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Original Research Article

Cryobiological effects of cryoprotectants on morphology of cumulus oocyte complexes (COCs) of sheep using vitrification

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Abstract

The aim of this study was to investigate the effect of different cryoprotectants like glycerol and DMSO individually and in combination of both on morphology of sheep oocyte using vitrification. The vitrification of sheep oocyte was performed using 40% glycerol and 40% DMSO individually and in combination of 20% glycerol + 20% DMSO. It was found that combination of cryoprotectants proved more efficient than using 40% glycerol or 40% DMSO alone. The morphological damages were found less (8%) using combination of 20% glycerol+20% DMSO as compared with using 40% Glycerol or 40% DMSO alone which was 16% and 15% respectively. Glycerol and DMSO when used alone found to induce adverse effect on the morphology of sheep oocyte. However combination of 20% glycerol + 20% DMSO found better to maintain the integrity and internal structure of sheep oocyte by vitrification.

Keywords: *Cryoprotectants (CPAs), Glycerol, DMSO, COCs, vitrification*

1. Introduction

Cryobiology, the effects of extreme low temperatures, opens new opportunities in the field of germplasm conservation. In future it may be possible to cryopreserve human and animal cells, whole organ, such as kidneys, hearts and livers, for subsequent transplantation, preserve corneas and other delicate tissue with minimal damage long enough to allow them to be shared all over the world and protect fragile and rare plants from extinction through ice free preservation (Gook 2011).

Oocyte cryopreservation is a panacea for conservation of extinct and endangered species. Furthermore oocyte cryopreservation make possibility of salvaging genetic material from prepubertal, infertile, pregnant or even from dead animals and creating oocyte bank. The preservation of ovarian tissue is cryobiologically challenging because it contains many cell types and specific extracellular matrix components (Hovatta 2005). The thousands breeds of small ruminants including sheep decreasing, according FAO nearly 20% of wild breeds of cattle, goat, pigs, horses and poultry are currently at risk of extinction and at least one live stock breed has become extinct per month from past many years that becomes the reason for complete loss of genetic characteristics for forever (Burekle 2007). The aim of germplasm conservation to preserve genetic diversity and gene combination in reverse able form and to keep particular gene of interest such as Booroola, fecundity gene in sheep (Mishra *et al*., 2009). Recently, it has been observed that the importance of the thawing temperature in ovine frozen ovarian fragments (Oskam *et.al.* 2010). Since immature oocyte are more resistant to freezing stresses so invitro maturation of oocyte after cryopreservation present best option for fertilization (Song *et.al.* 2010). Types and concentration of cryoprotectants are also important factors for post thaw survival rates of cryopreserved oocyte. Glycerol, Dimethyl sulfoxide (DMSO), propylene glycol (PG/PROH) and ethylene glycol (EG) have been conventionally used for the cryopreservation of oocyte (Gautam *et al.* 2008) and they are reported to have different biochemical nature as cryoprotectants. Many protocols have been standardized for oocyte preservation by many cryobiologists for numerous laboratory and domestic animals such as mouse, cow (Lim *et.al.*, 1992) and buffalo (Gautam *et al*., 2008).

During cryopreservation the extent of injury mainly depend on the size of the cell, permeability of membrane and quality of oocyte. However these factors vary with species to species, developmental stage and origin (Lechmak *et.al*, 1998). However these damages can be minimized using recently developed methods called vitrification. This method appears more promising and seems much effective than the classic slow freezing technique, but many factors in vitrification like concentration of cryoprotectants, cooling and osmotic stress need to be considered. Cryoprotectants causes oocyte to undergo osmotic dehydration prior to cooling, this treatment coupled with extremely high cooling rates prevents the formation of extracellular ice crystals within oocyte, which reduce morphological changes in oocyte architecture. The vitrification treatment does not affect the

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proportion of oocyte with intact morphology after warming although it has been reported that mammalian oocyte are very sensitive to high concentration of CPAs. Since cryoproctants are cytotoxic (Fahy *et.al*., 1999) therefore the selection of concentration and time of exposure to cryoprotactants is critical to minimize the toxic effects. The purpose of this study was to examine the morphological damages on cryopreserved COCs of sheep.

