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CRYOTOLERANCE OF IMMATURE AND MATURE OVINE OOCYTES SUBJECTED FOR VITRIFICATION

S. Sathesh Kumar*

*Associate Professor, Teaching Veterinary Clinical Complex, Veterinary College and Research Institute, Tamilnadu Veterinary and Animal Sciences University, Orathanadu – 614 625, Thanjavur
Department of Animal Biotechnology, Madras Veterinary College
Chennai – 600 007
E-mail: drsatheshkumar6@rediffmail.com

Abstract: The objective of the study was to determine the cryotolerance of different developmental stages of ovine oocytes subjected for vitrification. Oocytes were collected from slaughter house derived sheep ovaries and graded. A and B grade oocytes were subjected for two treatment groups. T1 – Vitrification of immature (Germinal vesicle GV stage) oocytes (n = 100) and T2 – Vitrification of MII stage oocytes (n = 105). The oocytes were vitrified using 3M propanediol (PROH). The frozen thawed oocytes were washed and treated with trypan blue stain for dye exclusion assay. It was found that out in the T1 group, 38 (38.0%) and 62 (62.0%) frozen rewarmed GV oocytes were live and dead respectively. On the other hand, in the T2 group, 30 (28.6%) and 75 (71.4%) vitrified MII oocytes were live and dead respectively. Thus it was concluded that GV stage oocytes survive better for vitrification than the MII stage oocytes.

Keywords: Ovine oocytes – GV and MII stage – vitrification - cryotolerance.

Introduction

Oocyte cryopreservation is a potentially valuable technique and certainly represents one of the most attractive developments in the field of assisted reproduction, with the aim of preserving female fertility and circumventing the ethical and legal drawbacks associated with embryo freezing. Oocytes are more difficult to freeze than embryos due to the fact that they are large, delicate spherical cells with a low surface area to volume ratio and low hydraulic conductivity (Leibo, 1980). There are two very different approaches to cryopreservation of mammalian cells, conventional slow-cooling and vitrification. Although these methods are drastically different, both can produce successful results on choosing the optimal method for each cell type. The future of oocyte cryopreservation lies with novel vitrification procedure, as slow freezing is associated with increased cellular trauma (Prentice and Anzar, 2011). Nevertheless, vitrification has the advantage of being low-cost, since it eliminates the need for programmable freezing equipment, and it is quicker and easier to perform compared to

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slow-cooling. The overall objective of this study was to determine the survivability of different developmental stages of ovine oocytes subjected for vitrification.

Materials and methods

Sheep ovaries were collected irrespective of their breed, age, body condition and stage of estrous cycle from Chennai Corporation abattoir and utilized for the study. The ovaries were transported in saline supplemented with 50µg/ml gentamicin at 37ºC in the thermos flask to the laboratory within 30 minutes of slaughter. The extra-ovarian tissues were removed and the ovaries were washed with saline. Each ovary was held firmly with an artery forceps in a 60 mm petridish containing oocyte collection medium and was sliced as per the standard technique. The oocytes were screened under a zoom stereomicroscope and transferred to 35 mm petridish containing oocyte collection medium and then graded as per Zeinoaldini *et al*. (2013). Grade A and B oocytes were randomly divided into two treatment groups and vitrified in two different developmental stages.

i) T1 – Vitrification of immature (Germinal vesicle GV stage) oocytes (n = 100) immediately after harvesting.

ii) T2 – Oocytes were subjected for *in-vitro* maturation as described by Chauhan *et al*. (1997). Oocytes with complete cumulus expansion were considered as matured (MII stage) oocytes. Vitrification of MII stage oocytes (n = 105) were carried out.

Vitrification procedure

The oocytes were partially dehydrated at room temperature (22-25°C) by sequential equilibration in 1M sucrose at five minutes interval. The oocytes were then placed in drops of freezing media containing 3M propanediol (PROH) and left for 3 minutes for equilibration. After equilibration the oocytes were immediately loaded into the middle of a 0.25ml French mini straw. First, 60µl of one M sucrose was aspirated into straw followed by 5mm air space, then 40µl of freezing media containing cryoprotectant along with 20-25 oocytes followed by 5mm air space and finally 60µl of one molar sucrose. The open end of the straw was sealed. Entire operation was carried out at ambient temperature. After filling, the sealed straws were dipped slowly into liquid nitrogen (to avoid cracking) within 45 seconds and transferred to goblets with liquid nitrogen and stored at -196°C in liquid nitrogen storage container for one to three weeks.

Evaluation of oocytes after rewarming

After one to three week of storage, the straws were rewarmed by waving in air followed by plunging into water bath at 37 ^{o}C for 30seconds. Removal of cryoprotectant was

carried out by using 0.25M sucrose for 5 minutes (Lim *et al.*, 1999). The oocytes were transferred to holding media (TCM plus 10% serum) for further studies.

Viability assay by Trypan blue staining: The cumulus oocyte complexes were washed four to five times in holding medium and freed from cumulus cells by repeated pipetting. The proportion of live and dead oocytes was estimated by trypan blue exclusion study as described by Gupta *et al*. (2002). The oocytes stained deep blue were classified as dead and those unstained as live.

Analysis of results

The survivability of the oocytes was arrived by calculating the percentage of live and dead oocytes in both the treatment groups. The values were compared and analysed.

Results

Recovery rate and quality of oocytes

A total of 570 oocytes were collected from 392 ovaries with a recovery rate of 1.5 oocytes per ovary. Out of the 570 oocytes recovered, 163 (28.6%), 184 (32.3%), 124 (21.7%), 79 (13.8%) and 20 (3.5%) were A, B, C, D and E grades respectively.

Post thaw viability

Perusal of viability assay results revealed that out of the 100 frozen thawed GV oocytes in the T1 group, 38 (38.0%) and 62 (62.0%) oocytes were live and dead respectively. On the other hand, in the T2 group, 30 (28.6%) and 75 (71.4%) vitrified MII oocytes were live and dead respectively.

Discussion

Based on the results it was found that GV stage oocytes survive better for vitrification than the MII stage oocytes. Shaw *et al.,* (2000) also reiterated that the GV oocytes seem to be less susceptible to cryoinjury than MII oocytes, because they are slightly smaller and are still in a quiescent stage of development with the chromatin in the diplotene phase of prophase I. Oocytes at the MII stage of development have undergone several developmental paths including both nuclear and cytoplasmic maturation, extrusion of the first polar body and the arranging of the chromosomes on the MII spindle. These immature oocytes also have a longer period to recover from cryoinjury because they have to mature in vitro prior to insemination or other manipulations. MII oocytes are vulnerable to cryoinjury due to the delicate spindle they possess. The mature oocyte of most mammalian species contains a spindle that is usually arrested in the metaphase stage of the second meiotic division. This spindle is made up of microtubules connected to maternal chromosomes which are vulnerable

to CPAs and changes in temperature. Damage to the meiotic spindle may change the position of the chromosomes and thus limit the fertilization capabilities of the oocyte (Luster, 2004; Tharasanit *et al.,* 2006).

Conclusion

It was concluded that GV stage immature ovine oocytes survive better the vitrification procedure than the MII matured oocytes. However further studies should be conducted to improvise the vitrification protocol inorder to increase the cryotolerance of ovine oocytes.

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