In vitro competence of vitrified bovine oocytes with open pulled straw

D J Dutta*, B C Sarmah, Hiramoni Dev and Himangshu Raj
Department of Veterinary Physiology, Faculty of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati 781022, India

Received 2 June 2014; revised 8 July 2016; accepted 12 July 2016

In vitro developmental competences of post thaw vitrified bovine cumulus oocyte complexes (COCs) were studied. Good qualities COCs were cryopreserved using vitrification solution comprising of 15 or 20% ethylene glycol (EG) + 15% or 20% dimethyl sulfoxide (DMSO) + 0.6 M sucrose in tissue culture medium 199 (TCM199) with 10% fetal bovine serum (FBS). Immediately they were plunged in LN₂ for ultra-rapid freezing using open pulled straw. Thawing and dilution were made stepwise. Post thaw normal vitrified and non vitrified oocytes were subjected to in vitro maturation and fertilization. The performance in respect of post thaw survivality and in vitro maturation on the basis of cumulus cell expansion was more than 80%. In comparison, the non-vitrified COCs group, in vitro maturation performance was 93.12%. The in vitro fertilization performances of vitrified immature and mature post thaw oocytes were recorded as 50.65% and 56.16% with 17.95% and 19.51% of blastocysts formation, respectively. Ultra rapid freezing protocol using vitrification with open pulled straw technique i.e. 7.5 or 10% EG + 7.5 or 10% DMSO for equilibration at room temperature for 3 min and 15 or 20% EG + 15 or 20% DMSO + 0.6M sucrose as vitrification solution within 1 min, on immature bovine oocyte yielded acceptable in vitro oocyte growth and embryonic development.

Keywords: Oocyte, vitrification, in vitro maturation, in vitro fertilization

Introduction

Global developmental scenario demand conservation of biodiversity particularly endangered species. Cryopreservation of male and female germplasm has the potential to be an important adjunct to reproductive biotechnology. Important advances have been made in cryopreservation of mammalian oocytes and embryo in recent years1-3. Controlled slow freezing continues to be the most widely used technique for cryopreservation of oocytes and in vivo and in vitro produced embryos. However, in the last decade the vitrification technique, a cryoprotectants system involving the addition of higher concentrations of cryoprotectants and ultra rapid cooling4 has been tested in various species with good results5-8. Different vitrification protocols have been experimented to cryopreserved in vivo and in vitro produced embryos or oocytes. These protocols differ in many ways, including the type and concentration of the cryoprotectants, number of equilibration steps, type of cryopreservation device used, time of exposure, and number of dilution steps at warming5,6,9-12 and it is widely believed that each of these factors can affect the results. The results obtained so far have been modest and procedures need substantial improvement. Development of a reliable method for cryopreservation of oocytes is therefore relevant for assisted reproduction for conservation of female animal genetic resources. Classical freezing protocol have been replaced by the most of recent vitrification methods including several modification, such as vitrification by open pull straw method5, glass capillaries13 and micro droplet14. A poor survival rate of most of the cryopreserved oocytes is mainly due to chilling injury, intracellular ice formation15 etc.

Retrieval of higher number of competent oocytes for in vitro maturation and in vitro fertilization to obtain superior transferable bovine embryos coupled with development of freezing technique through vitrification will entail more productivity from the non descript animals. The aim of this study was to test developmental competence of immature and in vitro matured oocytes to vitrification using open pulled straw.

Materials and Methods

Oocyte Recovery

Cattle ovaries were collected from slaughter house and within 1 1/2 – 2 hours processed as per routine
standard protocol. Oocytes were aspirated from 3 to 8 mm ovarian follicles with medium containing TCM-199 and supplemented with 200 mM L-glutamine solution, 0.4% bovine serum albumin (BSA) and antibiotics. The cumulus oocyte complexes (COCs) were categorised and morphologically evaluated under stereo zoom microscope. Homogenous and compact COCs were washed four times in holding media (Modified TCM-199, 200 mM L-glutamine solution, 10% fetal bovine serum (FBS), 0.8 M sodium pyruvate and 50 μg/ml gentamicin and 50 μM cysteamine) by gentle pipetting and were subjected to cryopreservation by vitrification or *in vitro* maturation. Freshly collected COCs were used for *in vitro* maturation and kept as control.