2. Materials and methods

2.1 Retrieval of COCs from sheep ovaries

Sheep ovaries were collected from slaughterhouse, New Delhi immediately after slaughter and washed twice with sterile normal saline supplemented with antibiotics at 32-37^{o}C. The ovaries were then transported to the laboratory. The ovaries were rinsed twice with warm saline containing antibiotics. Oocyte were collected by aspiration of surface follicles (2-8mm diameter) with a 19- gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM-199 + 10 % FBS). The contents of syringe which included aspirated oocyte, follicular fluid, granulosa cells and other debris were poured in Petri dishes. The oocyte were examined under Zoom Stereomicroscope at around 20X magnification. Cumulus Oocyte Complex (COCs) with multilayered cumulus cells and homogenous ooplasmic granulation were choosen. The oocyte were then shifted to 35 mm cell culture Petri dishes containing the washing medium (TCM-199+10%FBS+0.81mM sodium pyruvate). The aspirated oocyte were graded.

2.2 Cryopreservation of Oocyte

Cryopreservation of oocyte was performed using 40% Glycerol or 40% DMSO or combination of 20% Glycerol +20% DMSO by vitrification method.

2.2.1 Loading of Straw

The immature oocyte were divided in 3 groups, each containing 15-20 oocyte. The oocyte were grouped as group 1 (G40), group 2 (D40) and group 3 (G20+D20). After equilibration the oocyte were immediately loaded into the middle of 25 µl french mini straw. First 60µl of 0.5 ml sucrose was aspirated into the straw followed by 5 mm air space, then 40 ml of freezing media containing cryoprotectant and 15-20 oocyte, followed by 5 mm air space and finally 60µl of 0.5M sucrose. The open end of straw was sealed. The entire operations was carried with polyvinyl alcohol powder. Group1 (G40) and group2 (D40) was exposed to final concentration of cryoprotectant in a 3 steps by equilibrating the oocyte serially to 10%, 20%, and 40% of cryoprotectant. Briefly the oocyte were placed into culture dishes containing 2 ml of medium with G10 or D10 for 1 min and subsequently suspended to G25 or D25 for 30 sec before exposure to G40 or D40 solution. The oocyte of Group3 (G20) and (D20) were finally exposed to final concentration of cryoprotectant in 2 steps. The oocyte were placed in culture dishes containing 2 ml of TF medium with G10 or D10 for 1 min and were subsequently suspended to G10 or D10 for 30 sec. Group of around 20 oocyte were loaded into a 0.25 ml French straw suspended in a 100 µl microdrop of G20 or D20 solution and sealed with polyvinyl alcohol powder.

2.3 Freezing of straws by Vitrification

After filling sealed straws were dipped in liquid nitrogen. The first half of straw was rapidly immersed in liquid nitrogen and the rest part of straw was then immersed slowly to avoid bursting. The plunging in liquid nitrogen was done within 1 min of the final exposure of oocyte G 40 or D40 cryoprotectant solution. The straws were stored in liquid nitrogen for 60 days.

2.4 *Thawing*

After 60 days of duration of storage thawing was done by plunging straws into a water bath at 37^{0}C for 20 sec. The contents of straw were drained into a sterile 35 mm Petri dish after removal of cryoprotectant, the cumulus oocyte complex were washed and freed from cumulus cells by repeated pipetting and morphological evaluation was done under Zoom Stereo microscope.

3. Results

All the good quality oocyte were cryopreserved using individual or different concentration of cryoprotectants i.e. 40% Glycerol or 40% DMSO or combination of 20% glycerol+20% DMSO. Thawing of oocyte was done after 60 days from storage. Morphological assessment was done under Zoom Stereo microscope. The results are given in Table1. Morphologically normal oocyte after thawing was record 85% using glycerol 40% while the morphologically normal oocyte was using DMSO 40% were recovered 86% which is nearly equal. However percentage of morphologically normal oocyte recovered was significantly higher (92%) when a combination of both glycerol 20% and DMSO 20% was used. The morphologically normal oocyte recovery was 92% using combination of cryoprotectants than when used individually as given in Table1 and Fig1.

