Successful delivery derived from vitrified-warmed spermatozoa from a patient with nonobstructive azoospermia

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Objective: To report the clinical outcomes following intracytoplasmic sperm injection (ICSI) with vitrified sperm from patients with severe male factor infertility.
Design: Retrospective case series.
Setting: IVF unit of a medical center.
Patient(s): Three patients with severe oligozoospermia or nonobstructive azoospermia (NOA).
Intervention(s): Cryopreservation of limited numbers of spermatozoa with the use of Cryotop and Cell Sleeper as nonbiologic containers.
Main Outcome Measure(s): Four cycles underwent intracytoplasmic sperm injection (ICSI) with vitrified sperm.
Result(s): A total of 148 spermatozoa in 18 containers (8.2 sperm per container) were vitrified and 36 of them (5 containers) were warmed. Thirty-three sperm (92%) were retrieved successfully and injected individually into 17 mature oocytes. Fertilization was observed in 12 oocytes (71%), and all zygotes (100%) cleaved. A couple with NOA achieved a singleton pregnancy and concluded with full-term delivery of a healthy boy (2,632 g).
Conclusion(s): A successful delivery was achieved after transfer of a blastocyst derived from vitrified-warmed spermatozoa. A small number of vitrified sperm cells were used for ICSI to fertilize oocytes with predictable timing. (Fertil Steril® 2012;98:1423–7. ©2012 by American Society for Reproductive Medicine.)
Key Words: Severe male factor infertility, live birth, single sperm vitrification, Cryotop, Cell Sleeper

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Cryopreservation of human spermatozoa has now become an essential technique in the assisted reproduction laboratory. In cases of azoospermia, the use of cryopreserved testis-derived sperm cells for intracytoplasmic sperm injection (ICSI) has a beneficial option of avoiding multiple surgical procedures for sperm extraction (1). In recent studies, small numbers of spermatozoa have been frozen with the use of various types of containers (2–9), and successful pregnancies have been reported using a few spermatozoa stored in empty zona pellucida (10, 11). However, these approaches depend on biologic materials from human or animal sources and are the only currently available options for freezing a small number of sperm. The lack of an easily implemented technology has remained a major bottleneck for the cryopreservation of limited numbers of spermatozoa (12).

In our previous study (13, 14), we developed simple novel vitrification techniques for a single spermatozoon with the use of Cryotop (Kitazato Biopharma) and Cell Sleeper (Nipro). In the present study, we report a successful case of delivery after performing ICSI with vitrified sperm.

MATERIALS AND METHODS

Patients

From December 2007 to October 2011, a total of three couples underwent ICSI using vitrified-warmed spermatozoa. Two couples had been diagnosed

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with nonobstructive azoospermia (NOA), and testicular sperm were isolated surgically by microdissection testicular sperm extraction (micro-TESE). The other couple had been diagnosed with severe oligozoospermia, and a few motile spermatozoa were obtained from ejaculates (3–10 sperm/ejaculate). Each patient gave written consent to be involved in this study protocol, which was approved by a local Ethics Committee.

Media

As a basic medium, HEPES-buffered modified HFF99 (Fuso Pharmaceutical Industries) containing 20% serum substitute supplement (Irvine Scientific) was used. Freezing medium was SpermFreeze (FertiPro), which includes both glycerol and sucrose. Following the manufacturer's instructions, a mixture of SpermFreeze (0.7 mL) and basic medium (1.0 mL) was used for sperm vitrification.

