance in the establishment of gamete bank from which particular genetic combinations could be derived (Whittingham, 1977). Preservation of oocytes reduces the risk and expense involved in transport of live animals, hazards of disease transmission and also provides insurance against catastrophes and natural disasters. Buffalo is an important livestock, which provides milk, meat and work power. India has the largest population of buffalo in the world. It has 94.1 million heads of animals

(FAO, 2001) which constitutes more than 58 per cent of world buffalo population. In recent years, there has been increasing interest in IVEP for faster propagation of superior germplasm in buffalo because of low efficiency of superovulation and embryo transfer in this species. This may be due to some physiological features, peculiar of this species *viz.*, lower number of primordial follicles observed in buffalo ovaries, varying from 10, 000 to 19,000 (Samad and Nasseri, 1979) compared with 1,50,000 in cattle (Erickson, 1966), lower number of antral follicles throughout the whole estrus cycle (Kumar *et al.*, 1997) and high incidence of follicular atresia *i.e.* 82 per cent (Ocampo *et al.*, 1994) or 92 per cent (Palta *et al.*, 1998) as observed in slaughter house ovaries. Hence, establishment of a protocol that optimizes the survival of buffalo oocytes by vitrification is necessary to supply continuous source of oocytes during lean season. So, the present study has been conducted to assess the post thaw recovery of buffalo oocytes in different vitrification groups.

RESEARCH METHODOLOGY

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 preequilibrated 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 glassware, rests 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 70per 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.

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 CO_2 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^{-1} 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 h to 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. After thawing, oocytes from 0, 6, 12, 18 and 24 h were counted for post thaw recovery.

Statistical analysis :

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

RESULTS AND DISCUSSION

A sum of 3285 buffalo ovaries was collected from local slaughterhouse and used for this study. From 3285 ovaries, 2991 oocytes were harvested by follicle aspiration method. Out of 2991 oocytes, 2542 culturable quality oocytes were selected under stereomicroscope. An average of 0.91 oocyte per ovary was recovered and out of which, 0.77 culturable oocytes per ovary was selected (Table 1), Fully/partially denuded oocytes as well as oocytes with unevenly granulated cytoplasm comprised of around 15 per cent of total population. These oocytes were not included for this study.

A total of 2542 oocytes were used in this experiment, out of which 2220 oocytes were allotted into five different vitrification groups (495, 432, 457, 416 and 420 oocytes for 0, 6, 12, 18 and 24 h, respectively, Table 2). The oocytes were stored in LN_2, for at least 7 days and then thawed.

After thawing, 444 (89.70%), 384(88.89%), 418 (91.47%), 381 (91.59%) and 387 (92.14%) oocytes were recovered (Fig. A), whereas 51 (10.30%), 48 (11.11%), 39 (8.53%), 35 (8.41%) and 33 (7.86%) oocytes were lost during vitrification and thawing processes in 0, 6, 12, 18 and 24 h groups, respectively (Table 2).

Discussion :

In this study, buffalo oocytes were vitrified at different stages of maturation in 40 per cent w/v PROH and 0.25 M trehalose supplemented with 0.4 per cent w/v BSA in PBS to assess the post thaw morphology, survivability, nuclear status and developmental competence. PROH (low MW permeating cryoprotectant) has high glass forming tendency and wholly amorphous state of its aqueous solution has great stability at subzero temperature which limits the formation of ice crystals (Boutron and Kaufmann, 1979). Trehalose (low MW nonpermeating cryoprotectant) induces cellular dehydration through changes in osmotic pressure without toxic effect on cells (Crowe *et al.*, 1983). BSA (high MW nonpermeating cryoprotectant) suppresses the formation of small ice crystal (Franks *et al.*, 1977) and protects cell membrane (Leibo, 1988) during freezing.

The average number of harvested and culturable quality oocytes obtained for this study is 0.91 and 0.77, respectively. A major limiting factor in the IVEP in buffalo species is very poor recovery rate of immature oocytes from slaughterhouse ovaries. When, the method of oocyte retrieval employed is

Table 1 : Number of harvested and culturable oocytes obtained from buffalo ovaries

Number of Ovaries	Number of oocytes harvested	Number of culturable oocytes
3285	2991	2542

Number of harvested oocytes per ovary = 0.91
Number of culturable oocytes per ovary = 0.77

Table 2 : Impact of vitrification on buffalo oocytes vitrified at different stages of maturation *in vitro*

Group	Number of oocytes Vitrified	Recovered (A)	Lost (B)
0 h	495	444 (89.70)	51 (10.30)
6 h	432	484 (88.89)	48 (11.11)
12h	457	418 (91.47)	39 (8.53)
18 h	416	381 (91.59)	35 (8.41)
24 h	420	387 (92.14)	33 (7.86)

In parenthesis, percentage is shown
In (A) and (8), percentage is calculated out of vitrified oocytes
In (C) and (D), percentage is calculated out of recovered oocytes

aspiration of 2-8 mm follicles, the average recovery of total oocyte per ovary is 0.7 (Totey *et al.,* 1992), 1.7 (Das *et al.,* 1996) and 2.4 (Kumar *et al.,* 1997). Because of high incidence of atresia, mean recovery of good quality oocytes per ovary is further reduced *i.e.* 0.4 (Totey *et al.,* 1992), 0.9 (Das *et al.,* 1996), 1.76 (Samad *et al.,* 1998) and 2.4 (Gasparrini *et al.,* 2000). Variations in the oocyte yield among different studies are due to differences in geographical location in relation to status of animals slaughtered, season of ovary collection, number of ovaries processed and techniques employed by different technical personnel, Furthermore, criteria for selecting ovaries at slaughterhouse might have influenced the oocyte yield in different studies (Totey *et al.,* 1992; Palta and Chuahan, 1998).

The oocyte recovery rate in buffalo is therefore, much lower compared with cattle, from which 8-12 good quality oocytes are obtained on an average per ovary (Gordon, 1994). Low oocyte yield in buffaloes may be due to some physiological features, peculiar of this species lower number of primordial follicles observed in buffalo ovaries, varying from 10,000 to 19,000 (Samad and Nasseri, 1979) compared with 1,50,000 in cattle (Erickson, 1996), lower number of antral follicles throughout the whole estrus cycle (Kumar *et al.,* 1997) and high incidence of follicular atresia *i.e.* 82 per cent (Ocampo *et al.,* 1994) or 92 per cent (Palta *et al.,* 1998) as observed in slaughterhouse ovaries.

The post thaw recovery rate in different vitrification groups varied from 89 to 92 per cent in this study. The recovery rate of vitrified thawed oocytes has been reported to vary from 80 to 100 per cent in different species (Fuku *et al.,* 1992; Nowshari *et al.,* 1994) which is in agreement with the present study. Loss of oocytes during the process of freezing and thawing is well documented in several studies (Hamano *et al.,* 1992; Miyake *et al.,* 1993; Lim *et al.,* 1999; Dhali *et al.,* 2000 a,b; Luna *et al.,* 2001). Such loss of oocytes occur due to sticking of oocytes on inner wall ofstraw adhering to crack or rough surface (developed sometimes during thawing) or oocyte disintegration due to improper vitrification- Also, oocytes may be removed with sealed end when it is cutoff after thawing (Wright, 1985).

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