International Scholars Journals

International Journal of Animal Breeding and Genetics Vol. 7 (10), pp. 001-006, October, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals



*Full Length Research Paper*

# Efficiency of intracellular cryoprotectants on the cryopreservation of sheep oocytes by controlled slow freezing and vitrification techniques

**Dike I. P.**

Department of Biological Sciences, Covenant University, Ota, Ogun State, Nigeria. E-mail: ejdike@gmail.com, ejdike@yahoo.com. Tel: 0234- 8074658012.

Accepted 15 March, 2018

**Oocyte cryopreservation has encompassed various technical difficulties and thus remained a challenge to many cryobiologists. The effect of three widely used cryoprotectants and two cryopreservation techniques on the post thaw recovery rate of morphologically normal sheep oocyte, their fertilizability and developmental competence were analyzed. Ethylene glycol, propylene glycol and Dimethyl Sulfoxide (DMSO) at concentrations 5.5 M +1 Msucrose, 5.0 M+1.5 Msucrose and 4.5 M+0.5 Msucrose were used for vitrification, whereas for controlled slow freezing 1.5 M was used. Post thaw recovery and cleavage rate of 88.13, 93.20, 85 and 17.92, 20.84, 15.21% was obtained using controlled slow freezing, whereas vitrification yielded 89.8, 88.14, 91.33 and 21.54, 29.05, 16.38%. Propylene glycol and ethylene glycol were found to be more efficient cryoprotectants, showing a promising developmental capacity, irrespective of the technique employed. Though the rate of cleavage and blastocyst formation still remained well below the control levels, the study clearly indicates that the type of cryoprotectant and the method adopted, have a significant effect on cryopreservation; i.e. the rate of maturation, fertilization and development.**

**Key words:** Sheep oocytes, slicing, ethylene glycol, propylene glycol, DMSO, vitrification, controlled slow freezing.

## INTRODUCTION

Recently the procedures for *in vitro* maturation and *in vitro* fertilization (IVM-IVF) of oocytes have progressed to such an extent that embryos could be obtained on routine basis. If *in vitro* matured oocytes could be successfully cryopreserved then unfertilized oocytes which have greater potential for animal breeding and biomedical research, especially when confirmed with IVM, IVF and embryo culture systems should also be put up for cryopreservation. However, the overall successes of these freezing and thawing procedures have remained low (Asada and Fukur, 2000).

Though some success has been achieved in freezing oocytes from cattle by adapting embryo cryopreservation protocols (Aman and Parks, 1994; Agca et al., 1998; Asada and Fukur, 2000; Baka et al., 1995; Cooper et al., 1996), the effectiveness of existing procedures based on viable embryos per oocyte frozen remains low compared to use of fresh oocytes in cattle and other domestic animal species, as well as humans.

The various steps required for cryopreservation that is, cryoprotective agent [CPA] loading, cooling below 0°C, seeding, cooling to a low subzero temperature, freezing/storage, thawing, and CPA removal, may contribute individually or cumulatively to oocyte damage that in turn decreases fertilization and development rates (Fuku et al., 1992). Alterations in the cytological components of mammalian oocytes due to procedures required for cryopreservation have been reported for the mouse (Cooper et al., 1996; Johnson and Pickering, 1987), human (Baka et al., 1995; Pickering et al., 1990; Sathananthan et al., 1988; Testart et al., 1986), rabbit and cow (Agca et al., 1998; Asada and Fukur, 2000; Fuku et al., 1992; Fuku et al., 1995; Fry et al., 1997; Hamano et al., 1992; Hochi et al., 1998; Lim et al., 1999).

The developmental stages at which the oocytes are frozen greatly influence the post thaw survival rate. *In vitro* matured oocytes have been shown to be more permeable to cryo-

**Abbreviations: IVM,** *In vitro* maturation; **IVF,** *In vitro* fertilization; **CPA,** cryo-protective agents; **PG/ PROH,** propylene glycol; **EG,** Ethylene glycol; **DMSO,** dimethyl sulfoxide; **TL HEPES,** HEPES buffered Tyrode's Lactate; **TALP,** tyrode's lactate solution (TALP**)**; **ANOVA,** analysis of variance.

cryoprotectants than immature freshly collected oocytes. Thus the reports reveal that the fertilizability of mature oocytes was found to be superior to the imam-ture oocytes (Le Gal et al., 1993).