Table1. Morphologically normal oocyte recovered after 60 days of cryopreservation

Cryoprotectant	Concentration (%)	Oocytes frozen (n)	Morphologically normal oocytes recovered (n)	%of Morphologically normal oocytes recovered (n)
Glycerol	40	62	53	85
DMSO	40	65	56	86
Glycerol+DMSO	20 20	63	58	92

Cryopreservation induces different types of damages in oocyte like cracked zona pellucida, leaking of cellular contents, shrinking of cytoplasm, change in shape of oocyte and partially or fully removal of cumulus cells

Table 2: Different types of damages induced due to cryopreservation

Cryoprotectant	Concentration (%)	Oocyte damage	Type of damages					
		(n)	Cracked ZP (n)	Split in Two Halves (n)	Leaked contents (n)	Shrunken cytoplasm (n)	Change in shape (n)	partly/fully denuded (n)
Glycerol	40	9 (15%)	3.23	0	4.84	3.23	1.61	1.61
DMSO	40	9 (14%)	3.08	1.54	1.54	1.54	1.54	4.62
Glycerol+ DMSO	20+20	5 (8%)	1.59	0	0	1.59	1.59	3.17

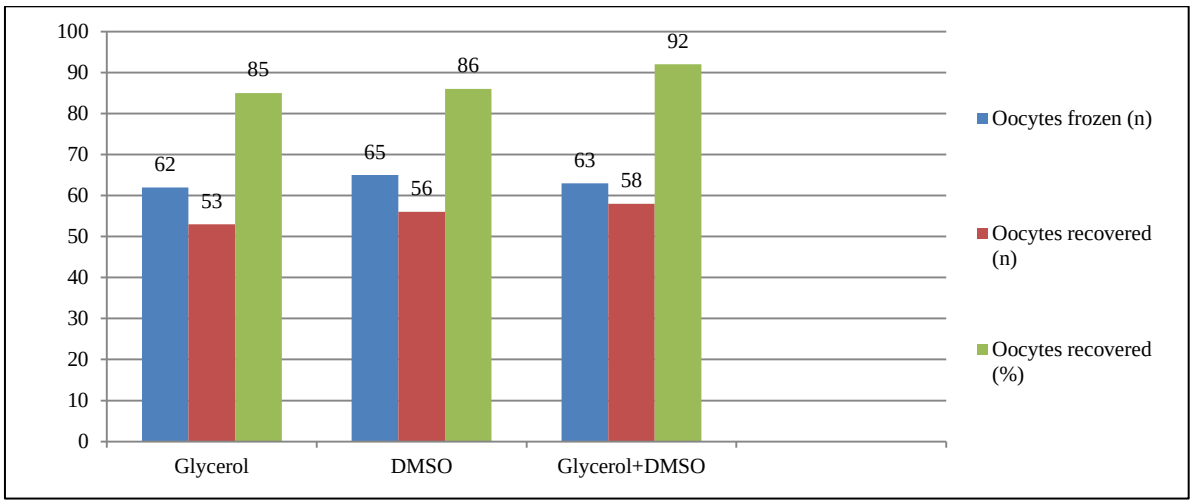

Fig1: Comparative bar chart showing morphologically normal sheep oocyte using glycerol, DMSO and combination of glycerol and DMSO