**Vitrification and Thawing**

Two vitrification solutions were prepared in media consisting of TCM-199 with 10% FBS. Vitrification solution I (VS I) consisted of 7.5% or 10% ethylene glycol (EG) + 7.5% or 10% dimethyl sulfoxide (DMSO) and vitrification solution II (VS II) consisted of 15 or 20% EG + 15% or 20% DMSO + 0.6 M sucrose. The immature bovine oocytes with cumulus cells were exposed to VS I for equilibration up to 3 minutes followed by 25-30 seconds in VS II at room temperature (22-25°C). The oocytes in VS II were immediately loaded to an open pulled straw preloaded with 0.6 M sucrose in holding medium with air gap in between and plunged into liquid nitrogen. The open bulled straws are standard 0.25 ml straw with one extremity pulled and thinned by heating. This increases the superficies/volume rate and hastens the cooling rate of small (2 µl) drop in which the oocytes is contained. 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 kept into the medium containing 0.6 M of sucrose in basic solution for 2 minute. Then, they were transferred successively into the holding medium in step-wise dilution containing 0.3 M and 0.15 M of sucrose for one minute in each. Following rehydration, oocytes were washed three times in holding medium. Morphological integrity of post thaw vitrified oocytes was assessed under inverted phase contrast microscope. Oocytes having fragmented zona pellucida and absent cytoplasmic contents were not considered. The remaining morphologically normal post thaw oocytes were taken for *in vitro* maturation.

**In Vitro Maturation**

The fresh or post thaw vitrified normal oocytes were matured in modified TCM-199, 200 mM L-glutamine solution, 10% FBS, 0.8 M sodium pyruvate and 50 μg/ml gentamicin and 50 μM cysteamine supplemented with p-FSH (5 μg/ml), 10% v/v follicular fluid, 1 μg/ml 17-β estradiol at 38.5°C in a humidified atmosphere of 5% CO₂ for 24 hours. For confirmation of maturation after 24 hrs the oocytes were evaluated for morphological change and *in vitro* maturation performance under stereo zoom microscope. The oocytes with an intact zona pellucida, plasma membrane and homogenous cytoplasm were considered as morphologically normal in the study. *In vitro* maturation performance was assessed on the basis of expansion of cumulus cells surrounding the homogenous oocytes.

**In Vitro Fertilization**

For *in vitro* fertilization, frozen bovine semen (2 straws each) was prepared for capacitation with swim-up technique using bovine oocytes washing medium. After washing, spermatozoa were added to bovine oocyte fertilization medium to make a final concentration of 2 × 10⁶ sperms/ml. *In vitro* matured oocytes of both the vitrified and non-vitrified groups were co-incubated with spermatozoa in bovine oocytes fertilization medium at 38.5°C, 5% CO₂ in air and saturated humidity for 20 hrs. The expected zygotes were washed in *in vitro* culture (IVC) medium (viz., mCR2aa containing 5% FBS and supplemented with 2% essential amino acids (v/v), 1% non essential amino acids (v/v), 1% α-glutamic acid, 0.3% BSA and 0.05 μg/ml gentamicin sulphate). Then zygotes were placed into IVC droplets and covered with mineral oil and incubated at 38.5°C under 5% CO₂ in humidified air. After 48 hrs culture the cleavage up to (2-8 cells) were recorded. Subsequently culture was continued for further development. Embryos in each group were observed under a microscope every 24 hrs following insemination. Development to 2 cell stage was assessed at 48 hrs after insemination and subsequently cultured for 7 days to evaluate the blastocysts formation. Medium was replaced with fresh medium in every 48 hrs of culture. The study was carried out on different dates with replicates of 10 times.

**Statistical Analysis**

The data were compiled and analysed using analysis of variance (ANOVA).
Results and Discussion

A total of 474 good quality bovine COCs were the subject of the experiment. Out of which 314 COCs were subjected to vitrification and the rest were non-vitrified. The performance in respect of post thaw survivability and *in vitro* maturation on the basis of cumulus cell expansion was more than 80% respectively (Fig. 1). In comparison the non vitrified COCs group, the study demonstrated 93.12% *in vitro* maturation performances as shown in Table 1. The vitrification thus makes minimal affect on the survival rates and the ability of the oocyte to mature *in vitro* in the present study. High proportions of bovine COCs retain their post-thaw morphology after a short exposure to high concentration of permeating cryoprotectants namely EG and DMSO with sucrose using open pulled straw for freezing. Previous studies on vitrified cattle oocytes had recorded the morphological survival rates between 65 to 94%\(^\text{16,17}\). The sensitivity of bovine oocytes to cryo injury and consequent survivality has been well described with variable results. The factors primarily responsible for cell damage are the type of cryoprotectants, concentration, equilibration time, cooling and warming procedures\(^\text{18}\). The maturation rate primarily depends on quality of oocytes, media ingredients and amount and incubation environment. Cooling germinal vesicle stage bovine oocytes might have minimal effect on the nuclear maturation or fertilization. The nuclear material is membrane bound hence there is very low risk of any chromosomal damage as compared to that with dividing cells such as within an embryo or maturing oocytes. Hurtt *et al*\(^\text{19}\) compared viability of immature and mature bovine oocytes vitrified in ethylene glycol based solution and recorded 60% and 70% nuclear and cytoplasmatic maturation rate. Faster membrane penetration of ethylene glycol makes it an ideal cryoprotectant combine with non penetrating cryoprotectant like sucrose that act as a stabilizer, minimizing the affect of high concentration of ethylene glycol\(^\text{20}\).