Types and concentration of cryoprotectants are also important factors for the post thaw survival rates of cryo-preserved oocytes. Glycerol, Dimethyl Sulfoxide (DMSO), propylene glycol (PG/ PROH) and ethylene glycol (EG) have been conventionally used for the cryopreservation of oocytes and they are reported to have different biochemical natures as cryoprotectants.

The procedures for successful cryopreservation of oocytes are available for numerous laboratory and domestic animals including mouse and cow (Lim et al., 1992).

Le Gal et al. (1993) cryopreserved goat embryos by the three step equilibrium procedure using ethylene glycol and obtained 25 per cent developmental rate *in vitro* (Le Gal et al., 1993). Martinez and Matkovic (1998) equilibrated sheep embryos with ten minutes interval and obtained 80% developmental rate (Martinez and M Matkovic, 1998).

*In vitro* matured bovine oocytes were vitrified using 2M DMSO, 1 M acetamide and 3 M propylene glycol and only 10% post thaw development was obtained (Hamano et al., 1992). However there is paucity of information regarding sheep oocyte cryopreservation procedures (Martinez and Matkovic, 1998; Nowshau et al., 1994).

Hence this study was programmed to assess the efficiency of three widely used cryoprotectants viz. ethy-lene glycol, propylene glycol and DMSO by the surviva-bility of post thaw cryopreserved oocytes based on morphological appearance, further maturation and its fertiliz-ation *in vitro* using two different cryopreservation techniques namely, Vitrification and Controlled Slow Freezing Methods.

## MATERIALS AND METHODS

### Sources of oocytes

Ovaries of mature ewes from southern India were collected immediately after slaughter from the regional abattoir in normal saline supplemented with 10 μ L gentamycin per 1 ml and transported at 37ºC to the laboratory in vacuum flask within one hour. Upon arrival at the laboratory, the ovaries were removed of extra ovarian tissues and washed 2-3 times with tap water and 7-8 times with 0.9% normal saline at 37-38ºC. The ovaries were then kept in a sterile beaker containing normal saline (37- 38ºC) supplemented with 10 μL gentamycin per 1 ml. The ovaries were sliced as per the standard slicing technique described by Dutta et al. (1993) and the follicular fluid collected was then transferred into 50 ml sterile conical tube and kept at 37ºC in water bath (10 min) for the oocytes to settle at the bottom due to gravity. The supernatant was then discarded and the pellet was diluted with HEPES buffered Tyrode's Lactate (TL HEPES) and transferred to 2 – 3, 90 mm Petri dishes for oocyte screening and graded under the stereo zoom microscope. Oocyte grading was done according to Fry et al. (1997).

### *In vitro* culture and maturation of oocytes

A and B grade oocytes (18-20 oocytes/droplets) were then transferred to pre-incubated maturation droplets. The oocytes were allowed to mature *in vitro* for 24-26 h in $CO_2$ incubator under 5% $CO_2$, 95% relative humidity and 38.5ºC temperature. After 24 h of *in vitro* culture of oocytes, the oocytes were examined under the zoom stereomicroscope and assessed for cumulus layer expansion.

### Cryopreservation of oocytes

Cryopreservation of oocytes was performed using three different cryoprotectants by the controlled slow freezing and vitrification method. Under each experiments ten trials were conducted

### Experiment 1: Controlled slow freezing

The oocytes were partially dehydrated at room temperature (22-25ºC) in 3 steps by sequential equilibration in 0.5, 1 and 1.5 M of each cryoprotectant, namely ethylene glycol, propylene glycol and DMSO, at 10 min interval (Otoi et al., 1993).

