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RESEARCH ARTICLE

IN-VITRO MATURATION OF POST-THAW VITRIFIED BOVINE IMMATURE OOCYTES

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A total of 319 fresh bovine ovaries were obtained from the local abattoir. COCs were cryopreserved using a vitrification solution comprising TCM-199 (HEPES modified) with 10% FBS and two cryoprotectants of 20% ethylene glycol (EG) and 20% dimethyl sulfoxide (DMSO). Vitrified COCs were stored in liquid nitrogen (LN_2) for 7 days and then thawed. Morphologically normal COCs were tested for *in-vitro* maturation to record morphological changes of COCs during vitrification. Freshly collected COCs were separately used for *in-vitro* maturation and kept as control. By aspiration method, 816 oocytes were recovered with an average of 2.72 ± 0.14 oocytes per ovary. The average recovery rate of grade A, B, and C oocytes were 8.67 ± 0.82, 5.77 ± 0.41 and 2.62 ± 0.31respectively. The proportions of COCs that were recovered in morphologically normal form following vitrification was 83.68% and the *in-vitro* maturation rate of vitrified COCs was 74.20 ± 1.88 % as compared to non vitrified control (87.09 ± 1.63 %). Vitrification of immature bovine oocytes using 10% EG + 10 % DMSO for equilibration and 20%EG + 20%DMSO with 0.6M sucrose as vitrification solution yielded acceptable *in-vitro* maturation rate.

Introduction

Cryopreservation of immature oocytes by vitrification can proved to be a valuable method for *in–vitro* maturation (IVM) and *in–vitro* fertilization (IVF) technology. Vitrification is an alternative that may increase oocyte survival after thawing. Vitrification is the solidification of a solution at low temperature without ice crystal formation. This phenomenon requires either rapid cooling rates (Rall, 1987) or the use of concentrated cryoprotectant solutions, which depress ice crystal formation and increase viscosity at low temperature (Vajta, 2000). Several studies demonstrated that ethylene glycol would be the ideal cryoprotectant (Wahid *et al*. 2011), because it penetrates membranes faster than glycerol (Cha *et al*. 2000) and is less toxic than other permeable cryoprotectants (Cha *et al*. 2000, Martino *et al*.1996 and Dinnyes*et al*.2000). Freezing solutions, containing permeable (usually ethylene glycol) and non-permeable cryoprotectants, seem to be more advantageous than solutions containing just a permeable cryoprotectant (Shaw *et al*. 2000). The disaccharides sucrose and trehalose are the most common non-permeable cryoprotectants used for oocyte cryopreservation. Sucrose acts as a stabilizer, minimizing the effects of high concentrations of ethylene glycol (Fagundes, 2002). The present experiment was undertaken to assess the morphological changes and *in- vitro* maturation performance of immature vitrified bovine oocytes.

MATERIALS AND METHODS

Oocyte collection and vitrification

Indigenous cattle ovaries were collected from slaughter house and transferred to the laboratory within $1^1/_2$ – 2 hours of slaughter in a thermo flask maintaining 37° C in normal saline solution incorporated with penicillin. The ovaries were washed with sterile normal saline solution (NSS) and kept in the incubator maintained at 37° C and 5% CO_2 for half an hour for warm up. Oocytes were retrieved from ovaries by manual aspiration of 2 to 6mm follicles with an 18G needle on a 10 ml syringe containing a small volume of Medium 199 (Sigma M7528) with earles salts, 25mM HEPES, Sodium bicarbonate and supplemented with 200mM L-glutamine solution, 0.4% BSA and antibiotics. Oocytes were categorised as 'A', 'B', 'C' and 'D' after morphological evaluation under stereo zoom microscope and according to cumulus cell characteristics (Dutta *et al*. 1998). Homogenous and compact cumulus-oocyte complexes (COCs) were washed four times in holding media (Medium 199 with earles salts, 25mM HEPES, Sodium bicarbonate, 200mM L-glutamine solution, 10% FBS, 0.8M Sodium pyruvate and 50µg/ml Gentamicin and 50µM Cysteamine) by repeated pipetting and were subjected to cryopreservation by vitrification.

Two vitrification solutions were prepared in media consisting of TCM-199 (HEPES modified) with 10% FBS. vitrification solution I (VS I) consisted of 10% ethylene glycol (EG) + 10% dimethyl sulfoxide (DMSO) and vitrification solution II (VS II) consisted of 20 % EG + 20% DMSO + 0.6M sucrose. The immature bovine oocytes with cumulus cells (COCs) were exposed to VS I for 1 minute followed by 25-30 seconds in VS II. The oocytes in VS II were loaded in 0.25 ml French straw 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 expelled into the holding medium with 0.6M of sucrose and held for 1 minute. Oocytes were then transferred successively into a holding medium with 0.5, 0.33 and 0.17 M of sucrose for one minute in each solution. At the end of the last rehydration, oocytes were washed successively (three times) in holding medium. Post thaw vitrified oocytes were then examined for their morphological integrity (Dutta *et al*. 1998). Briefly, the recovery rate was defined as the number of oocytes counted after the end of rehydration, in relation to the total of oocytes vitrified. Oocytes with fractured zona pellucida and with loss of the cytoplasmic contents were discarded. The remaining oocytes were subjected to IVM. Freshly collected COCs were separately used for *in-vitro* maturation and kept as control.

