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.

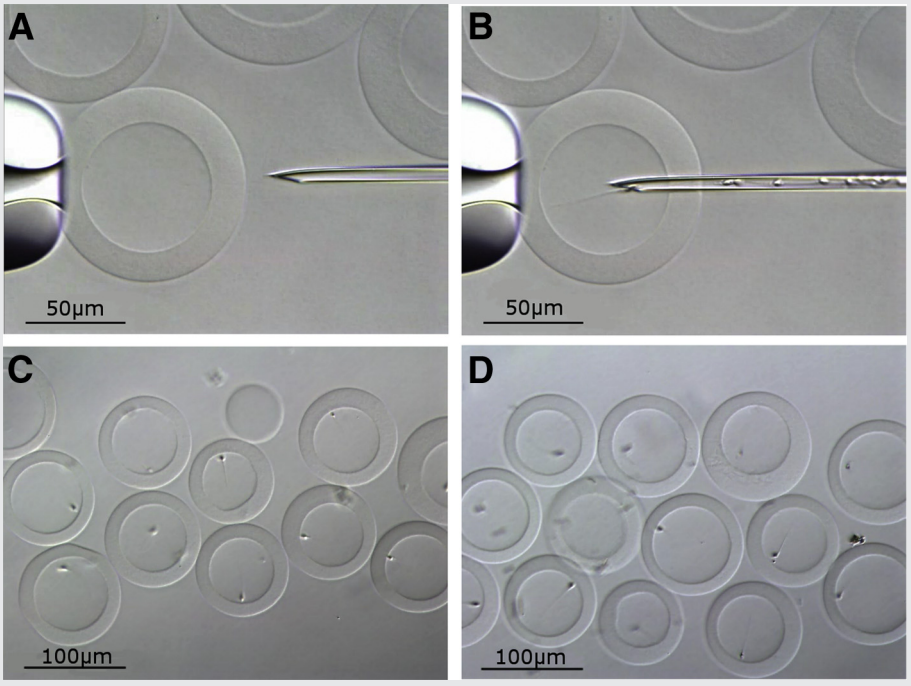
Two types of devices were prepared. One was a polycarbonate sheet (PS) that was 0.1 mm thick and cut 0.8–1.0 mm wide and 8–10 mm long. The sheet was fixed on a plastic straw for easier handling (Fig. 2A). The other device was made of a nylon mesh sheet (MS) with a hole in one side measuring 60 μm. It 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

FIGURE 1



Sperm injection into an agarose capsule. (**A**) Agarose capsule held by a holding pipette before injection. (**B**) Injection pipette inserted into an agarose capsule. A single sperm was injected into the capsule. (**C**) Agarose capsules containing a sperm before freezing. (**D**) Agarose capsules after thawing.

Araki. Single sperm cryopreservation using capsule. Fertil Steril 2015.

mineral oil. The top of the sheet was pulled out from the LN_2 and immersed immediately into the prewarmed mineral oil at 37°C. After thawing, the sheet was transferred onto the drop of the medium and held there for several seconds to separate the capsules from the sheet. Sperm motility in the capsules was checked by an inverted microscope. The motility was classified as progressively motile, nonprogressively motile, or immotile. Progressively meant that there was forward movement of the sperm and nonprogressively meant a barely motile sperm. The survival of the immotile sperm was investigated using the hypoosmotic swelling test. Briefly, the capsules that contained the immotile sperm were transferred into a hypotonic solution of HEPES-HFF99 medium that was mixed with the same volume of pure water. The survival of spermatozoa was determined if their tail swelled.

Statistics

The obtained data were analyzed by the χ^2 test. A P value of $<.05$ was considered statistically significant.

