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RESEARCH ARTICLE
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In vitro blastocyst development of post-thaw vitrified bovine oocytes

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Abstract

Aim: To evaluate the developmental competence of post-thaw vitrified bovine cumulus-oocyte complexes (COCs) *in vitro*.

Materials and Methods: A total of 129 COCs were cryopreserved using vitrification solution comprising of 15% ethylene glycol (EG) + 15% dimethyl sulfoxide (DMSO) + 0.6 M sucrose in medium TCM-199 with 10% FBS. Immediately, within a minute they are plunged into liquid nitrogen using 0.25 ml straws. Thawing was made with a step wise dilution method. Post-thaw normal vitrified and non-vitrified oocytes were subjected to *in vitro* maturation and *in vitro* fertilization.

Results: Post-thaw survival percentage of vitrified oocytes was 88.37% and maturation performance of vitrified oocytes on the basis of cumulus expansion was 81.58% as compared to non-vitrified control 93.85%. The *in vitro* fertilization performance of vitrified oocytes was 49.46% as compared to the non-vitrified ones (63.11%). Similarly, blastocyst formation of vitrified oocytes was 21.74% as compared to 32.47% in non-vitrified oocytes.

Conclusion: Vitrification of immature bovine oocytes using 7.5% EG + 7.5% DMSO for equilibration and 15% EG +15% DMSO + 0.6 M sucrose as vitrification solution yielded better *in vitro* fertilization and blastocyst formation rate.

Keywords: cumulus-oocyte complexes, *in vitro* fertilization, *in vitro* maturation, oocytes, vitrification

Introduction

Retrieval of a higher number of competent oocytes for *in vitro* maturation and *in vitro* fertilization (IVM – IVF) to obtain superior transferable bovine embryos coupled with development of freezing technique through vitrification will entail more productivity from the non descript animals. Oocyte cryopreservation is of paramount importance for assisted reproductive technologies (ART). Unique characteristics of mammalian oocytes in respect of permeability of cryoprotectants and water through plasma membrane make them vulnerable for conventional freezing protocols as compared to embryo freezing. Recent advances in freezing techniques like vitrification procedure with modifications in equilibration time, concentration of cryoprotectants, volume and dilution protocol resulted in a higher survival of different stages of oocytes to maturation and fertilization *in vitro* [1]. The vitrification procedure for freezing technique of oocytes may be more advantageous as compared to conventional freezing protocol. Lesser impact of cryoprotectants as regard to oocytes status with further development *in vitro* creates new frontiers in ART [2]. Vitrification of immature bovine oocytes using glass micropipettes under normal or super-cooled LN_2 resulted in viable blastocysts and live calves following in vitro embryo production [3]. Although oocyte competence with vitrification procedure seems to improve, additional experiments are required to ascertain its efficacy in oocytes towards its developmental competence *in vitro*.

Hence the present study was conducted to evaluate the developmental competence of bovine immature oocytes post vitrification.

Materials and Methods

Oocyte recovery: Cattle ovaries were collected from slaughter house and within $1^1/_2$ – 2 hours, they are processed as per the routine standard protocols. Oocytes were aspirated from 3 to 8mm ovarian follicles with medium containing TCM-199 and supplemented with 200mM L-glutamine solution, 0.4% BSA and antibiotics. The cumulus-oocyte complexes (COCs) were categorised and morphologically evaluated under stereo zoom microscope [4]. Homogenous and compact COCs were washed four times in holding media (Modified TCM-199, 200mM L-glutamine solution, 10% FBS, 0.8M Sodium pyruvate and 50µg/ml Gentamicin and 50µM Cysteamine) by gentle pipetting and were subjected to cryopreservation by vitrification.