Sperm Preparation

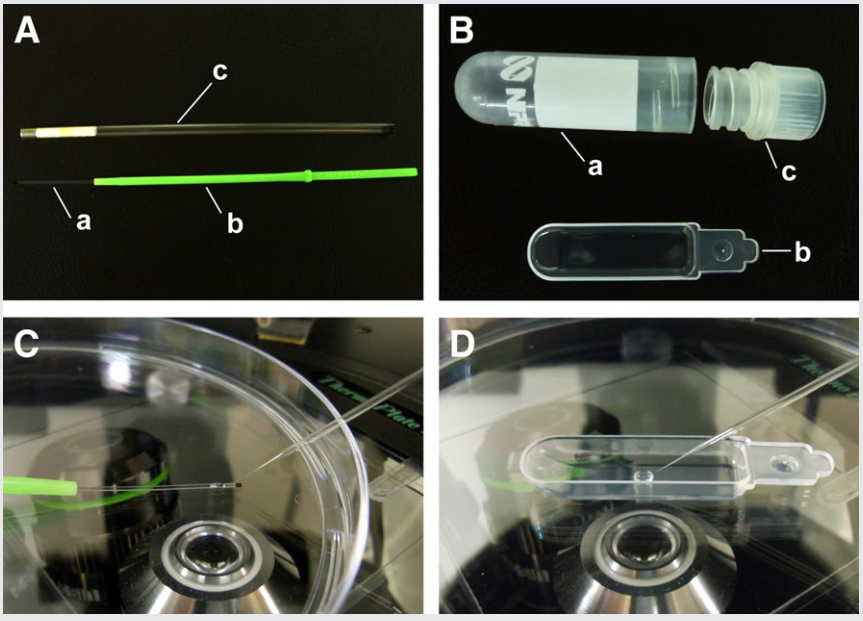
The seminiferous tubules from patients with NOA were isolated surgically by micro-TESE and placed into 100 μL basic medium on a glass-bottom dish (Wilco Wells). Immediately, the tissues were dissected using by stainless steel blades and spread on a dish. Ejaculated sperm from the patient with severe oligozoospermia were washed with basic medium by centrifugation procedures at 760*g* for 15 minutes. After removing the supernatant, 100 μL of the sperm pellet was spread on a glass bottom dish.

Recovered spermatozoa were diluted with a further 100 μL basic medium containing 7.2 mmol/L pentxifylline (Sigma Chemical) and covered with oil. After 3–10 minutes of culture at 37°C, the spermatozoa were searched using an inverted microscope (Olympus IX-71; ×100–200 magnification) equipped with a relief contrast system and a 21-inch monitor. The found spermatozoa were collected using ICSI injection pipettes (Kitazato Biopharma), which had an inner diameter of >4 μm and were equipped with a micromanipulator, and stored in a quite small volume (<0.1 μL) of basic medium until vitrification procedures.

Cryotop Method for Single Sperm Vitrification

Cryotop consists of a fine polypropylene strip attached to a plastic handle and is equipped with a cover straw (Fig. 1A). In accordance with the previously described report (13), individual sperm cells were transferred to a droplet of freezing medium (1 μL) on the Cryotop strip using the ICSI pipettes at room temperature (Fig. 1C). Immediately, the Cryotop strip was placed at 4 cm (−120°C) above the surface of the liquid nitrogen (LN_2) for 2 minutes and then directly exposed to sterilized LN_2. The Cryotop strip was covered with the hard plastic straw during storage in the cryotank. For warming, the Cryotop strip was placed directly in a flat droplet of basic medium (2 μL), which was covered by oil in a Falcon 1006 dish (Becton Dickinson) at 37°C. To prevent sperm from being left on the Cryotop strip, it was washed with two further drops (2 μL). Each droplet was carefully checked and the recovered sperm were transferred to 8% polyvinylpyrrolidone (Irvine

FIGURE 1



(A) Cryotop consists of a fine polypropylene strip (a), a plastic handle (b), and a cover straw (c). (B) Cell Sleeper vial (a) is equipped with an inner tray (b) and a screw cap (c). Sperm were loaded on (C) a Cryotop strip or (D) a tray of Cell Sleeper with the use of the ICSI pipettes equipped with a micromanipulator.

Endo. A live birth from vitrified sperm. Fertil Steril 2012.

Scientific) drops. After being washed three times by ICSI injection pipettes, they were stored until ICSI procedures.

Cell Sleeper Method for Single Sperm Vitrification

Cell Sleeper is a type of vial used as cell-cryopreservation container, which is equipped with an inner tray and is sealed with a screw cap (Fig. 1B). In accordance with the Cell Sleeper method (14), individual sperm were transferred with the use of the ICSI pipettes to a freezing medium (3.5 μL) deposited on the tray (Fig. 1D). Immediately, the tray was put into a vial and sealed with a screw cap. The vial was cooled in vapor (−120°C) of LN_2 for 2.5 minutes and then submersed in LN_2. The Cell Sleeper was mounted on a cane and stored in a cryotank. On the day of oocyte retrieval, Cell Sleeper was warmed at room temperature for 1 minute and the tray taken out immediately from the vial. The sperm on the tray was covered with oil immediately and incubated at 37°C for 2 minutes. The droplet was observed carefully and the recovered sperm was retrieved individually as described above.