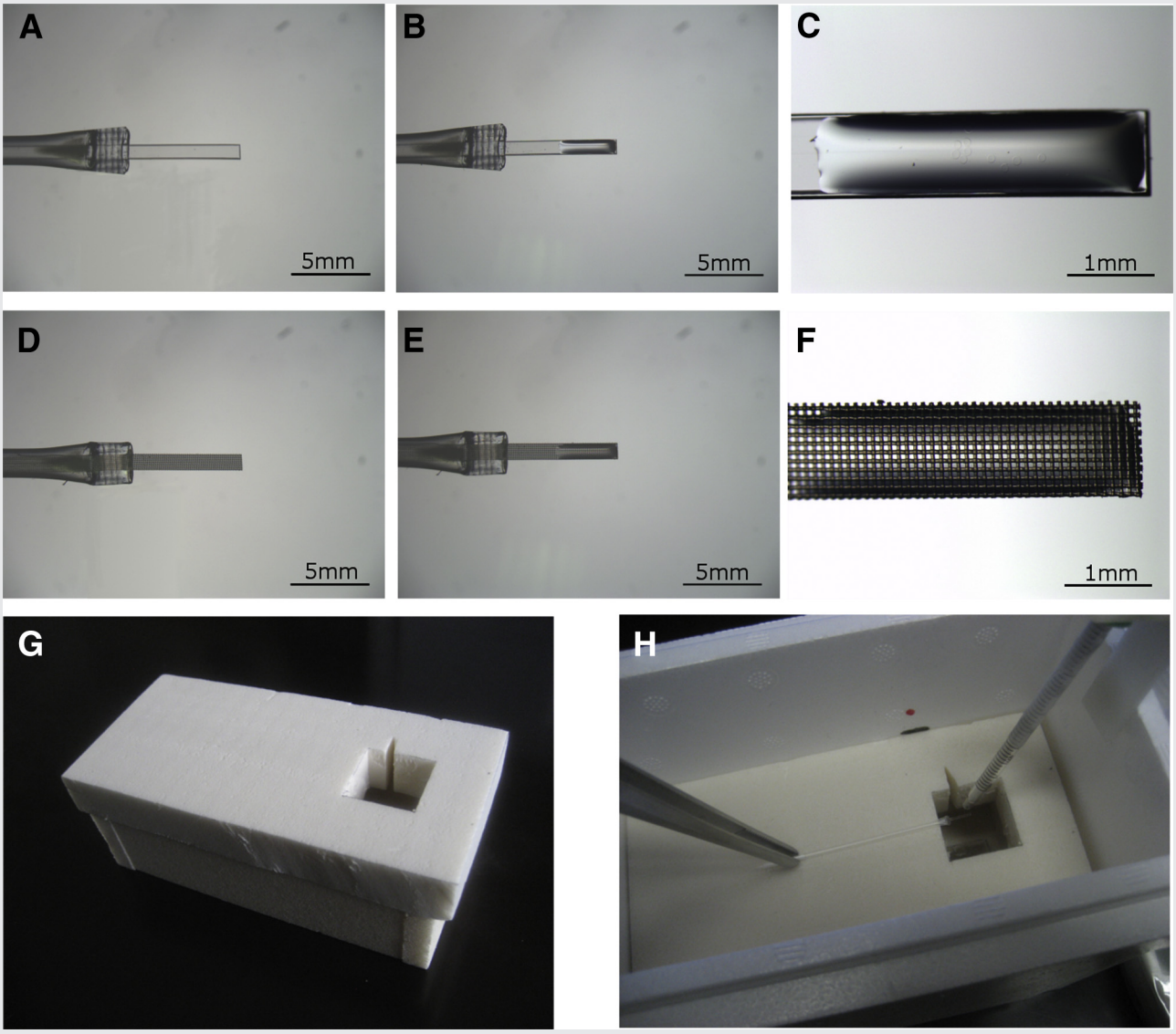
RESULTS

After thawing, the capsules fell to the bottom of a dish in the drop of the medium. The collection of the capsules was relatively easy because we did not have to look for spermatozoa specifically but for capsules of the size of the mammalian oocyte. The recovery rate of capsules was 91.5% (75/82) using PS (Table 1). Spermatozoa were never lost from the capsules; therefore, the recovery rate of the spermatozoa was the same 91.5% (75/82). Using MS, the recovery rates of capsules and spermatozoa were 98.3% (59/60) and 96.7% (58/60), respectively. Sperm motility rates were 85.3% (64/75) using PS and 82.8% (48/58) using MS just after thawing. The rates of progressive spermatozoa were 78.1% (50/64) and 77.1% (37/48). Immortality was observed in 14.7% (11/75) spermatozoa using PS and 17.2% (10/58) spermatozoa using MS. However, the rates of sperm with cell membrane survival were 81.8% (9/11) and 50.0% (5/10) by the hypoosmotic swelling test. Finally, the total survival rates containing motile and hypoosmotic swelling test-positive spermatozoa were 97.3% (73/75) in the PS group and 91.4% (53/58) in the MS group. The results were not significantly different between the PS and the MS group. No damaged capsules were observed after thawing in both groups.

DISCUSSION

The ICSI has been theoretically used to fertilize an oocyte with only one sperm (15). It has been possible for patients who have a few spermatozoa present in the testes to conceive healthy babies with the development of the TESE technique (16, 17). The sperm samples obtained from the testes were cryopreserved until an oocyte was available for the TESE treatment. Sperm may not always be collected by TESE and this avoids unnecessary surgery in women. However, it is possible for no sperm to be

FIGURE 2



Devices for freezing capsules. (**A**–**C**) A polycarbonate sheet fixed on plastic straw. (**D**–**F**) A mesh sheet fixed on plastic straw. (**B**, **C**, **E**, **F**) Cryoprotectant solution contained capsules on the top of the sheets. (**G**) The special tool was made using a styrene foam board. It had a square 2.5-cm hole on the top of a styrene foam box. (**H**) The box floated on liquid nitrogen vapor within a bigger box for freezing the capsules.

Araki. Single sperm cryopreservation using capsule. Fertil Steril 2015.