### Experiment 2: Vitrification

The oocytes were partially dehydrated at room temperature at 5 min interval. The oocytes were then placed in drops of freezing media containing the cryoprotectants namely, ethylene glycol, propylene glycol and DMSO, and left 45 s for equilibration (Nowshau et al., 1994).

### Loading of the straws

After equilibration the oocytes were immediately loaded into the middle of a 0.25 μ l French mini straw. First 60 μl of 0.5 M sucrose was aspirated into the straw followed by 5 mm air space, then 40 μl of freezing media containing cryoprotectant and 20-25 oocytes, followed by 5 mm air space and finally 60 μl 0.5 M sucrose. The open end of the straw was sealed. The entire operations were carried out at ambient temperature.

### Freezing of straws by controlled slow freezing

After filling, the sealed straws were loaded in a controlled biological all freezers and frozen by adopting the slow freezing protocol.

### Freezing Protocol

Seeding was done at - 5ºC and held fast for 5 min at this temperature. The straws were then plunged into liquid Nitrogen ($LN_2$) at -196ºC for storage. After freezing in a programmable freezer, all the frozen straws were immediately transferred into goblets with liquid nitrogen and stored at -196ºC in LN2 storage container (Lim et al., 1997) for three weeks (Table 1).

### Freezing of straws by vitrification

After filling, the sealed straws were dipped slowly into liquid nitro- in $LN_2$ storage containers for three weeks.

### Thawing

After three weeks of storage, the straws were thawed first in air at room temperature for 3 s, until no ice crystals were visible. The straws were gently shaken to dislodge the air bubbles and the con-

**Table 1.** Freezing protocol.

| | Start temp | Rate of fall/min | End temp |
|---|---|---|---|
| **Ramp1** | **Ambient Temp** | - 1ºC | - 5ºC |
| 2 | - 5ºC | - 0.3ºC | - 30ºC |
| 3 | - 30ºC | - 10ºC | - 80ºC |

tents mixed uniformly. The straws were then plunged into the water bath at 37ºC for 30 s.gen within 45 s and transferred to $LN_2$ goblets and stored at 196ºC

The removal of the cryoprotectants was carried out by the single step sucrose dilution procedure. The oocytes were expelled to 0.5 M sucrose solution and then transferred to the maturation medium (Lim et al., 1997).

#### Post thaw morphological evaluation of frozen thawed oocytes

After removal of cryoprotectants, the cumulus oocytes complexes were washed four times in Fertilization Tyrode's lactate solution (TALP) medium and freed from cumulus cells by repeated pipetting. Morphological evaluation was done under zoom stereomicroscope. Morphologically damaged oocytes which were identified as oocytes with dark granulated or fragmented cytoplasm, indistinct vitelline were removed and 10-12 morphologically normal oocytes were selected to be placed in the fertilization droplets.

#### *In vitro* fertilization of frozen thawed oocytes

Testes from adult rams collected from the slaughterhouse were washed thoroughly, after removing tunica albugenia, with tap water and 0.9% saline supplemented with gentamycin. The cauda epididymis was sterilized with 70% alcohol and incised deeply using a surgical blade. Then using a syringe the sperm TALP medium was used to collect the seminal fluid. The semen collected were assessed for motility of the sperms and then processed for sperm separation by Percoll method (Papis et al., 1995).

The sperms were layered on the top of the percoll gradient. The tube was then centrifuged at 800 rpm for 30 min. The hazy layer was recovered immediately after centrifugation with a sterile pipette. Sperms were again washed with 10 ml of sperm TALP at 400 rpm for 10 min and the supernatant was removed until 100 µl was left in the tube. The motility of the sperms was assessed under lower (10x) magnification of the inverted phase contrast microscope. The concentration of sperms was adjusted to 2 x 10 sperms/ml using haemocytometer.

#### Co-Incubation of sperm and oocytes

The pre incubated fertilization droplets with oocytes were inseminated with sperms at a concentration of 2-10 sperms/ml.

Now two sets of oocytes were incubated, that is freshly harvested oocytes and the thawed oocytes. These oocytes were then incubated with the sperms for 18-20 h at 38.5ºC, 5% $CO_2$ and 95% relative humidity. After 24 h of fertilization, the presumptive zygotes were observed under the zoom stereomicroscope or phase contrast microscope for evidence of cleavage.

