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RESEARCH ARTICLE

DEVELOPMENTAL COMPETENCE OF POST-THAW VITRIFIED BOVINE OOCYTES

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Abstract

The cumulus oocyte complexes (COCs) were collected from the visible follicles from immediate slaughter house cattle ovaries. Good quality oocytes were considered for vitrification using two permitting cryoprotectants *viz.* ethylene glycol (EG) and dimethylsulphoxide (DMSO). Equilibration solution (ES) was prepared by mixing equal amount of 10% EG+10%DMSO in basic solution comprising TCM-199 with 0.3%BSA and vitrification solution consist of 20% EG+20%DMSO and 0.6M sucrose in basic solution. The immature oocytes were exposed to ES for three minutes, and then transferred to the VS and immediately loaded in a 0.25ml straw and plunged in liquid nitrogen within 1 minute for storage. Post thawed normal oocytes following dilution in step-wise manner were then subjected to *in-vitro* maturation (IVM) in TCM-199 supplemented with 10% follicular fluid, sodium pyruvate, L-glutamine and 10% FBS at 38.5^{o}C in 5% CO_2 in humidified air for 24 hrs. The mean recovery rate of oocytes per ovary through aspiration was 3.48±1.25 and through aspiration followed by slicing was 4.29±1.01. The post thaw survival rate of oocytes subjected to cryopreserved by vitrification was 91.06% .There was significantly ($p<0.05$) higher i*n-vitro* maturation rate based on cumulus expansion in non-vitrified group (93.33%) than in vitrified group (85.89%). On the other hand there was also significant difference ($p<0.05$) in nuclear maturation rate in non-vitrified group (92.86%) than in vitrified group (82.35%). Bovine ovarian oocytes could be successfully matured *in-vitro* following conventional vitrification technique for cryopreservation using two permitting cryoprotectants (EG and DMSO) and a non-permeable cryoprotectant (Sucrose).

Introduction

The availability of viable, developmentally competent oocytes has been a major problem preventing progress in the development of *in-vitro* fertilization (IVF), embryo culture and related reproductive technologies. Development of a reliable method for cryopreservation of oocytes is therefore relevant for assisted reproduction. Classical freezing

protocol have been replaced by the most of recent vitrification methods including several modification, such as vitrification by open pull straw method (Vajta *et al.* 1998), glass capillaries (Hochi *et al.* 1998) and micro droplet (Landa, 1990). Vitrification requires either rapid cooling rates or the use of concentrated cryoprotectant solution which depresses ice crystal formation and increase viscosity at low temperature (Vajta, 2000). Supply of superior bovine germplasm within a short span of time is of paramount importance, which is highly relevant in the North East Region of India in particular. Retrieval of higher number of competent oocytes for *in–vitro* maturation and *in-vitro* fertilization to obtain superior transferable bovine embryos coupled with development of freezing technique

through vitrification will entail more productivity from the non descript animals. Therefore, the present experiment has been proposed to study the developmental competence of bovine oocytes following vitrification.