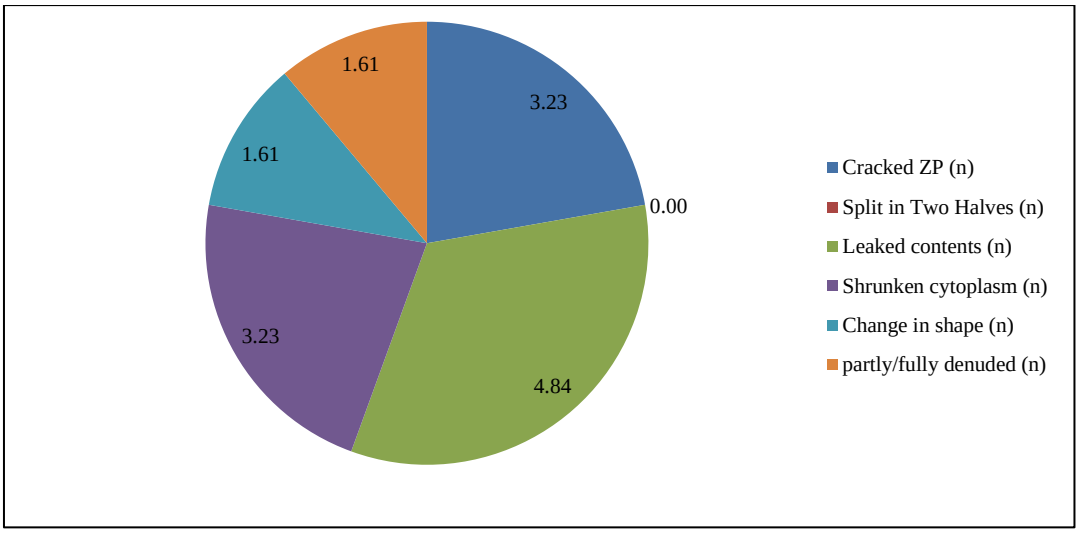

Fig 2. Different types of morphological damages (%) in sheep oocyte using 40% glycerol as cryoprotectant

layer from oocyte. The observations are shown in Figs (2-7). The minimum damages (8%) are observed by using combination of glycerol 20% + 20% DMSO as cryoprotectant. However the damages are higher using 40% glycerol and 40% DMSO as cryoprotectant (given in Table2).

4.0 Discussion

Availability of viable sheep oocyte can be assured by preserving sample in liquid nitrogen. However, this technique is time consuming and expensive. Vitrification,

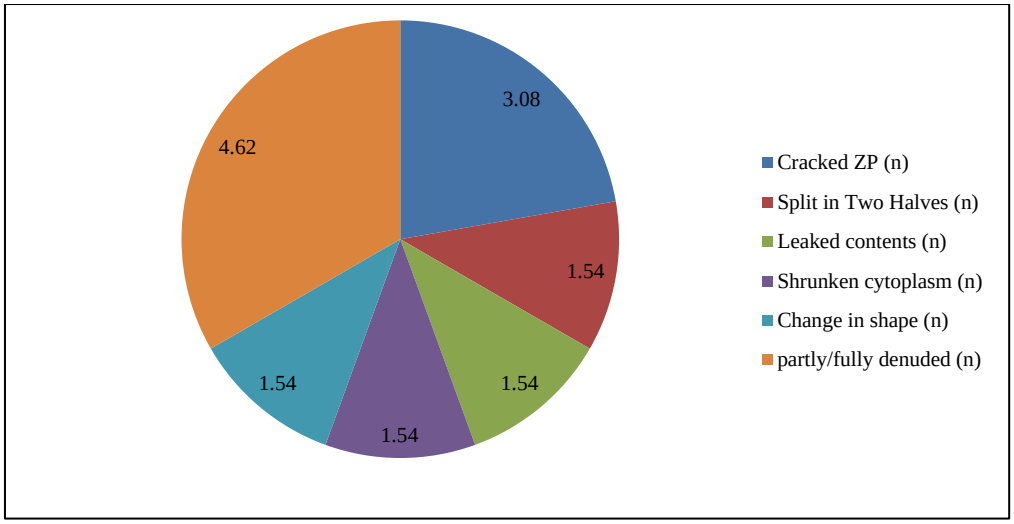

Fig3. Different types of morphological damages (%) in sheep oocyte using 40% DMSO as cryoprotectant