#### Statistical analysis

Statistical analysis was carried out using one way- Analysis Of Variance (ANOVA), a procedure for determining whether significant differences exist between two or more sample means. A difference of significant was considered when P-value was less than 0.05 ($P<0.05$) using the Instat Computer package.

## RESULTS

All the good quality oocytes (A and B) recovered through the slicing technique were utilized for *in vitro* maturation. The maturation rate obtained was 76%. Assessment of maturation of oocytes was carried out after 24 h of *in vitro* culture of oocytes. The oocytes were examined under the microscope and assessed for cumulus cell layer expansion. The obtained results were grouped into experiments for better analysis i.e. Experiments 1 and 2.

### Experiment 1: Effect of cryoprotectants

The post thaw recovery rate obtained for ethylene glycol, propylene glycol and DMSO by controlled freezing method were 88.13, 93.2 and 85.03% respectively out of which 67.60, 66.60, 72.14% of morphologically abnormal oocytes were obtained (Figure 1). The post thaw recovery rate obtained for ethylene glycol, propylene glycol and DMSO by vitrification method were 89.8, 88.14 and 91.33% respectively. The recovery of morphologically abnormal oocytes for ethylene glycol, propylene glycol and DMSO were 69.65, 71.65 and 71.77% respectively (Figure 2).

The control, non- cryopreserved oocytes showed a higher post thaw recovery rate of 95.52 and 93.44%, out of which morphologically abnormal oocytes were recorded to be 42.74, 44.54% for the controlled slow freezing and vitrification process respectively.

Oocytes were assessed for cleavage and developmental competence for 7 to 8 days post insemination *in vitro.* The developmental competence was evaluated by assessing the cleavage rate. Oocytes obtained after the controlled freezing procedure indicated that the cleavage rate and percentage of oocytes that developed to the two -cell, four-cell and blastocyst stages were significantly higher in oocytes frozen in ethylene glycol than those in propylene glycol and DMSO. While those oocytes obtained after the vitrification process, on fertilization, showed that the cleavage rate and the percentage of oocytes developed to the two-cell, four-cell and blastocyst stages were found to be significantly higher in the oocytes frozen in propylene glycol than those in ethylene glycol and DMSO.

Thus the percentage of cleavage rate of oocytes frozen

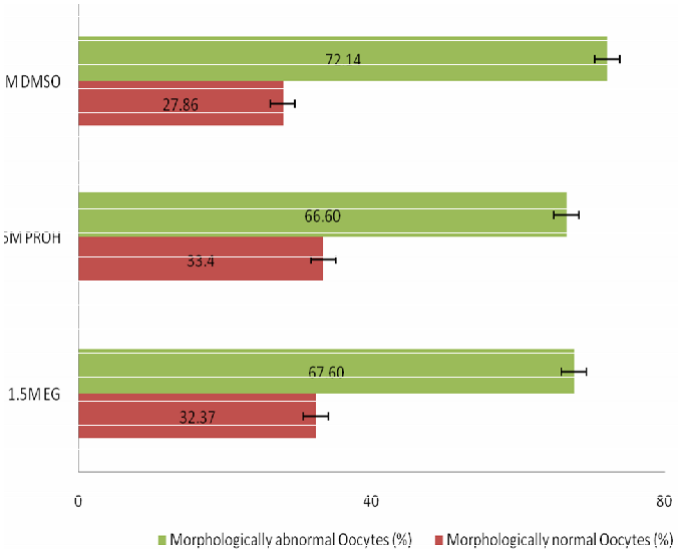


**Figure 1.** Post thaw recovery rate of sheep oocytes by controlled slow freezing using ethylene glycol, propylene glycol and dimethyl sulfoxide.