Materials and Methods

Indigenous cattle ovaries were collected from local slaughter house within 2 hrs. of slaughter. The cumulus oocyte complexes (COCs) were collected from the visible follicles measuring 3-8mm diameter by aspiration alone and aspiration with slicing technique as per Soren *et. al.* (2013). The cumulus-oocyte complexes were categorised depending on the compact cumulus cells surrounding the zona pellucida and evaluated under stereo zoom microscope (Dutta *et al,* 1998). Good quality oocytes were considered for subsequent vitrification and *in-vitro* maturation study. Two permitting cryoprotectants *viz.* ethylene glycol (EG) and dimethylsulphoxide (DMSO) were used in the present experiment for vitrification. Equilibration solution (ES) was prepared by mixing equal amount of 10% EG+10% DMSO in basic solution comprising TCM-199 with 0.3%BSA and vitrification solution consist of 20% EG+20% DMSO and 0.6M sucrose in basic solution. The immature oocytes were exposed to ES for three minutes, and then transferred to the VS solution and immediately loaded in a 0.25ml straw and plunged in liquid nitrogen within 1 minute for storage. Thawing was done in water bath at 37^{o}C for a minute and expelled the straw content in diluting medium containing TCM-199 with 0.6M sucrose and subsequent dilution in step wise manner. Post thaw survivability rate was evaluated. Post thawed normal oocytes were then subjected to *in-vitro* maturation (IVM). Culture media for IVM consist of TCM-199 supplemented with 10% follicular fluid, sodium pyruvate (0.0009g/10ml), L-glutamine (0.001g/10ml) and 10% FBS(5µl/ml). The oocytes were incubated in IVM culture media droplet covered with sterile mineral oil (50ul/10-12oocytes) maintaining at 38.5^{o}C in 5% CO_2 in humidified air for 24 hrs. For confirmation of maturation after 24 hours the oocytes were evaluated for morphological change and *in- vitro* maturation performance under stereo zoom microscope. The oocytes were defined as morphologically normal if it possessed an intact zona pellucida and plasma membrane and homogenous cytoplasm. *In- vitro* maturation performance was confirmed on the basis of cumulus cell expansion and nuclear maturation (Martins *et al*. 2005). The experiment was replicated nine (9) times on different dates. The results obtained were subjected to statistical analysis to determine difference among groups using student 't' and Chi-square test as per Snedecor and Cochran (1994).

Results

In the present study the result indicated that COCs recovered per ovary by aspiration with slicing technique from slaughtered bovine ovaries yielded a significantly ($p<0.01$) higher mean number of oocytes recovered per ovary (4.29) than by aspiration alone (3.48). Similarly the mean recovery of oocytes per visible antral follicle (3 to 8 mm in diameter) also yielded significantly higher number on aspiration with slicing technique as compared to aspiration alone (Table 1). In the study, a total of 358 immature bovine good quality oocytes were cryopreserved by vitrification where 326 were found to be morphologically normal following post-thaw assessment with 91.06% survivability (Table 2).

The *in-vitro* maturation performance of post thawed vitrified and non- vitrified bovine immature oocyte based on cumulus cell expansion revealed significantly ($p>0.01$) higher IVM rate has been recorded on non-vitrified control group (93.33%) than vitrified group (85.89%) in the study (Table 2).In the present study a total of 17 numbers of post-thaw vitrified mature oocytes irrespective of grade were considered for observing nuclear maturation out of which 14 numbers were found to be nuclear matured. The rate of *in-vitro* maturation performance of vitrified oocytes on the basis of nuclear maturation was 82.35%.

Table 1: Recovery performance of oocytes collected from slaughter house bovine ovaries using two collection techniques

Collection techniques	No. of ovary	No. of antral follicle	No. of recovered oocytes	No. of Oocytes per ovary		No. of oocytes per antral follicle	
				Mean	SD	Mean	SD
Aspiration Alone	86	319	262	3.48	1.25	0.78	0.23

Aspiration + Slicing	116	387	478	4.29**	1.01	1.16**	0.32
t-value				2.25 (Significant at p<0.01)		4.24 (Significant at p<0.01)	

Table 2: Post-thaw survivability and *in-vitro* maturation performance of vitrified cumulus oocytes compared with non-vitrified control

Groups	No of oocytes subjected to vitrification/ as control	Post thaw survivability of control / vitrified oocytes % (No.)	*In-vitro* maturation performance of vitrified oocytes on the basis of cumulus cell expansion % (No.)
G1 (Control)	120	100.00[a] (120)	93.33[a] (112)
G2 (Vitrified)	358	91.06[b] (326)	85.89[b] (280)

values in same column with different superscript differ significantly, Chi-square test (p<0.05)