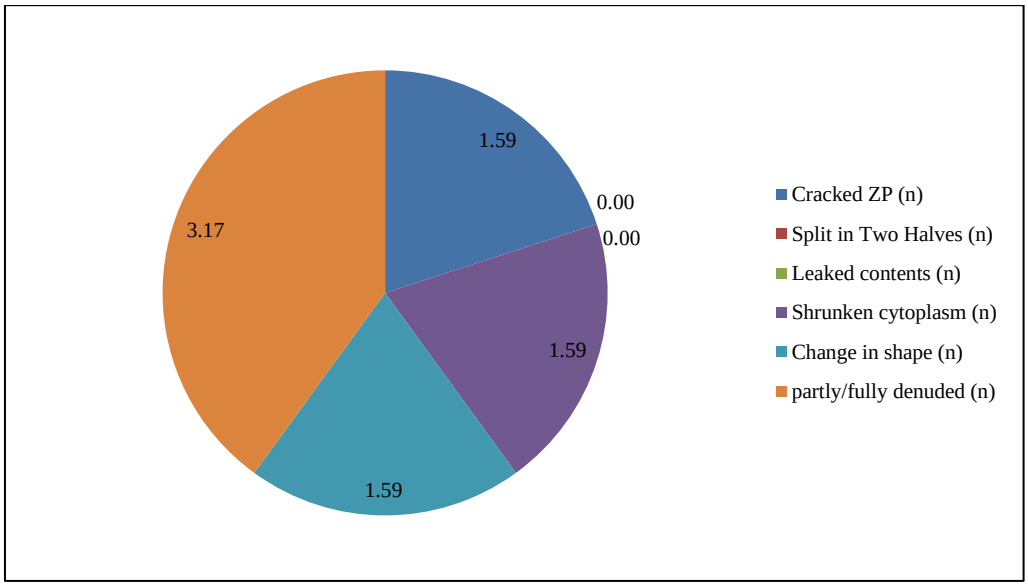

Fig4. Different type of morphological damages (%) in sheep oocyte using 20% glycerol + 20% DMSO as cryoprotectant

