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Vitrification of Germinal Vesicle Stage Oocytes

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Summary

In order to cryopreserve germinal vesicle (GV) stage oocytes, we first need to develop a novel container for keeping large quantities of GV oocytes, because of collecting them as cumulus oocytes complexes (COCs) that have bigger size and larger volume than oocytes themselves, and second modify a protocol for optimizing vitrification of them. In this mini-review, we describe our recent progress for attaining these objectives. When 65 bovine COCs having GV oocytes could be placed on a sheet of nylon mesh, and plunged directly into liquid nitrogen for vitrification, the recovery rate was significantly higher compared with that in 15 ones on the electron microscope (EM) grid as a control, followed by obtaining the resultant cleavage and developmental rates after in vitro fertilization and culture (IVFC) without significant difference. Using bovine and murine oocytes, we found that a step-wise manner to expose them with the vitrification solution increased rates of in vitro maturation, subsequent development to blastocysts and hatching/hatched blastocysts after IVFC. Our results show that nylon mesh is an alternative material for cryopreserving large quantities of bovine GV oocytes, and that a step-wise exposure to cryoprotectants may have befit for decreasing disadvantage during vitrification.

Cryopreservation of mammalian female gametes can be applied to various processes in a wide range of species, i.e., for breeding and reproduction of farm animals, preservation of genetic variants in laboratory animals, conservation of wild species and treatment of infertility in humans.

The first successful cryopreservation of embryos was performed by a slow cooling method that performed ice seeding at a few degrees below the freezing point, followed by cooling the samples very slowly (0.3-0.4°C/min) to −80°C before storage in liquid nitrogen. Following this success, the slow cooling method was shown to be effective for embryos of several species, but has some disadvan-

tages in that it needs time as long as 2–3 h for cooling, and that special equipment and liquid nitrogen are required to control the cooling rate. In 1985, when many researchers were making efforts to simplify the cryopreservation method, Rall and Fahy devised an extremely rapid method called as vitrification, by which 8-cell murine embryos were plunged directly into liquid nitrogen from a temperature above 0°C, thus taking only a few seconds for cooling (Rall and Fahy, 1985). Since then, vitrification has been widely used, and is now regarded as a potential alternative to traditional slow-rate freezing.

Recent in vitro progress in utilization of ovaries derived from an abattoir, on the other hand, seems to make the storage of oocytes clinically important as it would allow IVF groups to store unfertilized eggs rather than fertilized embryos. Oocytes, however, are sensitive to low temperature, and it is difficult to cryopreserve them compared with embryos. In fact, although procedures for long-term storage of cryopreserved metaphase II (MII) oocytes have been reported (Martino *et al.*, 1996), these results have been regarded as unsatisfactory. In MII oocytes, microtubular spindles, to which chromosomes can attach, are highly sensitive to temperature changes (Aman and Parks, 1994), and hence chromatid disjunctions may occur during cooling, resulting in aneuploidy after fertilization (Eroglu *et al.*, 1998). Since oocytes in the GV stage do not have any microtubular spindles (Wu *et al.*, 1999), cryopreservation of GV oocytes may be an alternative approach to the storage of female gametes.

For successful freezing of cells, the most important thing is the total elimination of ice crystal injury inside of the cytoplasm. When vitrification is applied to cryopreserve embryos, it is expected that ice crystal formation would be expectedly prevented by use of high concentrations of cryoprotectants that can draw water out of the cytoplasm via high rates of cooling and warming rates. From the first trials until the present, many modifications in the process of vitrification have been tested, especially paying attention to the fact that the solution of cryoprotectants may have toxic effects resulting in irreversible damage to the cytoskeletal organization of the cells (Rall, 1987). Thus, the main objective of the different approaches has been to minimize toxic, osmotic and other injuries to cells.

In this mini-review, we will introduce our recent protocols to vitrify GV stage oocytes in bovine and mice.

