Effects of cryoprotectants and cryopreservation on germinal vesicle-stage cumulus–oocyte complexes of rhesus monkeys

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Objective: To determine the effect on rhesus germinal vesicle-stage oocytes enclosed within cumulus cells (COCs) of cryopreservation either by slow, equilibrium cooling or by rapid, non-equilibrium cooling.
Design: Experimental study.
Setting: University primate research center.
Subject(s): Twelve female rhesus monkeys.
Intervention(s): Monkeys were stimulated with recombinant FSH, and COCs were aspirated from follicles by an ultrasound-guided procedure.
Main Outcome Measure(s): Rhesus COCs were examined by confocal microscopy to evaluate integrity of microtubules and intactness of transzonal processes between cumulus cells and the oocytes.
Result(s): Exposure to 1.5 mol/L propylene glycol + 0.3 mol/L sucrose caused disruption of microtubules in all but 1 of 24 COCs and of transzonal processes in more than half of the COCs. Cryopreservation of 11 COCs by slow freezing disrupted microtubules and transzonal processes in all of them. Exposure alone to 2.7 mol/L ethylene glycol + 2.2 mol/L dimethylsulfoxide + 0.5 mol/L sucrose caused disruption of microtubules and transzonal processes in 7 of 19 COCs. Cryopreservation of COCs by rapid, non-equilibrium cooling caused disruption of microtubules and transzonal processes in 14 of 20 complexes.
Conclusion(s): Maturation of rhesus COCs at the germinal vesicle stage may be seriously impaired because intracytoplasmic microtubules and transzonal processes are likely to be irreversibly damaged by cryopreservation. (Fertil Steril® 2008;90:805–16. ©2008 by American Society for Reproductive Medicine.)

Key Words: Cumulus–oocyte complexes, rhesus monkeys, cryoprotectants, cryopreservation

If oocytes collected from rhesus monkeys during the breeding season can be successfully cryopreserved, they could be used for research throughout the year. However, as is true for oocytes of other species, oocytes collected from hormonally stimulated female monkeys vary in their state of maturation. The relevance of this is that mammalian oocytes are extremely sensitive to cryopreservation (1), and the meiotic status of oocytes influences their sensitivity to cryopreservation and their ultimate development (2, 3).

Despite this sensitivity, oocytes at the metaphase II (MII) stage of development of mice and many other species, other than those of rhesus monkeys, have been successfully cryopreserved. Success in this case means that the oocytes were fertilized and the resultant zygotes developed into live young (see the first experimental reports and recent reviews [4–15]).

More than 25 years ago, the sensitivity of oocytes to cryopreservation was partially explained by the observation that, when ovulated oocytes were cooled to 0°C, their microtubules underwent depolymerization and their microtubule organizing centers became dispersed (16). Since then, analogous observations have been reported for oocytes of many species, including those of mice (17–19), cattle (20, 21), and humans (22, 23). Even cooling from approximately 37°C just to room temperature may have a deleterious effect on development after fertilization of oocytes of sheep (24) and humans (25). Recently, we showed that the organization of tubulin and chromosomes of rhesus oocytes may be similarly damaged when the oocytes are cooled to 0°C (26). Our observations complemented an earlier study of the cryobiology of macaque oocytes, in which the effect of glycerol and cooling on microfilaments and transzonal processes (TZPs) were examined (27).

The sensitivity of the meiotic spindle and microtubules to cooling and chilling renders MII-stage oocytes difficult to

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cryopreserve. Because cryopreservation methods that use slow, controlled-rate cooling inevitably expose oocytes to damaging temperatures near 0°C for several minutes, it has been suggested that non-equilibrium cooling at very high rates may circumvent this problem of microtubule sensitivity to chilling (28, 29). In principle, rapid cooling methods may be more effective because oocytes would be cooled through the damaging range of temperatures (+10° to −10°C) so rapidly that there would be insufficient time for microtubules to undergo depolymerization before the entire system solidified at low subzero temperatures at which further changes could not occur. Such rapid cooling methods may cause oocytes and the medium in which they are suspended to vitrify, a method first described for embryo cryopreservation by Rall and Fahy (30, 31). It has also been hypothesized that germinal vesicle (GV)-stage oocytes may be easier to cryopreserve because a meiotic spindle has not yet formed within them (32–35). In fact, rhesus oocytes at the GV stage seem to tolerate chilling to 0°C, and some of them undergo maturation after chilling; these can be fertilized and will develop into blastocysts (36).