Ovarian Stimulation and Oocyte Collection

The female partners received ovarian stimulation treatment by a combination of recombinant FSH (Follistim, Merck) and GnRH antagonist (Ganirelix; Merck). Vaginal ultrasound–guided follicle puncture was conducted 36 hours after the hCG (Mochida Pharmaceutical) injection. The retrieved oocytes were denudated enzymatically with recombinant human hyaluronidase (Origio) and mechanically by pipetting with narrow glass pipettes. The vitrified-warmed sperm cells were injected into the oocytes in accordance with the previously reported method (15).

Oocytes and embryos were cultured in Global medium (Lifeglobal) supplemented with recombinant human albumin (Vitrolife) at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Embryo transfers were performed on day 3 or 5. Clinical pregnancy and implantation were observed by ultrasonic detection of the gestational sac in the uterine cavity at 4–6 weeks after transfer.

RESULTS

Patient 1

The patient was a 29-year-old woman. Her 30-year-old husband with NOA underwent micro-TESE. A total of two motile sperm and 10 nonmotile sperm were retrieved, and all sperm cells were vitrified in two Cell Sleeper vials. On the day of oocyte retrieval, six mature oocytes were retrieved and then all sperm were warmed. Ten nonmotile sperm were recovered and two were lost. Sperm were injected individually into oocytes, and unused spermatozoa were recryopreserved. After ICSI, all injected oocytes were activated by calcium ionophore A23187 (10 micro-mol/L; Sigma) treatment for 15 minutes. Normal fertilization was observed in five oocytes (83%), and all of the zygotes cleaved. A single expanded blastocyst was transferred on day 5, which resulted in a singleton pregnancy, which concluded with full-term delivery of a healthy boy (2,632 g) at 38 weeks 3 days; the infant has had normal physical profiles up to the time of writing.

Patient 2

The patient was a 34-year-old woman, and her 34-year-old husband had been diagnosed with severe oligozoospermia. Total of 55 motile sperm from seven ejaculates were vitrified with the use of Cryotop and Cell Sleeper. In the first cycle, six mature oocytes were retrieved and then seven sperm stored in a Cryotop were warmed. Six nonmotile sperm were recovered and one was lost. After ICSI, normal fertilization was observed in three oocytes (50%), and all of the zygotes cleaved. A single poor-quality morula was transferred into the patient's uterus on day 5 but no pregnancy resulted. In the second cycle, one oocyte was collected and seven vitrified sperm (7/7; 100%), which were stored in a Cryotop, were recovered. A normal fertilized zygote was obtained after ICSI, and an excellent-quality embryo (8-cell stage) was transferred on day 3 but failed to implant.

Patient 3

The patient was a 37-year-old woman. Her 37-year-old husband had been diagnosed with NOA, and 81 motile sperm collected by micro-TESE were vitrified using ten Cryotops. On the day of oocyte retrieval, four mature oocytes were retrieved and a Cryotop containing ten sperm was warmed. All sperm were recovered but had lost motility. After ICSI, normal fertilization was observed in three oocytes (75%), and all of the zygotes cleaved. A single poor-quality blastocyst was transferred on day 5 but did not implant.

All of the couples refused to undergo chromosome analysis. A total of 148 spermatozoa obtained from three patients

TABLE 1

Vitrification of small numbers of sperm using Cell Sleeper and Cryotop.

Patient	Diagnosis	Sperm origin	Container (n)	Vitrified spermatozoa (n)	Sperm per container (mean)
1	NOA	Testis	Cell Sleeper (2)	12	6.0
2	Oligo	Ejaculate	Cell Sleeper (6)	41	6.8
		Ejaculate	Cryotop (2)	14	7.0
3	NOA	Testis	Cryotop (8)	81	10.1
Total			18	148	8.2

Note: NOA = nonobstructive azoospermia; Oligo = oligozoospermia.

Endo. A live birth from vitrified sperm. Fertil Steril 2012.

TABLE 2

Recovery and motility of vitrified-warmed spermatozoa.

Patient	Cycle	Warmed container (n)	Warmed spermatozoa (n)	Collected spermatozoa (n)	
				Motile	Nonmotile
1	1	Cell Sleeper (2)	12	0	10
2	1	Cryotop (1)	7	0	6
	2	Cryotop (1)	7	0	7
3	1	Cryotop (1)	10	0	10
Total		5	36	0	33

Endo. A live birth from vitrified sperm. Fertil Steril 2012.

were vitrified individually in 18 containers (Table 1) and underwent ICSI using vitrified sperm. On the day of oocyte pick-up, 36 sperm stored in five containers were warmed and 33 (92%) recovered (Table 2). From a total of four clinical results, fertilization was observed in 12 (71%) and all of the zygotes (100%) cleaved (Table 3). A couple with NOA achieved a singleton pregnancy and delivered a healthy boy.