Vitrification and thawing: Two vitrification solutions were prepared in media consisting of TCM-199 with 10% FBS. Vitrification solution I (VS I) consisted of 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) and vitrification solution II (VS II) consisted of 15 % EG + 15% DMSO + 0.6M sucrose. The immature bovine oocytes with cumulus cells were

Table-1. Effect of cryoprotectants exposed / vitrified immature oocytes on survival percentagr and maturation *in vitro*

Group	No. of COCs	Post thaw survivability / recovery performance		Maturation performance on the basis of cumulus expansion	
		No.	%	IVM	%
Non-vitrified	130	130	100.00	122	93.85[a]
Vitrified	129	114	88.37	93	81.58[b]

Values in maturation performance column with different superscripts differ significantly.Chi square test (P<0.05)

Table-2. *In vitro* fertilization performances of vitrified and non vitrified bovine immature oocytes.

Group	No. of oocytes	*In vitro* fertilization performance		Cleavage performance			
		No.	%	2-4 cell %	8-16 cell %	Morula %	Blastocysts %
Non-vitrified	122	77	63.11[a]	77(100)	47(61.03)	25(32.47)	25(32.47)
Vitrified	93	46	49.46[b]	45(97.83)	22(47.83)	10(21.74)	10(21.74)

Values in *in vitro* fertilization performance column with different superscripts differ significantly. Chi square test (P<0.05)

exposed to VS I for equilibration upto 3 minutes followed by 25-30 seconds in VS II at room temperature (22-25°C). The oocytes in VS II were immediately loaded into a 0.25 ml French straw preloaded with 0.6 M sucrose in holding medium with air gap in between and plunged into Liquid Nitrogen (LN_2). The straws were stored for a period of 7 days and then thawed in 37°C water bath for 30 seconds. After immersion in the water bath, oocytes were gradually rehydrated in sucrose solution. Oocytes were kept into the medium containing 0.6M of sucrose in basic solution for 1 minute. Then they are transferred successively into holding medium in stepwise dilution pattern containing 0.3 M and 0.15 M of sucrose for one minute in each. Following rehydration, oocytes were washed three times in holding medium. Morphological integrity of post thaw vitrified oocytes was assessed under inverted phase contrast microscope [4]. Oocytes having fragmented zona pellucida and absence of cytoplasmic contents were not considered. The remaining morphologically normal post thaw oocytes were taken for IVM. Freshly collected COCs were separately used for *in vitro* maturation and kept as control.

In vitro maturation: The fresh or post-thaw vitrified normal oocytes were matured in Modified TCM-199, 200mM L-glutamine solution, 10% FBS, 0.8M Sodium pyruvate and 50µg/ml Gentamicin and 50µM Cysteamine supplemented with p-FSH (5µg/ml), 10% v/v follicular fluid, 1µg/ml 17-β estradiol at 38.5°C in a humidified atmosphere of 5% CO_2 for 24 hours. For confirmation of maturation after 24 hours the oocytes were evaluated for morphological changes and *in vitro* maturation performance under stereo zoom microscope. The oocytes with an intact zona pellucida, plasma membrane and homogenous cytoplasm were considered as morphologically normal in the study. *In vitro* maturation performance was assessed on the basis of expansion of cumulus cells surrounding the homogenous oocytes [5].

***In-vitro* fertilization:** For *in vitro* fertilization, frozen bovine semen (2 straws each) was prepared for capacitation with swim-up technique using B.O. washing medium [6]. After washing, spermatozoa were added to B.O. fertilization medium to make a final concentration of $2x10^6$ sperm/ml. *In vitro* matured oocytes of both the vitrified and non vitrified groups were co-incubated with spermatozoa in B.O. fertilization medium at 38.5°C, 5% CO_2 in air and saturated humidity for 20-22 hrs. The expected zygotes were washed in culture (IVC) medium (*viz.*, mCR2aa containing 5%FBS and supplemented with 2% essential amino acids (v/v), 1% non essential amino acids (v/v), 1% α- glutamic acid, 0.3% BSA and 0.05 µg/ml gentamicin sulphate). Then zygotes were placed into IVC droplets and covered with mineral oil and incubated at 38.5°C under 5% CO_2 in humidified air. After 48hrs culture the cleavage up to 2-8 cells were recorded. Subsequently culture was continued for further development. Embryos in each group were observed under a microscope every 24hrs following insemination. Development to 2 cell stage was assessed at 48 hrs after insemination and subsequently cultured for 7 days to evaluate the blastocysts formation. Medium was replaced with fresh medium after every 48 hrs of culture. The study was carried out on different days with replicates of 10.

