Single human sperm cryopreservation method using hollow-core agarose capsules

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Objective: To develop an efficient cryopreservation method using a single sperm.
Design: Experimental study.
Setting: Laboratory of a private institute.
Patient(s): A fertile donor.
Intervention(s): We produced hollow-core capsules with agarose walls. A single human sperm was injected into each capsule as per the conventional intracytoplasmic sperm injection (ICSI) method. The capsules that contained the spermatozoa were cryopreserved on polycarbonate or nylon mesh sheets using nitrogen vapor. Before their use, the capsules were thawed and recovered. The motile spermatozoa in the capsules were counted.
Main Outcome Measure(s): The recovery rates of the agarose capsules and the spermatozoa in these capsules after thawing and the mortality and survival rates of the spermatozoa.
Result(s): The recovery rates of the capsules were 91.5% (75/82) using polycarbonate sheets (PS) and 98.3% (59/60) using mesh sheets (MS) after thawing. The recovered capsules were not at all damaged. The recovery rates of the spermatozoa were 91.5% (75/82) using PS and 96.7% (58/60) using MS. Sperm motility rates were 85.3% (64/75) and 82.8% (48/58), whereas the survival rates of the immotile spermatozoa by the hypoosmotic swelling test were 81.8% (9/11) and 50.0% (5/10); furthermore, the total survival rates of the spermatozoa were 97.3% (73/75) and 91.4% (53/58) using PS and MS, respectively. There was no significant difference between the results obtained using PS and MS.
Conclusion(s): A cryopreservation method for a single sperm using an agarose capsule has been developed. The method is expected to be useful in ICSI treatment in patients with few spermatozoa. (Fertil Steril® 2015;104:1004–9. ©2015 by American Society for Reproductive Medicine.)
Key Words: Agarose capsule, a single human sperm, cryopreservation, polycarbonate sheets, nylon mesh sheets

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With the development of the testicular sperm extraction (TESE) technique, it became possible to treat azoospermic patients with the absence of spermatozoa in the semen. Particularly, performing the micro-TESE for patients with nonobstructive azoospermia (NOA) has a high recovery rate compared with performing the conventional TESE, and it has contributed in enabling patients with NOA to conceive babies (1, 2). However, sperm recovery rates are still 32%–63%, with frequent instances of no sperm recovery (3). In many cases, if spermatozoa were found in the testicular tissue, the cryopreserved suspension would include spermatozoa and other cells. When the oocytes were collected, the tissues were thawed and used for the intracytoplasmic sperm injection (ICSI). However, it is difficult to find spermatozoa in the suspension if it is comprised of only a few. Therefore, it is necessary to develop a method to cryopreserve a few spermatozoa and to not lose them. Previous attempts to achieve this have been made.

It has been reported that it was possible to cryopreserve a few spermatozoa in the empty zona pellucida (ZP) of human or mouse oocytes (4). Healthy children were produced using this method (5). There were some reports

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about the use of the ZP from several mammalian species to store human spermatozoa. However, the ZP method had some issues. The number of empty human zona was limited because it is desirable to use eggs of the patient's partner, and there was a concern about the safety of using heterogeneous oocytes such as mouse, hamster, or others.

Various containers have recently been used to preserve a few spermatozoa. For example, spermatozoa were introduced in a drop on a culture dish surface and frozen (6). Some investigators have reported that spermatozoa have been cryopreserved in a very small drop on a tip of CryoLoop or CryoTop. These devices had been developed to freeze embryos (7–9). However, it is difficult to find all spermatozoa immediately after thawing using these methods because the spermatozoa are very small compared with embryos. Therefore, we attempted to produce hollow-core capsules with agarose walls, which were similar in size to mammalian oocytes, and to cryopreserve a single sperm injected in an agarose capsule.

MATERIALS AND METHODS

Reagents

The HFF99 culture medium and HEPES-HFF99 culture medium were purchased from Fuso Pharmaceutical Industries, Ltd. OptiPrep was purchased from Cosmo Bio Co., Ltd. The other reagents were purchased from Wako Chemical Industries, Ltd.