Discussion

Recovery of significantly higher number of oocytes using aspiration with slicing technique as compared to aspiration alone shows that the commonly practiced method of aspirating the visible antral follicles in bovine might failed to recover the oocytes from the follicle that are embedded deeply within the cortex of the slaughter bovine ovaries. The result of this study was in agreement with the earlier observations of Takagi *et al.* (1992) in bovine and Hoque *et al.* (2011) in goat ovaries. The variation in respect of oocyte recovery using different collection technique might be due to species, ovarian status, and collection technique, processing and searching of oocytes under microscope (Hoque *et al.* 2011). The post thaw survival of vitrified oocytes recorded in the study was 91.06% and the remaining post thaw oocytes were either had fractured zona pellucida, loss of cytoplasmic content during warming and dilution of cryoprotectant in the treatment. Earlier observations on the percentage of mammalian oocytes found to be morphologically normal were varied from 88-97% using different vitrification protocol (Dhali *et al.*, 2000; Atabay *et al.*, 2004 & Martins *et al.*, 2005). The high proportions of COCs retained their morphology in the present study might be resultant of a short exposure to high concentration of permitting and non-permitting cryoprotectant and step-wise rehydration with sucrose. Similar were expressed by Martins *et al.* (2005) and Sharma and Purohit (2008) in mature bovine oocytes. Im *et al.* (1997) indicated that oocytes maintain for long periods in cryoprotectant solutions will have reduced viability due to bio chemical (inactivation of enzymes needed for meiotic progression) and /or biophysical events (lipid elution from membrane) instead of osmotic stress. Wahid *et al.* (2011) expressed that Ethylene glycol based freezing and vitrification solution, most organelles retrained their normal morphological status following cryopreservation and thawing. Among the morphologically damaged oocytes undergo during freezing and thawing were zona pellucida crack, oocytes shrinkage and splitting (Sharma *et al.* 2010). The presnt results corroborated with the previous studies on mammalian vitrified oocytes (Nandi *et al.*, 2001 and Rangasamy *et al.*2008). Cumulus enclosed bovine oocyte survived vitrification better in 10%EG+10%DMSO as equilibration solution for 3 minutes and 20% EG+20% DMSO and 0.6M sucrose as vitrification solution in the present study. Martins *et al.* (2005) recorded highest maturation rate using vitrification solution as ethylene glycol with sucrose with an equilibration time of 5 minutes and a equilibration solution with 20% ethylene glycol. Similarly Kuchenmeister and Kuwayama (1997) vitrified immature oocytes in 0.25ml straw and obtained a cleavage rate of 42%. The reduction in the developmental ability of post thaw vitrified oocytes could be due to toxic effects of

cryoprotactants and osmotic injury. In addition the possibility of deleterious effects on chromosomes and other cytoplasmic structures cannot be ruled out since such effects have been shown during cryopreservation of mouse and human oocytes. The freeze thaw process is known to induce an alteration in the physico-chemical properties in the intra-cellular lipids 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 (Sharma and Purohit 2008). In comparison, Sharma and Loganthasamy (2007) found 60.0% oocyte maturation rate following vitrification of buffalo oocyte. Similarly Hadi *et al.* (2011) recorded 51% maturation rate of vitrified immature bovine oocytes. In respect of non vitrified oocyte, Wang *et al.* (2007) reported maturation rate up to 84.1% for bovine oocytes. Better maturation rate in the present study might be resultant of immature oocytes (germinal vesicle stage) enclosed in cumulus cells provide a rigid structure to oocytes and thus protect them against osmotic shock during cryopreservation. It has been reported that survival and developmental rate of bovine oocytes were higher when vitrified with enclosed cumulus cells than partially denuded cells (Prentice- Biensch *et al.*, 2012).The nuclear membrane at the GV stage of oocyte might also be helpful in overcoming the cryo stress during vitrification. An important characteristic of the GV stage oocyte is that the genetic material is contained at early prophase within the contours of a nucleus. Since no spindle is present, this type of oocyte is assumed to be less prone to micro tubular and chromosomal damage (Massip, 2003).

Conclusion

The study demonstrated that bovine immature oocytes could be successfully matured *in-vitro* following conventional vitrification technique for cryopreservation using two permitting cryoprotectants (EG and DMSO) with morphologically normal matured oocytes.

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