MORPHOLOGICAL SURVIVABILITY OF IMMATURE AND MATURE BOVINE OOCYTES VITRIFIED BY CRYOLOOP METHOD

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Abstract

Successful cryopreservation of oocyte using vitrification ensures availability of large number of oocytes for in vitro maturation (IVM) and in vitro fertilization (IVF).Ovaries were collected from 24 slaughtered cattle and culture grade oocytes were selected by aspiration. Group I consisted of thirty eight oocytes vitrified using cryoloop and matured in vitro, while group II consisted of thirty three oocytes vitrified after maturation. The oocytes were evaluated for morphological damages and viability. In group I, 52.63 per cent oocytes were morphologically viable, whereas in group II per cent of viable oocytes was 66.67. Viability per cent of live oocytes did not differ (P > 0.05) significantly between two groups. The results indicated that meiotic stage of oocyte had no influence on morphological survivability of bovine oocytes subjected to cryoloop vitrification.

Key words*: Aspiration, cryoloop, bovine oocytes, viability*

As a part of development in cryobiology, several cryopreservation methods such as conventional slow freezing and vitrification have been put forward to preserve oocytes of many species which resulted in birth of live offspring. The conventional slow freezing method causes osmotic shock and intracellular ice crystallization which ultimately results in cell damage (Vajta *et al.,* 1998). To overcome problems associated with conventional freezing an alternate method of vitrification was developed.

Vitrification is the solidification of an aqueous solution into glass-like state by increase in viscosity, without the formation of ice crystals. During vitrification oocyte were initially pre-equilibrated in a cryoprotectant of lower strength and was followed by a very short incubation in higher concentration of cryoprotectant.

Viability of oocytes after vitrification is influenced by meiotic stage of the oocytes at which it is cryopreserved (Otoi *et al.,* 1995). The vitrification of immature and mature oocytes has been performed in different species with variable results. The present study was undertaken to assess morphological survivability of immature and *in vitro* matured bovine oocytes following cryoloop vitrification.

Materials and Methods

Ovaries were collected from slaughtered cattle of various South Indian breeds like Kangayam, Khillari, Hallikar and

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crossbred cattle from Thrissur corporation slaughter house and transported to the laboratory in a thermoflask containing freshly prepared normal saline solution at 36-38^{0}C. The ovaries were washed several times in sterile normal saline solution at 37^{0}C to remove excess blood and tissue debris.

Oocytes were retrieved from surface follicles of 2 to 8mm size by aspiration in Cumulus Oocyte Complex (COC) handling media prepared with Dulbecco's Phosphate Buffered Saline (DPBS) enriched with five per cent heat inactivated (56°C for 30 min) day zero estrus cow serum and 0.5 per cent BSA (maintained at 39^{0}C).

Oocytes were examined under zoom stereomicroscope at 40X magnification and culture grade oocytes were selected based on the number of cumulus layer and ooplasm character. Culture grade immature oocytes from group I were vitrified using cryoloop, whereas oocytes from group IIwere subjected to *in vitro* maturation and matured oocytes were vitrified using cryoloop technique (Mavrides and Morroll, 2002). Maturation was assessed by cumulus cell expansion at 40X magnification under zoom stereomicroscope.

In vitro maturation of oocytes

Medium used for *in vitro* maturation of oocytes was TCM-199 supplemented with 10 per cent foetal calf serum (v/v), 10 mg/ml of FSH and antibiotics (100 IU/ml of penicillin and 50 mg/ml of streptomycin). Fifty microliter maturation drops were prepared with this media in 35mm sterile petri dishes and sterile mineral oil was layered over these drops. After repeated washing, five to seven oocytes were loaded gently into separate maturation drops and incubated for 22h at 39^{0}C temperature, five per cent CO_2 tension and maximum humidity in a standard air jacketed CO_2 incubator.