The subsequent *in vitro* fertilization performance of vitrified immature and mature post thaw oocytes in the present study were recorded as 50.65% and 56.16% with 17.95% and 19.51% of blastocyst formation respectively (Table 2) (Fig. 2, 3 & 4). The results are in agreement with the findings of Vajta *et al*\(^\text{5}\) and Papis *et al*\(^\text{21}\) using vitrified matured bovine oocytes. The improved success rate was attributed to the increased cooling rate during oocyte vitrification and step wise dilution method. Reports have been found that after thawing using the four step dilution methods with 2.5 minutes interval

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of COCs</th>
<th>Post thaw survivability / recovery performance</th>
<th>Maturation performance on the basis of cumulus expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percent</td>
<td><em>In vitro</em> maturation</td>
</tr>
<tr>
<td>Control</td>
<td>160</td>
<td>160</td>
<td>100</td>
</tr>
<tr>
<td>VS I (7.5% &amp; 15%)</td>
<td>158</td>
<td>146</td>
<td>92.40</td>
</tr>
<tr>
<td>VS II (10% &amp; 20%)</td>
<td>156</td>
<td>144</td>
<td>92.31</td>
</tr>
</tbody>
</table>

Values in maturation performance column with different superscripts differ significantly (P < 0.05)

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of mature oocytes</th>
<th>In <em>in vitro</em> fertilization performance</th>
<th>Cleavage performance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percent</td>
<td>2-4 cells (%)</td>
</tr>
<tr>
<td>Control</td>
<td>76</td>
<td>48</td>
<td>63.16(^a)</td>
</tr>
<tr>
<td>Vitrified immature (VS I)</td>
<td>77</td>
<td>39</td>
<td>50.65(^c)</td>
</tr>
<tr>
<td>Matured vitrified (VS I)</td>
<td>73</td>
<td>41</td>
<td>56.16(^b)</td>
</tr>
</tbody>
</table>

Values in *in vitro* fertilization column with different superscripts differ significantly (P < 0.05)
the vitrified oocytes developed to the two cells (71-100%), four cells (71-93%), eight cells (46-71%) and blastocyst (23-36%) stages. Step wise dilution might be helpful to reduce osmotic injury of vitrified oocytes after thawing. The cryopreservation capacity of oocytes at the germinal vesicle stage is lower as compared to metaphase I through metaphase II stages of oocytes have been well documented. However, notably well adapted vitrification program could lessen the differences in the capacity of in vitro development of different maturational stages of oocytes following cryopreservation. Vitrification is an attractive alternative to slow freezing methods. It requires both ultra rapid cooling rates and very high cryoprotectant concentrations to prevent ice crystal formation and to increase viscosity at low temperatures. The strategy that has had the greatest impact on the advancement of vitrification is that of increasing the cooling rate to > 20 000°C/minute to obtain vitrification with the lowest possible concentration of cryoprotectants. Such high rates of cooling are achieved by suspending the oocyte in a tiny volume (often < 0.1 μl) of the vitrification solution, then placing the sample in direct contact with liquid nitrogen at -196°C. Vitrified samples are warmed rapidly to avoid intracellular crystallisation, although the effect of warming rates on survival remains to be examined systematically. Oocyte vitrification in tiny volumes of solution in open containers has safety implications that must be addressed.

**Conclusion**

Vitrification with open pulled straw procedures i.e. using 7.5% ethylene glycol and dimethyl sulfoxide (DMSO) for equilibration and 15% EG + 15% DMSO + 0.6 M sucrose as vitrification solution, on immature bovine oocyte yielded acceptable in vitro oocyte growth and development competence.

**Acknowledgement**

Authors are thankful to Department of Biotechnology (DBT), Government of India for their financial support towards smooth running of the research.

**References**

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