present after thawing in patients who have only a few spermatozoa, particularly in NOA. Even if spermatozoa are found after thawing, we often find that the spermatozoa are immotile. In this situation, it is impossible to know whether the immotile sperm would survive before freezing. It has been reported that the fertilization rate was very low with the injection of immotile sperm (18) obtained from the testes after freezing. Therefore, TESE is performed simultaneously with oocyte pickup. Another method is useful when the oocytes are recovered and cryopreserved in advance, and if sperm can be recovered, the oocytes were thawed and used for ICSI. However, sperm freezing is preferred to avoid unnecessary oocyte handling and freezing because it is physically and economically challenging for the patient, considering the low recovery rates even with micro-TESE (3).

Therefore, the development of a method to cryopreserve a few spermatozoa has become important. At present there have been few reports of babies born using several sperm cryopreservation methods (19). This fact suggests that the present methods of small sperm sample preservation are not ideal. One of the problems is the loss of spermatozoa after thawing. In a previous report with a high recovery rate (20), the investigators cryopreserved sperm in the ZP after using an irradiating laser on their tails. They described a recovery rate of 92%. In the present study, it was easier to obtain the sperm because the capsules were the size of an oocyte. The

TABLE 1

Recovery and survival rates of sperm frozen into agarose microcapsules.

Variable	Polycarbonate sheet	Nylon mesh sheet
No. of freezing capsules	82	60
No. of sperm in a capsule	1	1
Total number of freezing sperm	82	60
Recovery rates of capsules	91.5% (75/82)	98.3% (59/60)
Recovery rates of sperm	91.5% (75/82)	96.7% (58/60)
Rates of motile sperm	85.3% (64/75)	82.8% (48/58)
Progressively motile rates	78.1% (50/64)	77.1% (37/48)
Nonprogressively motile rates	21.9% (14/64)	22.9% (11/48)
Rates of immotile sperm	14.7% (11/75)	17.2% (10/58)
HOS test positive	81.8% (9/11)	50.0% (5/10)
Total survival rates of sperm[a]	97.3% (73/75)	91.4% (53/58)
Total recovery rates of survival sperm	89.0% (73/82)	88.3% (53/60)

Note: All *P* values are not significant. HOS = hypoosmotic swelling.
[a] The total number of motile and HOS-positive sperm.

Araki. Single sperm cryopreservation using capsule. Fertil Steril 2015.

recovery rates were high. Most spermatozoa remained in the capsules after recovery. The recovering rates were 91.5% using PS or 96.7% using MS. This was a high recovery rate compared with other reports.