1. Development of Container for Vitrification of Large Quantities of Oocytes

The cooling rate is an important parameter for the success of vitrification. The original vitrification method, which was developed to cryopreserve 8-cell murine embryos (Rall and Fahy, 1985), used a standard straw for holding the

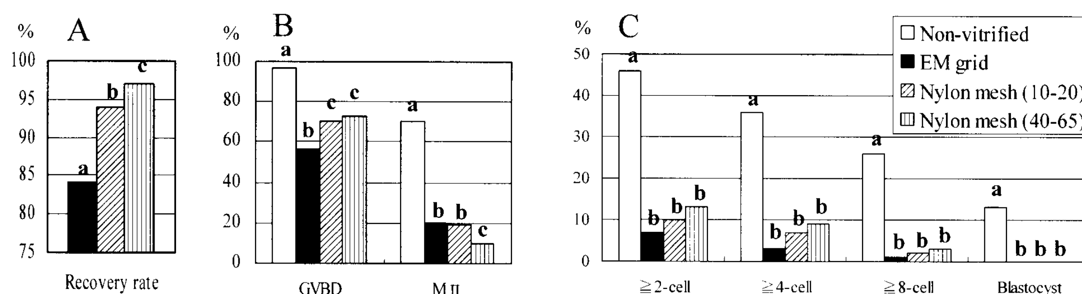

FIG. 1. The rates of normal oocyte recovery for vitrified COCs (A), in vitro maturation (B), and development after in vitro fertilization (C) for bovine COCs after vitrification and warming. [a–c]Values with different superscripts within each parameter differ significantly ($p<0.05$).

embryos during cooling, storage, and warming, although the use of the straw imposed limitations of the maximum cooling and warming rates to less than 2000°C/min. To increase cooling and warming rates, alternative methods that allow direct contact between the medium containing the embryo and liquid nitrogen have been developed using either EM grids (Martino *et al.*, 1996) or cryoloops (Lane *et al.*, 1999), each of which results in improved survival rates after vitrification. Recently, we have demonstrated that a sheet of nylon mesh (pore size 60 μm) can be useful for vitrifing large quantities of bovine GV oocytes. Usually, bovine GV oocytes are collected as COCs, so that their size is larger than the size of an oocyte only, and consequently cryoloops are not applicable for their vitrification. Since EM grids can be used for larger numbers of embryos compared with other unsealed container systems (Martino *et al.*, 1996), we compared EM grids and nylon mesh (Matsumoto *et al.*, 2001). A thin bar grid T400H mesh (pore size 55 μm) was used as a physical support EM grid. Bovine COCs were exposed to a vitrification solution, EFS40, which consisted of 40% (v/v) ethylene glycol, 18% (w/v) Ficoll-70 and 0.3 M sucrose in PB1, loaded either on a sheet of nylon mesh or on an EM grid, and then plunged directly into liquid N_2. The number of the COCs loaded was 10–20 or 40–65 per nylon mesh, and 10–15 per grid. Over 90 percentage of the oocytes vitrified on the nylon mesh were recovered which was higher than the recovering of those on the EM grid (84.2%) ($p<0.05$) (Fig. 1). A higher recovery rate after vitrification on the nylon mesh was obtained for 40–65 COCs (97.4%) than in the 10–20 COCs (94.3%) ($p<0.05$). After in vitro maturation of vitrified COCs, the rate of germinal vesicle breakdown (GVBD) was higher in the group using the nylon mesh than in that using the EM grid ($p<0.05$). The developmental rates beyond 2- and to 8-cell stages were not significantly different between the oocytes vitrified on the nylon mesh and on the EM grid. Although only the maturation rate to MII in the 40–65 COCs vitrified on the nylon mesh was lower than that in those vitrified on the EM grid ($p<0.05$), neither cleavage nor developmental rate was significantly different

between them. Considering that recent progress in assisted reproductive technology, cloning, stem cell biology and so on has been achieved, a large number of oocytes will be required. Therefore, when GV oocytes are obtained as COCs, our method to vitrify GV oocytes using nylon mesh would be helpful in facilitating application of these technologies.