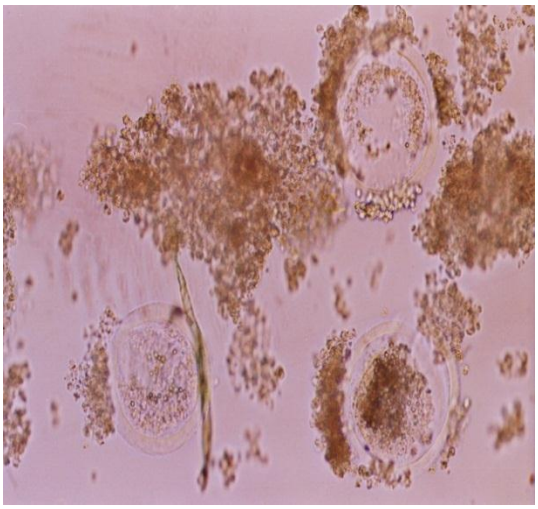

Fig.5: Partially denuded oocyte

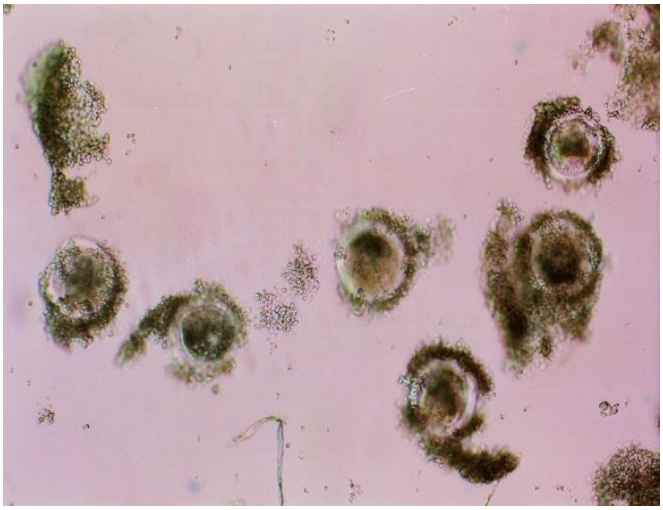

Fig.6: Oocyte after thawing

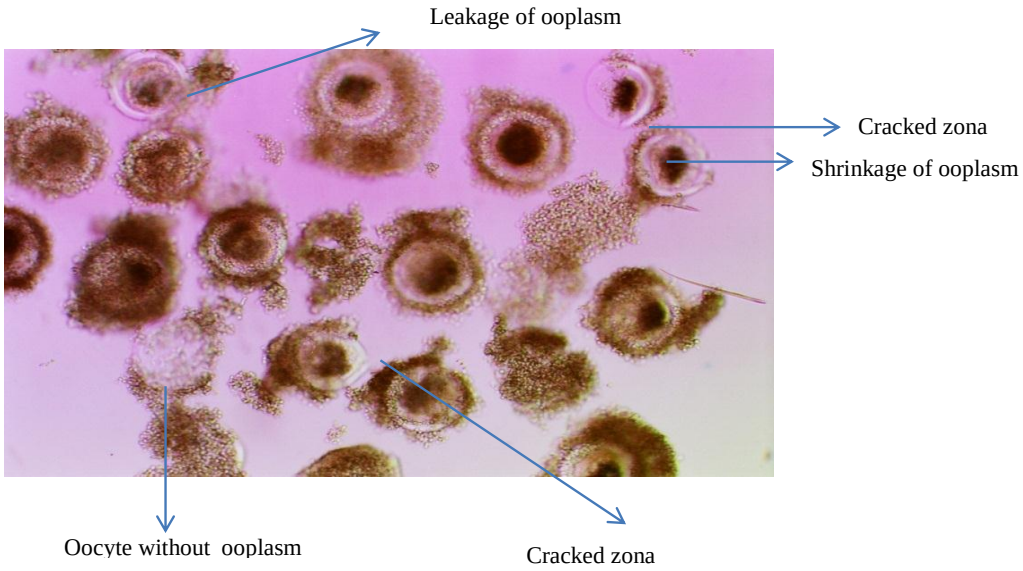

Fig.7: Different types of damages after cryopreservation

which is relatively new technique need less expertise, low cost and provide freedom for transportation of oocyte at far place for cryopreservation. Vitrification needs addition of cryoprotactants before storage. The cryoprotactants act like antifreeze. They also increase the viscosity, thus solution becomes amorphous ice despite crystallizing (Bhat *et.al*, 2005). Therefore an increase in the viscosity and depression of antifreezing temperature are major aspect to allow vitrification. The combination of cryoprotactants induce less toxic effects and proved more suitable than independent cryoprotactants . From study it is proved that glycerol and DMSO alone are not effective cryoprotactants. It was observed glycerol and DMSO provide 85% and 86% morphologically normal oocyte. However when used in combination the morphologically normal oocyte percentage was recoverd 92%.Which is similar to those recoreded previously (81.4%-95%) in vitrified goat oocyte (Garg *et.al.* 2007). Earlier study showed that the caprine oocyte and embryos vitrified by solid surface vitrification had significantly low survival rate than controls. While the survival rate of cryopreserved vitrified oocyte and embryos did not differ significantly from the control (Begin *et.al*, 2003). In this study the morphologically normal oocyte were similar with the findings of (Agarwal 1999) who found only 32 oocyte exhibiting abnormal morphological changes from 304 vitrified goat oocyte recovered. Similarly from the earlier study it was found that vitrification of the sheep oocyte using cryoprotectant (propylene glycol and sucrose) showed a high post thaw recovery rate .These findings are in line with that of (Men *et.al.* 1997) who reported that oocyte cryopreserved using vitrification have poor post thaw recovery which were cryopreserved using only one type cryoprotectants. In conclusion DMSO in combination with Glycerol found more effective in Glycerol 20% + 20% DMSO concentration. This can also be concluded that DMSO and glycerol combination is suitable for maintaining the integrity of the internal structures and morphology of oocyte.

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