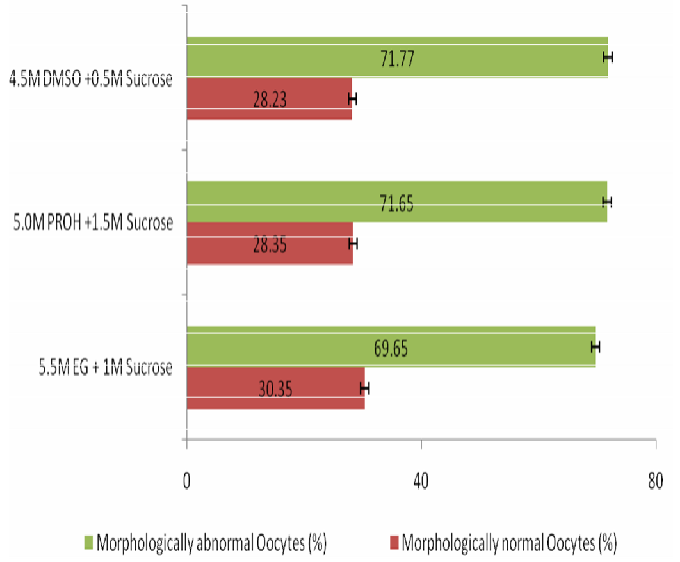


**Figure 2.** Post thaw recovery rate of sheep oocytes by vitrify-ation method using ethylene glycol, propylene glycol and dimethyl sulfoxide.

by controlled freezing and vitrification methods using ethylene glycol, propylene glycol and DMSO were 17.92, 20.84, 15.21% and 21.54, 29.05, 16.38% respectively (Tables 2 and 3).

However the freshly collected oocytes showed a mean cleavage rate of 46.76% which was significantly higher than that of the oocyte frozen with the cryoprotectants namely, ethylene glycol, propylene glycol and DMSO. This high cleavage rate was consistent for both cryo-preservation techniques.

**Experiment 2: Effect of cryopreservation method**

The mean percentage, post thaw recovery rate for controlled slow freezing and vitrification, using ethylene glycol, propylene glycol and DMSO were 88.13, 93.20 and 85% and 89.80, 88.14 and 91.83% respectively (Figures 1 and 2)

The data obtained revealed that the two methods did not register any significant variation in the post thaw recovery rate of the oocytes.

Oocytes were assessed for cleavage and developmental competence for 7 to 8 days post insemination *in vitro.* The developmental competence was evaluated by assessing the cleavage rate.

The percentage of cleavage rate of oocytes frozen by controlled freezing (Table 2) and vitrification methods (Table 3) using ethylene glycol, propylene glycol and DMSO were 17.92, 20.84, 15.21% and 21.54, 29.05, 16.38% respectively.

The values obtained for vitrification were found to be slightly higher than that of controlled slow freezing method with the exception of propylene glycol

The cleavage rate of the non-frozen oocytes was 34.76% by controlled slow freezing and 32.33% by vitrify-cation.

## DISCUSSION

Availability of viable, cryopreserved sheep oocytes would allow greater flexibility in the use of *in vitro* fertilization and related technologies by providing developmentally competent oocytes when needed. The effectiveness of existing procedures based on viable embryos per oocyte frozen remains low compared to use of fresh oocytes. It was recorded in earlier studies, that the maturation and developmental competence of frozen immature oocytes were inferior to the matured oocytes (Lim et al., 1992).

Results of this study demonstrate that this can be overcome to a certain extent by selecting the appropriate cryoprotectant and cryopreservation method.

The experiment carried out to study the effect of cryo-protectants on the post thaw recovery rate, revealed that despite the type of freezing method used all three cryoprotectants, namely ethylene glycol, propylene glycol and DMSO had similar post- thaw recovery rates. Propylene glycol and DMSO seemed more effective than ethylene glycol, though the difference was not highly significant.