Vitrification of oocytes

The holding medium (HM) consisted of HEPES-buffered TCM-199 supplemented with 20 per cent FCS. The oocytes were equilibrated in 7.5 per cent ethylene glycol with 7.5 per cent dimethylsulfoxide in HM for 3 minutes followed by the exposure to vitrification medium (VM) containing 16.5 per cent ethylene glycol, 16.5 per cent dimethylsulfoxide and 0.5 M sucrose in HM for 45-60 seconds. Three to five oocytes were gently placed onto a cryoloop pre-loaded with a thin film of vitrification solution created by surface tension. The loaded cryoloop was immediately plunged into liquid nitrogen and then screwed on the cryovial already immersed in liquid nitrogen. After a period of 5 days, cap of the cryovial submerged in liquid nitrogen was carefully unscrewed and opened and the cryoloop containing the vitrified oocytes was transferred immediately into warming solution, which consisted of HM supplemented with 1.25 M of sucrose for 3 minutes pre-warmed at 39^{0}C. The oocytes were then transferred stepwise to HM supplemented with 0.5 M, 0.25 M and 0.125 M of sucrose for 30 seconds and finally to the original holding medium. After thawing and cryoprotectant removal, oocytes were examined for morphological characteristics under inverted microscope at 100X magnification.

Results and Discussion

In the present study, 20 oocytes out of 38 immature oocytes vitrified in group I were viable (52.63 per cent), whereas 22 oocytes out of 33 mature oocytes (66.67 per cent) were viable in group II (Table 1 and Fig. 1). Eventhough there is higher proportion of morphologically viable oocytes in group II compared with group I, no significant difference was observed between these two groups.

Group I oocytes exhibited more proportion of damaged oocytes (26.32 per cent) compared with group II (18.18 per cent). The damages observed in the present study were shrinkage of ooplasm, partial cumulus loss, complete cumulus loss and abnormal shape, among them shrinkage of ooplasm contributes to higher proportion (Table 2 and Figure 2).

The results obtained in the present study is in agreement with the results recorded by Abd-Allah *et al.* (2009) in which the percentage of mature vitrified oocytes that survived vitrification were higher compared with immature vitrified oocytes. Yang *et al.* (2003)

Table 1. Morphological survivability of bovine oocytes subjected to cryoloop vitrification

Group	Total no. of oocytes vitrified	No. of oocytes recovered	No. of morphologically normal oocytes (oocytes survived)	No. of damaged oocytes
GROUP I (Immature Vitrified)	38	30	20 (52.63)	10 (26.32)
GROUP II (Mature Vitrified)	33	28	22 (66.67)	6 (18.18)

Any value in the same column between two groups differ non-significantly (χ^2 test). Figures in parentheses indicate percentage.

Table 2. Types of morphological damages following vitrification of mature and immature oocytes

Group	No. of oocytes vitrified	No. of damaged oocytes (%)	Types of morphological damages (%)			
			Shrinkage of ooplasm	Partial cumulus loss	Complete cumulus loss	Abnormal shape
Group I (Immature vitrified)	38	10 (26.32)	4 (40)	3 (30)	2 (20)	1 (10)
Group II (Mature vitrified)	33	6 (18.18)	4 (66.67)	1 (16.67)	1 (16.67)	-

Any value in the same column between two groups differ non-significantly (χ^2 test). Figures in parentheses indicate percentage.

obtained morphological survivability similar to those obtained in the present study, following vitrification of immature bovine oocytes in straws.

Metaphase II oocytes are less susceptible to cryodamage, because they display a more flexible cytoskeleton. Moreover the hydrolytic conductivity of immature bovine oocytes is only half that of *in vitro* mature oocytes.

Mavrides and Morroll (2002) obtained very high survival rate for vitrified germinal vesicle (GV) stage bovine oocytes, whereas Dinnyes *et al.* (2000) obtained higher survival rate after vitrification of mature bovine oocytes by solid surface vitrification method. Higher survivability of goat MII stage oocytes was also reported by Begin *et al.* (2003) after cryoloop vitrification.