IVM of vitrified oocyte

The fresh or post thaw vitrified normal oocytes were matured in holding media supplemented with p-FSH (5µg/ml), 10% v/v Follicular fluid, 1µg/ml 17-β estradiol at 37-38.5°C in a humidified atmosphere of 5% CO_2 for 24 hours. For confirmation of maturation, the oocytes were taken out from incubator after 24 hours and 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).

RESULTS AND DISCUSSION

Recovery performance of different categories of bovine oocytes collected from slaughter house ovaries were presented in Table 1. The overall mean recovery rate of different types of oocytes through aspiration method was 8.67± 0.82 for 'A' grade, 5.77± 0.41 for 'B' grade, 2.62± 0.31 for 'C' grade and 1.02 ± 0.21 for 'D' grade respectively. The overall mean oocyte recovered per ovary was 2.72± 0.14. The results were in accordance with the findings of earlier studies (Haque *et al*.2011) observed that the total mean number of cumulus oocyte complex (COCs) per ovary as well as the number of abnormal COCs per ovary by aspiration technique was 3.28 and 0.80, respectively. Hussain *et. al*. 2005 reported that the average number of good quality oocytes was 3.9±0.21 per ovary. The number of COCs recovered in summer (3.41 ± 0.15) was significantly lower than winter (4.4 ± 0.07). Various workers used different collection techniques for oocytes with varying degree of success. Varisanga *et. al.* 1998 recorded 6.0 ± 1.6 to 9.5 ± 2.4 good quality oocytes recovered per ovary from different bovine ovarian morphological classes. The recovery of good quality oocytes (A and B) in the present study were comparable as the number of normal COCs were found to be significantly higher in 2-6 mm diameter follicles than others, moreover aspiration techniques produce less debris and required less washing as compared to other recovery techniques (Haque *et al*.2011).

The morphological and *in-vitro* maturation performance of fresh / vitrified oocytes were presented in Table 2. Out of these the proportions of COCs that were recovered in morphologically normal form following vitrification was 89.59%. The *in-vitro* maturation rate of vitrified COCs was 74.20 ± 1.88 % as compared to non vitrified control (87.09 ± 1.63%), whereas *in-vitro* maturation rate in respect of morphologically normal post-thaw oocytes were 88.96 ± 1.74%. The findings of the present experiment are in broad agreement with Kuchenmeister & Kuwayama,

1997, Sharma & Purohit, 2008 and Wahid *et al*. 2011. The maturation rate depends on oocyte quality, sufficiency and efficacy of the media and optimization of incubation period. Hurtt *et. al*. 2000 compared viability of immature and mature bovine oocytes vitrified in Ethylene Glycol based solution and recorded 60% & 70% nuclear and cytoplasmic maturation rate. The performance of maturation of fresh or post-thaw vitrified oocytes in the study might be attributed to normal quality COCs subjected to a vitrification protocol with optimum cryoprotectant with a stabilizer. Faster membrane penetration of ethylene glycol make it as an ideal cryoprotectant combine with non penetrating cryoprotectant like sucrose that act as a stabilizer, minimizing the affect of high concentration of ethylene glycol (Martins *et al.*2005).

Table-1. Recovery performance of different categories of bovine oocytes collected from slaughter house ovaries.

Groups	No.s of Ovary collected	Oocytes/ Ovary Mean±SE	Total Oocytes (No.s)	Categories of oocytes			
				A Mean±SE	B Mean±SE	C Mean±SE	D Mean±SE
Test	154	2.33 ±0.12	346	8.28 ± 0.98	4.95 ± 0.41	2.09 ± 0.46	1.14 ± 0.30
Control	165	3.06 ± 0.22	470	9.00 ± 1.29	6.5 ± 0.65	3.08 ± 0.41	0.91 ± 0.31
Overall	319	2.72 ± 0.14	816	8.67 ± 0.82	5.77 ± 0.41	2.62 ± 0.31	1.02 ± 0.21

Table -2. Morphological and *in-vitro* maturation performance of fresh / vitrified oocytes.

Groups	No.s of oocytes fresh/ Cryo-preserved	Number of normal/ post-thaw oocytes (%)	Morphologically Normal (%)	*In-vitro* maturation performances on the basis of				
				In-vitro matured oocytes Nos.	Normal Mean± SE	Collection Mean± SE	Cryo-preserved Mean± SE	Post Thaw Mean± SE
Test	288	269 (93.40%)	241 (89.59%)	214	88.96 ± 1.74	62.29 ± 2.20	74.20 ± 1.88	79.27 ± 2.15
Control	408	408 (100%)	408 (100%)	357	87.09 ± 1.63	75.18 ± 1.88	---	---

Vitrification of immature bovine oocytes using 10% EG + 10 % DMSO for equilibration and 20%EG + 20%DMSO with 0.6M sucrose as vitrification solution yielded acceptable *in-vitro* maturation rates.

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