However, reports of live young produced by fertilization of cryopreserved GV-stage oocytes are rare (37, 38). Physiological and biophysical differences between oocytes at the GV stage and the MII stage may require that cryopreservation methods be adapted to take account of these differences (2, 28, 33, 39, 40). For example, oocytes at the MII stage do not rely on support from surrounding cumulus cells, and microtubules that are present within the cytoplasm are mainly associated with the meiotic spindle. In contrast, during maturation GV oocytes require communication with cumulus cells through microfilament-rich TZPs so as to develop to the MII stage, and microtubules form an extensive network throughout the ooplasm of GV oocytes (41–44).

For GV oocytes to undergo maturation into MII oocytes after vitrification, TZPs by which cumulus cells and the oocyte itself communicate must remain intact after the oocyte is exposed to the high concentrations of cryoprotectants required for vitrification. Furthermore, the cytoskeleton of microtubules within the ooplasm itself of GV oocytes must also withstand all of the several steps of cryopreservation. Recently, some of the causes responsible for the failure of rhesus oocytes to undergo maturation have been identified (45); and methods to induce in-vitro maturation of oocytes have been improved, making the capability to store immature oocytes by cryopreservation increasingly valuable. For that reason, we have begun to investigate some aspects of cryopreservation of rhesus GV-stage oocytes. In a preliminary study, we found that, although rhesus GV oocytes would tolerate chilling and exposure to ethylene glycol (EG) used as a cryoprotective additive (CPA), they were damaged or killed if frozen at −7° or −35°C . Those results suggested that rhesus oocytes may be less susceptible to damage by chilling or exposure to a CPA but are nevertheless injured when the suspending medium itself undergoes a phase change upon freezing.

The research described here is part of our continuing study to derive a method to cryopreserve rhesus oocytes. In this study, to determine the possible efficacy for rhesus oocytes, we examined two methods (slow, equilibrium cooling; rapid, non-equilibrium cooling; for definitions see Leibo [46] and Mazur [47]) that have been used successfully to cryopreserve mature human oocytes. Using a method commonly used to freeze human oocytes (6, 48, 49) but with one important difference, we evaluated the effects of "slow freezing" on rhesus oocytes suspended in propylene glycol plus sucrose. Most slow-cooling methods described in the literature for human oocytes specify that the oocytes are first incubated in CPAs at room temperature. However, as discussed by Bernard and Fuller (50), it has been known since 1980 that exposing oocytes to temperatures near 20°C for 30 minutes causes depolymerization of microtubules. To avoid that deleterious effect, we exposed the oocytes to the CPAs at 37°C. We also studied the effects of vitrifying oocytes by very rapid, non-equilibrium cooling in a mixture of dimethylsulfoxide (DMSO) plus EG plus sucrose (51, 52). The effects of these various treatments on rhesus cumulus–oocyte complexes (COCs) were evaluated by confocal microscopy to examine the morphology of microtubules and TZPs of the oocytes.

MATERIALS AND METHODS

Oocyte Recovery

Adult female rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center. Animal protocols were reviewed and approved in advance by the Animal Care and Use Committee of the University of California, Davis; all studies were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. After onset of menstruation, 12 adult female rhesus monkeys (age range 3.8–15.6 years) were hormonally stimulated by one of two stimulation protocols. Nine animals were stimulated by twice-daily injections of 37.5 IU (75 IU total per day) recombinant human FSH (Ares-Serono, Randolph, MA; or Organon, West Orange, NJ) for 7 days. Three animals were stimulated by twice-daily injections of 37.5 IU recombinant cynomolgus monkey FSH for 7 days. Preliminary studies revealed no significant differences in the effectiveness of the two FSH preparations. In other experiments, oocytes aspirated from females stimulated with either hormone preparation have been successfully fertilized by in vitro fertilization. Oocytes were aspirated from follicles the morning after the last dose of recombinant FSH by an ultrasound-guided procedure (53). Aspirates containing oocytes were maintained at 35°C within a temperature-controlled isolette at all times during experiments. Aspirates were transferred onto a 24-mm diameter, 70-μm pore-size filter (Netwell Inserts 3479; Corning, Acton, MA), and blood cells were rinsed away with fresh Tyrode's lactate (TL) medium + *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer + 0.1 mg/mL polyvinyl alcohol (PVA) medium (TL-HEPES-PVA), and the COCs were recovered