Statistical analysis: The data were compiled and the performance of maturation and *in vitro* fertilization between vitrified and non vitrified groups were compared by chi-square test.

Results and Discussion

In the present study, a total of 259 good quality bovine COCs were the subject of the experiment. Of which, 129 COCs were subjected to vitrification and the rest were non-vitrified and served as control. The performance in respect of post-thaw survival rates and *in vitro* maturation on the basis of cumulus cell expansion was more than 80% respectively. In comparison the non vitrified COCs group, the study demonstrated 93.85% *in vitro* maturation performances (Table-1). The vitrification thus had minimal effect on the survival rates and the ability of the oocyte to mature *in vitro* in the present study. High proportions of bovine COCs retain their post-thaw morphology after a short exposure to high concentration of permeating cryoprotectants namely EG and DMSO with sucrose

using 0.25 ml straws for freezing. Previous studies on vitrified cattle oocytes had recorded the morphological survival rates between 65 to 94% [7,8]. The sensitivity of bovine oocytes to cryo injury and consequent survival has been well described with variable results. The factors primarily responsible for cell damage are the type of cryoprotectants, concentration, equilibration time, cooling and warming procedures [9]. The maturation rate primarily depends on quality of oocytes, media ingredients and amount and incubation environment. Cooling the Germinal Vesicle (GV) stage bovine oocytes had no effect on the nuclear maturation or fertilization. The nuclear material is membrane bound; thus there is a very low risk of any chromosomal damage as compared to that with dividing cells such as within an embryo or maturing oocytes. Hurtt et al. [10] compared viability of immature and mature bovine oocytes vitrified in Ethylene Glycol based solution and recorded 60% & 70% nuclear and cytoplasmic maturation rate. Faster membrane penetration of ethylene glycol make it as an ideal cryoprotectant combined with non-penetrating cryoprotectants like sucrose that act as a stabilizer, minimizing the affect of high concentration of ethylene glycol [11].

The subsequent *in vitro* fertilization performance of vitrified post-thaw *in vitro* matured oocytes in the present study was recorded as 49.46% with 21.74% of blastocyst formation. The results are in agreement with the findings of Vajta et al. [12] and Papis et al. [13] who used vitrified matured bovine oocytes. The improved success rate was attributed to the increased cooling rate during oocyte vitrification and to the step wise dilution method. Earlier reports show that after thawing using the four step dilution method with 2.5 minutes interval the vitrified oocytes developed to the two cells (71-100%), four cells (71-93%), eight cells (46-71%) and blastocyst (23-36%) stages [14]. Step wise dilution might be helpful to reduce osmotic injury of vitrified oocytes after thawing. The cryopreservation capacity of oocytes at the GV stage is lower as compared to metaphase I through metaphase II stages of oocytes have been well documented [15, 16]. However, notably well adapted vitrification program could lessen the differences in the capacity of *in vitro* development of different maturational stages of oocytes following cryopreservation [17, 18].

Conclusions

Effect of suitable vitrification procedures i.e. using 7.5% EG+ 7.5% DMSO for equilibration and 15%EG +15%DMSO+0.6 M sucrose as vitrification solution, on immature bovine oocyte yielded better *in vitro* oocyte growth and development competence.

Authors' contributions

All authors contributed equally in this study.

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Competing interests

The authors declare that they have no competing interests.

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