2. Step-wise Exposure to Cryoprotectants

Vitrification is a physical process in which cryoprotectants (the vitrification solution) form a glassy solid, so that the vitrification solution must be sufficiently concentrated to avoid crystallization when cooled but must not produce chemical toxicity or osmotic injury during equilibration or dilution (Rall and Fahy, 1985). When this approach is applied to cryopreserve oocytes and embryos, their viability and developmental ability should be considered as the functional aim. In this regard, step-wise addition of cryoprotectants may be considered to minimize damage due to extreme cell volume expansion (Rall, 1987). In addition, during removal of cryoprotectants, the water influx may cause extreme swelling and subsequent damage of cellular membranes. For preequilibrium before vitrification, murine GV oocytes were treated as COCs using four different manners of exposure to the vitrification solution in six experimental groups (Aono *et al.*, 2003). The COCs were transferred to the vitrification solution, which was composed of 15% ethylene glycol, 15% dimethyl sulfoxide and 0.5 M sucrose, either in a by single step manner (non-preequilibrium) or in a step-wise manner (single-, two- or ten-step preequilibrium). After an ultrarapid vitrification and storage in liquid nitrogen, the COCs were warmed, washed by diluting the

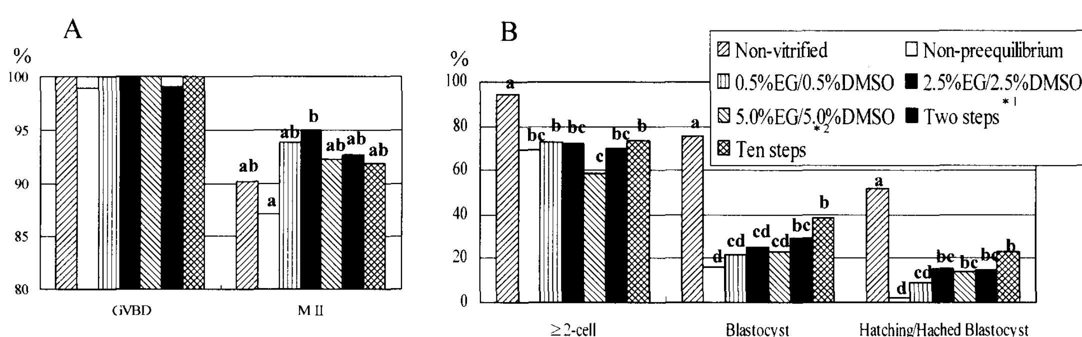

FIG. 2. The rates of in vitro maturation (A), and development after in vitro fertilization (B) for murine COCs after vitrification and warming. *[1]The GV oocytes were treated with 0.5% EG/0.5% DMSO followed by 2.5% EG/2.5% DMSO for 5 min, respectively. *[2]The GV oocytes were treated in a step-wise manner with increasing concentration from 0.25 to 2.5% for 1 min, respectively. [a–d]Values with different superscripts within each parameter differ significantly ($p<0.05$).

vitrification solution in five steps, and then subjected to *in vitro* maturation, fertilization and culture. Higher rates of survival and maturation to MII were obtained and followed by efficient production of blastocysts when the oocytes were vitrified with an increased frequency of preequilibration with the vitrification solution (Fig. 2). These results suggest that the process of gradual equilibration with the vitrification solution seems to adjust the permeability of cellular membranes, so that the connection between the oocytes and cumulus cells in COCs may be sufficiently maintained, and that rapid changes in osmotic pressure may be decreased or avoided.

Conclusions

In this review, we introduced our vitrification method using a sheet of nylon mesh as an innovative container for easily handling large numbers of bovine GV oocytes as COCS. In addition, vitrification performed using step-wise equilibration was shown to improve the viability and production of blastocysts after cryopreservation of murine GV oocytes. Our protocol using a nylon mesh and step-wise equilibration provides a simple and inexpensive method for vitrification (Abe *et al.*, 2003). The EM grid can be used for vitrification of bovine COCs (Martino *et al.*, 1996), bovine blastocysts (Park *et al.*, 1999) and human zygotes (Park *et al.*, 2000), and we hope that the nylon mesh will also be applicable to many types of mammalian oocytes and embryos.

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