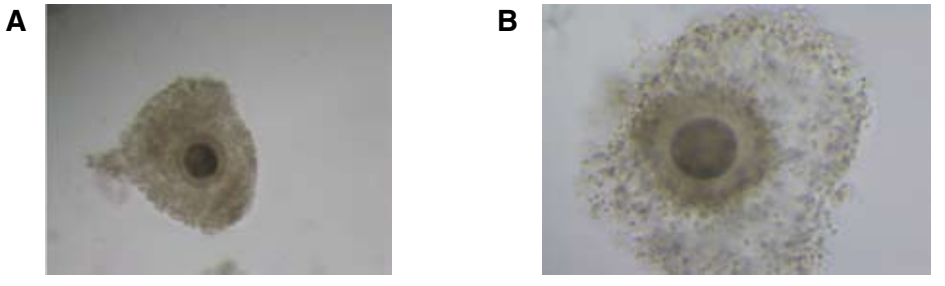

Fig. 1. Morphologically normal immature and mature oocytes after vitrification (100X). A- Immature oocyte and B- Mature oocyte

As per Quan *et al.* (2014) higher morphological survivability was obtained for goat mature MII oocytes compared to immature GV stage on cryoloop vitrification, which is attributed to reduced tolerance to the cryoprotectant toxicity compared with mature MII stage oocytes. However, Le Gal and Massip (1999) obtained lower values for immature vitrified and mature vitrified cattle oocytes compared with present study and found that the ability of oocytes to survive cryopreservation is not affected by meiotic stage in which oocyte cryopreserved.

The postvitrification abnormalities observed with immature oocytes compared to mature oocytes in the present study are in close relation with the earlier studies of Ali *et al.* (2014) attributable to the reduced tolerance of germinal vesicle stage oocytes to cryopreservation induced damage.

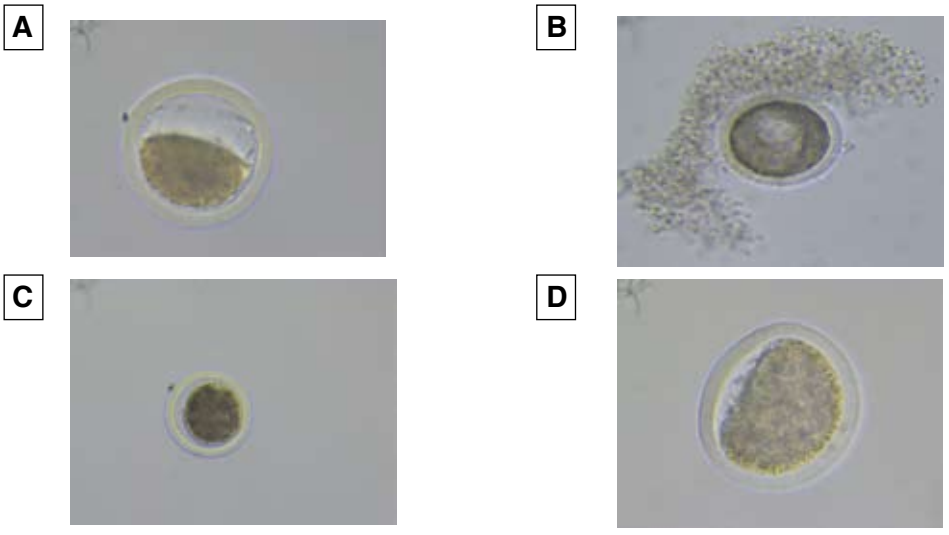

Figure 4.2 Morphological damages caused by vitrification (100X). A- Shrinkage of ooplasm, B-Partial cumulus loss, C- Complete cumulus loss, D- Abnormal shape

The results of the present study confirm the view that morphological survivability of mature bovine oocytes is better compared to immature oocytes following cryoloop vitrification.

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