DISCUSSION

Our clinical data show a successful delivery derived from vitrified-warmed spermatozoa from a patient with NOA. Limited numbers of spermatozoa were vitrified with the use of Cryotop and Cell Sleeper, which are already commercially available, as nonbiologic containers.

Our simple methods can vitrify and warm only the numbers of sperm cells needed for ICSI without significant loss. Having a method of reliable sperm storage for severe male factor patients may reduce multiple testicular surgical operations and prevent an ICSI failure owing to an unexpected lack of spermatozoa. Another important advantage of these methods is that individually frozen sperm are recovered efficiently without time-consuming searches. Most sperm are recovered within 30 minutes and used for ICSI with predictable timing. Searching for a few spermatozoa from ejaculate or testis on the day of oocyte retrieval is laborious and time consuming, which may seriously affect the ICSI outcome of the cycle (11).

In the present clinical cases, similar sperm recovery rates were seen in both Cryotop (23/24, 96%) and Cell Sleeper (10/12, 83%) groups. The fertilized oocytes were cleaved and developed, regardless of whether sperm were stored in Cryotop or Cell Sleeper. Although poor-quality sperm lost their motility after warming, viability seemed to be maintained, because oocytes were fertilized after ICSI. We consider that sperm quality is important to maintain their motilities after vitrification. It is well known that sperm collected by micro-TESE have slightly lower motility after cryopreservation than sperm obtained from men with normal sperm quality (3). In our laboratory studies (13, 14), the healthy ejaculated sperm could be vitrified in Cryotop and Cell Sleeper with similar motility rates (29%–44%) after warming.

The only delivery was achieved after oocyte activation with a calcium ionophore. We had expected the oocyte activation procedure to enhance the fertilization abilities of oocytes and sperm cells. Because all warmed spermatozoa were immobilized by freezing, we anticipated that the sperm's ability to fuse with the oocyte would be impaired. Fortunately, normal fertilization was observed after oocyte activation and the transfer of one blastocyst stage embryo resulted in singleton pregnancy and live birth. Oocyte activation was not used for the other patients: The oocytes fertilized, but no pregnancies were achieved after embryo transfer. The present study is based on a very small sample size, and further investigations are required to determine if oocyte activation is required for fertilization after ICSI of sperm vitrified in the Cell Sleeper.

In this study, we recovered 32 vitrified-warmed spermatozoa and only morphologically normal sperm were selected for ICSI. We did not attempt to assess the sperm membrane viability by the hypo-osmotic swelling test before ICSI or in sperm not selected for ICSI, because the nonselected sperm were morphologically abnormal (i.e., sperm with heads separating from tails, sperm with coiled tails). Most injected sperm were motile just before vitrification, and we expected the sperm would maintain their ability to fertilize oocytes throughout this process. For ICSI procedures, spermatozoa must be immobilized, and they look "dead" in a conventional sense. Along these lines, Hoshi et al. (16) reported the successful delivery after ICSI using freeze-killed spermatozoa. And Kusakabe et al. (17) reported that both mouse and human sperm killed by freeze-drying techniques maintain a normal karyotype and that ICSI of these sperm in the mouse model can lead to development of normal fetuses.

In conclusion, this is a rare case of a successful delivery after transfer of a blastocyst derived from ICSI using limited numbers of sperm stored in novel containers. We believe that our simple methods are suitable and clinically useful for the cryopreservation of small numbers of sperm. Further

TABLE 3

Results of embryo development and clinical pregnancy after intracytoplasmic sperm injection using vitrified sperm.

Patient	Cycle	Oocyte injected (n)	2PN fertilization [n (%)]	Embryo cleaved [n (%)]	Embryo transferred (n)	Outcome
1	1	6	5 (83)	5 (100)	1	Single live birth[a]
2	1	6	3 (50)	3 (100)	1	No pregnancy
	2	1	1 (100)	1 (100)	1	No pregnancy
3	1	4	3 (75)	3 (100)	1	No pregnancy
Total		17	12 (71)	12 (100)	4	

[a] Male baby (2,632 g).

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studies of clinical applications with extremely poor sperm specimens are necessary to confirm these findings.

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