Though DMSO offered a high post thaw recovery rate, it showed a lower fertilizability capacity. Thus indicating that though the post thaw rate of oocytes treated in DMSO was in line with those treated in ethylene glycol and propylene glycol, the post thaw fertilizability rate was greatly impaired, implying that these cryoprotectants may have some adverse cytological effects which in turn impair the rate of fertilization. Studies by Vincent et al. (1990) also confirmed these effects of DMSO on the

**Table 2** Fertilizability of oocytes frozen thawed by controlled slow freezing using ethylene glycol, propylene glycol and DMSO.

| Cryoprotectants | No. of Oocytes utilized for IVF | Cleavage rate in % |
|---|---|---|
| 1.5M EG | 101 | 17.92 [a] |
| 1.5M PROH | 83 | 20.84 [b] |
| 1.5M DMSO | 94 | 15.21 [a] |
| Control | 105 | 34.76 [c] |

* Groups with different superscript in the same column are significantly different (P<0.05).

**Table 3.** Fertilizability of oocytes frozen thawed by vitrification using ethylene glycol, propylene glycol and DMSO.

| Cryoprotectants | No. of Oocytes utilized for IVF | Cleavage rate in % |
|---|---|---|
| 5.5M EG + 1M Sucrose | 107 | 19.54 [a] |
| 5.0M PROH +1.5M Sucrose | 120 | 21.05 [a] |
| 4.5M DMSO +0.5M Sucrose | 115 | 16.38 [b] |
| Control | 120 | 32.33 [c] |

* Groups with different superscript in the same column are significantly different (P<0.05).

organization of microfilaments in the mouse oocyte (O'Neil et al., 1997; Vincent et al., 1990.

Thus from the study, it was deduced that DMSO, though effective, seems to show a greater number of abnormal oocytes and low fertilizability rate. Hence the normal oocytes obtained after cryopreservation with DMSO, may have cytological abnormalities. Hence propylene glycol and ethylene glycol were deemed to be better cryoprotectants, especially when combined with the vitrification technique.

However the data obtained revealed a significant difference (P<0.05) in the post thaw fertilizability rate of oocytes which were cryopreserved using propylene glycol.

Propylene glycol has also proven to be more effective when combined with the vitrification technique thereby yielding a higher rate of cleavage, thus indicating that the cytological effects caused by this cryoprotectant may be considerably lower than that caused by the other two. This was in line with previous studies which indicate propylene glycol and DMSO as effective cryoprotectants of mouse oocytes and embryos. Majority of oocyte freezing studies in mouse have used DMSO and Propylene glycol (Testart et al., 1986). Propylene glycol, in most cases is the cryoprotectant of choice for freezing human eggs because of its greater permeability, reduced toxicity, and improved success in embryo storage (Testart et al., 1986; Vincent et al., 1990). This is in concordance with results obtained in the study, since propylene glycol showed the highest rate of normal development after cryopreservation.

The relative lack of success with cryopreservation of oocytes is not surprising. But it is amazing that some embryos tolerate the drastic changes in extra- and intracellular conditions that accompany freezing and thawing. While physical considerations have been very helpful in elucidating the major causes of cellular damage during freezing and thawing and in determining optimal cooling and re-warming rates, more subtle biological factors may have to be taken into account to overcome the problems associated with freezing unfertilized eggs and certain other cells. This, in turn, may lead to overall improvements in cryopreservation procedures. But from the study, it is clear that the type of cryoprotectant and the method adopted, may have a significant effect on cryopreservation; that is, the rate of maturation, fertilization and development. It is also hypothesized that further studies analyzing the effect of these three cryoprotectants by chromosomal studies, will help analyze the reason behind the high ratio of abnormal cells after cryopreser-vation, thus evaluating the efficiency of these intracellular cryoprotectants, not only at the cellular level, but also at the chromosomal level.

However, even with these improvements, the rate of cleavage and blastocyst formation still remains well below control levels. A better understanding of how to overcome cytological damage associated with conventional procedures, in addition to the effects of maturation stage (Papis et al., 1995) and membrane permeability characteristics [21-28] (Park et al., 1997; Paynter et al., 1988; Pickering et al., 1990; Sathananthan et al., 1992; Sathananthan; Testart et al., 1986; Vincent et al., 1990; Williams et al., 1992), should provide a fundamental basis for the improve-ment of developmental potential of cryopreserved oocytes which can be adopted for mammalian species.

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