Gel material has been used for sperm cryopreservation. In one report (21), the spermatozoa were frozen by alginate acid gel. However, this method produced a gel ball with a sperm suspension; therefore, it was not suitable for freezing a very small number of spermatozoa. In addition, an important point when using a method that enveloped sperm with gel material is the speed that the cryoprotectant solution permeated into the gel. The agarose gel was found to have a mesh structure by a transmission electron microscope (22), and was permeated immediately by the cryoprotectant solution. In fact, it was possible to freeze sperm in the agarose capsules by immersing for several minutes as shown in the present study. In addition, the desired device for sperm preservation would not be toxic to mammalian cells. It was reported that mammalian cells were cultured in an agarose gel (23). Thus, the agarose gel material has low toxicity. In the case of cryopreservation of a few sperm, we must consider the method of transfer. If there are many spermatozoa, the transfer can be done using a pipette as a group. However, in the case of one or several spermatozoa, a pipette will not be useful for handling. Therefore, a few spermatozoa have been usually transferred by injection pipette for ICSI. However, using this method, it is possible to move several spermatozoa by glass pipette handling after injection into the agarose capsules. This method also enables spermatozoa to be transferred through several different types of solutions.

This is an excellent method of cryopreserving oocytes or embryos using narrow thin sheets such as CryoTop (24). This device is suitable to handle small cells and has become popular in assisted reproductive clinics. In addition, it was recently successful for sperm cryopreservation by the CryoTop (9). Although the CryoTop is superior for cryopreservation of few spermatozoa, the spermatozoa were loaded into a small freezing drop on the top of the sheet by a manipulator in air. It is necessary to transfer spermatozoa quickly from the culture drop to the freezing drop before the drop evaporates. In addition, it was reported that sometimes it takes a long time (1–1,800 seconds) to search for spermatozoa after thawing (8). However, in the present study, capsules were placed on the sheet with a glass capillary, similar to the embryo vitrification method, to enable the expeditious transfer of a dozen sperm. In addition, we were able to look for them in a short time (10 seconds) after thawing.

Consequently, using the agarose capsules is also a good method, similar to the sheet device. In the present study, we tried the MS and also a flat plastic sheet (PS). The recovery and survival rates of the sperm using the MS were satisfactory. A MS has been used to freeze mammalian immature eggs (25), and to cryopreserve human oocytes (26). An advantage of using mesh was the ability to adjust the amount of solution on the sheet, aspirate and eject using glass capillary from the side of the MS opposite to the one with capsules. A limitation of this method was the inability to see the capsules because it was not clear compared with polycarbonate.

In the preliminary examination, we tried freezing using the conventional LN_2 vapor method, wherein the sheet with capsules was placed 4 cm above the surface of LN_2 without the special devices used in this study. However, we did not obtain satisfactory survival rates. The overall survival rate was 45.2% (42/93), the survival rate of progressively motile sperm was 11.8% (11/93), and the survival rate of nonprogressively motile sperm was 33.3% (31/93). Therefore, we attempted using the styrene foam boat with a square hole. As a result, the survival rates of sperm after thawing highly improved. This may have been associated with the use of fertile donor sperm. When using a patient's sperm from TESE, the sperm may be nonprogressive and become immotile after freezing. However, it may be useful to confirm the survival of the immotile sperm just before freezing, because the spermatozoa are usually immobilized by touching their tails with ICSI. Therefore, we need to confirm the condition of each sperm before freezing, and the agarose capsule method can allow this. It may be better for the good quality sperm to be separated from the dead cells in the sperm

suspension because the dead cells may provide a negative influence to the normal sperm (27). Usually, in case of ICSI treatment with oligozoospermia it may be productive that only selected spermatozoa are cryopreserved while they are fresh.

A cryopreservation method for a few spermatozoa using an agarose capsule was established with PS or MS. Although this study described the methodology, this method is expected to be useful in ICSI treatment in patients with few spermatozoa. Therefore, it is necessary to investigate whether the method is useful in patients.

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