Preparation of Human Sperm Samples

The semen samples were obtained from a fertile donor who gave his informed consent. The semen was mixed in equal amounts with the HEPES-HFF99 medium that contained 12% glycerol and 0.1% methylcellulose. The mixtures were put into 2.0-mL tubes, frozen in liquid nitrogen (LN_2) vapor, and stored in LN_2. The stored semen samples were thawed in warm water at 40°C by shaking (10). Spermatozoa were separated from the seminal plasma and cryoreagent using the density gradient centrifugation technique (11). Briefly, OptiPrep at concentrations of 16% and 24% (vol/vol) was prepared into HEPES-HFF99. To prepare the gradient for sperm purification, 1 mL of 16% OptiPrep was pipetted into the bottom of the centrifuge tube and 1 mL of 24% OptiPrep was carefully layered under the bottom fraction. The semen samples were added over the layer and centrifuged at 710 × *g* for 20 minutes. The resulting pellets were washed once in 3 mL of HFF99 supplemented with 0.3% human serum albumin (HSA) and centrifuged at 410 × *g* for an additional 5 minutes. Finally, the motile spermatozoa were collected using the swim-up method. The study was approved by the Ethics Committee of our institute.

Production of Agarose Capsules

Agarose capsules were produced using the methods noted in previous reports with some modifications (12–14). Briefly, 0.5% (wt/vol) calcium carbonate was dispersed in 4% (wt/vol) alginate acid water. The solution was mixed with mineral oil containing 3% (vol/vol) lecithin and 0.5% (vol/vol) acetic acid. Small spheres were formed in the mixture. The calcium carbonate in the alginate acid solution was dissolved by acetic acid in mineral oil that reacted with alginate acid. Consequently, the spheres developed into gel beads. The beads were recovered and washed with pure water and transferred into a 2% (wt/vol) agarose solution. The agarose solution containing the alginate acid beads was mixed with mineral oil again, and gel spheres were formed by cooling the solution on ice. Finally, the alginate acid gel in the agarose gel beads was dissolved in 50 mM of sodium citrate solution. The small beads were collected through a nylon mesh sheet. The capsules had hollow-core structures with an agarose wall (outer diameters, 80–120 μm; inner diameters, 60–100 μm).

Sperm Cryopreservation by Agarose Capsules

We used conventional ICSI equipment for injecting the spermatozoa into the capsules with a sharp glass needle pipette. Several agarose capsules were immersed in a HEPES-HFF99 culture medium that contained 0.3% HSA. Motile spermatozoa were aspirated into an injection pipette under observation by an inverted microscope (IX-70, Olympus), and a single sperm was inserted into the agarose capsule in the same way as that done during injecting oocytes by conventional ICSI (Fig. 1A and B). The capsules were transferred into a drop of cryoprotectant solution that was mixed with HEPES-HFF99, which contained 6% glycerol with 0.05% methylcellulose. These were mixed by pipetting. After completely replacing the solution by transferring some drops, the cryoprotectant solution that contained the capsules was placed on the tip of each of the two devices.

Two types of devices were prepared. One was a polycarbonate sheet (PS) that was 0.1 mm thick and cut 0.8–1.0 mm wide and 8–10 mm long. The sheet was fixed on a plastic straw for easier handling (Fig. 2A). The other device was made of a nylon mesh sheet (MS) with a hole in one side measuring 60 μm. It was also cut and fixed like the PS (Fig. 2D). The capsules were placed on each device sheet with 0.25–0.5 μL of cryoprotectant solution (Fig. 2B,C,E,F). In the case of the MS, the solution volume could be adjusted by aspiration and ejection from the side of the MS opposite to the one with capsules.

For freezing, a special tool was made using styrene foam. It had a square 2.5-cm hole on the bottom of a styrene foam box (Fig. 2G). The box was inverted and floated on LN_2 into another larger styrene foam box. Before freezing, the temperature in the box was increased by breathing. When the temperature at the center of the hole was just 0°C by a digital thermometer (SK-1100, Sato Keiryoki), the sheet with the capsules was laid immediately on the floating boat with the tip of the sheet located over the hole (Fig. 2H). The solution was gently frozen with the LN_2 vapor and the sheet was immersed into LN_2 after 10–30 seconds.

Sperm Thawing

A 20-μL drop of HEPES-HFF99 medium that contained 0.3% HSA was prepared on a bottom of a dish and covered with

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