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A novel method for cryopreservation of individual human spermatozoa

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Abstract The purpose of this study is to develop a novel method for the cryopreservation and efficient post-thaw recovery of individual or small numbers of human spermatozoa. Spermatozoa equilibrated in cryoprotectant buffer were injected with an intracytoplasmic sperm injection (ICSI) needle into a droplet of cryoprotectant on a homemade cryoleaf. The droplet was of cryoprotectant and seminal plasma at a ratio of 1:1. The sperm-loaded cryoleaf was slowly lowered over and stored in liquid nitrogen. Spermatozoa were thawed in a 37°C oil bath without dilution and centrifugation. To test the fertilizing ability of these spermatozoa, the recovered spermatozoa were injected by ICSI into 1-d-old or in vitro-matured human oocytes. Fresh spermatozoa from the same semen samples served as controls. The trials were performed in two separate experiments. In the first set of experiments, 92 spermatozoa were thawed and carefully investigated. The spermatozoa from percutaneous epididymal sperm aspiration had a motility recovery of 92.9% (13/14); ejaculated spermatozoa had a motility recovery of 61.5% (48/78), and only 1.3% (1/78) was lost. Together in the first and second set of experiments, the fertilization rates for the fresh and frozen–thawed spermatozoa were 67.6% (25/37) and 60.6% (40/66), respectively ($P=0.052$). The mean embryo cleavage rates in the fresh and frozen–thawed groups were 88% (22/25) and 85% (34/40), respectively ($P=0.990$). This cryopreservation method for individual or small numbers of human spermatozoa was efficient and simple. These findings make this method a promising technique for the clinical application of ejaculated sperm from oligozoospermic patients.

Keywords Sperm · Cryopreservation · Droplet · Oil bath

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Introduction

Men suffering from azoospermia can usually be treated by intracytoplasmic sperm injection (ICSI) by using surgically isolated spermatozoa from their testis or epididymis (Hewitson et al. 2002; Park et al. 2003). However, the failure of surgical extraction of spermatozoa always results in cancelation of the treatment cycle. Furthermore, certain diagnostic and therapeutic extraction procedures may have negative effects on testicular function (Cohen et al. 1997). In the case of testicular sperm extraction (TESE), surgical procedures are not only costly and invasive but can also cause transient and even permanent adverse physiological effects (Cohen et al. 1997). In some cases, repetition of these procedures can be avoided by cryopreservation of

spermatozoa, but this is only possible when sufficient numbers of functional cells are isolated (AbdelHafez et al. 2009). Although survival of spermatozoa and the birth of live offspring are possible after cryopreservation of sperm-rich epididymal and testicular aspirations, conventional sperm freezing is often impossible when there are limited numbers of spermatozoa, mainly due to sperm loss during the process of thawing, which requires repeated centrifugation and washing (AbdelHafez et al. 2009). Cryopreservation of individual or small numbers of human spermatozoa may replace the need for repeated surgical retrieval of spermatozoa. However, it has been a challenging task to develop a simple, convenient, and efficient cryopreservation technique for individual or small numbers of spermatozoa (Nicopoullos et al. 2004; AbdelHafez et al. 2009).

In recent years, several carriers have been used to store isolated spermatozoa. Cohen et al. (1997) first introduced single-sperm cryopreservation within an empty zona pellucida to freeze slowly (Cohen et al. 1997). This technique has become the most widely used methodology for single-sperm freezing (Cohen et al. 1997; Borini et al. 2000; Hsieh et al. 2000; Levi-Setti et al. 2003) and involves the insertion of small groups of spermatozoa (and even single cells) into evacuated zonae. However, empty zonae must be prepared by evacuation of oocyte cellular contents, which is a time-consuming activity. Just et al. (2004) developed spherical *Volvox globator* algae as an easy vehicle for cryopreservation of functional, motile sperm cells (Just et al. 2004). The post-thaw recovery rate in cases of severe male infertility was 100%, and the number of motile sperm after thawing was at least 60%. Desai et al. (2004) reported successful cryopreservation of individual human spermatozoa with a vitrification cryoloop by directly plunging a copper cryoloop loaded with sperm suspension into liquid nitrogen (Desai et al. 2004). Microquantities of spermatozoa cooling in cryoloops exhibited overall motility and viability characteristics similar to those of control samples frozen in cryovials. Furthermore, individually selected spermatozoa that were cryopreserved in loops were easily warmed, and post-thaw motility was generally good. Herrler et al. (2006) developed a method for freezing small quantities of spermatozoa in polymerized alginic acid capsules, and the capsules could be thawed to recover the spermatozoa (Herrler et al. 2006). Cryopreservation of human spermatozoa from the use of this technique resulted in a decrease in motility of 18.3% relative to that of fresh spermatozoa. Isaev et al. (2007) reported freezing spermatozoa in agarose microspheres (Isaev et al. 2007); once thawed, 78% of the recovered spermatozoa were motile. Sereni et al. (2008) presented a new procedure for the freezing of testicular fine-needle aspiration-recovered spermatozoa in azoospermic patients (Sereni et al. 2008). Upon thawing, the spermatozoa recovery was 100%, with motility of 2.3% (versus 3.6% before freezing). However, the post-thaw procedures involve dilution and washing, resulting in a loss of spermatozoa. To date, there remains no consensus as to the ideal carrier for cryopreservation of individual or small quantities of spermatozoa for clinical purposes (AbdelHafez et al. 2009).

The purpose of this study was to describe a new method for spermatozoa cryopreservation, which allows the freezing and recovery of individual or small numbers of spermatozoa. In this method, a homemade cryoleaf was chosen as the vehicle for sperm cryopreservation, and spermatozoa were injected into a droplet of diluent loaded onto a cryoleaf with an ICSI needle with the aid of micromanipulation. To thaw spermatozoa and avoid loss of spermatozoa due to centrifugation and washing, the cryoleaf was directly plunged into a 37°C oil bath. The validity of this technique was assessed by the rate of sperm recovery upon thawing and the rate of fertilization of human oocytes.

Materials and Methods

Equipment, culture media, and tools. All micromanipulation was performed in HEPES (hydroxyethylpiperazine ethane sulfonic acid)-buffered, synthetic serum substitute (SSS)-supplemented human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA). Micromanipulation was performed in shallow Falcon 3001 Petri dishes (BD Biosciences, Franklin Lakes, NJ) using eight HEPES droplets (5 μL each) surrounding a polyvinylpyrrolidone (PVP, Irvine Scientific)-containing droplet (5 μL, sterile 10% (*w/v*) lyophilized PVP reconstituted with modified HTF), according to the methods of Cohen et al. (1997) and Palermo et al. (1995). The ICSI procedures were performed at 37°C by using a Nikon IX-70 inverted microscope (at ×40 magnifications) equipped with Hoffman interference optics and connected to a 14-inch monitor (Nikon, Tokyo, Japan). The ICSI needle (MIC-35-35) and holding were purchased from the Humagen Company (Charlottesville, VA). The ICSI was performed as described previously (Hewitson et al. 2002; Park et al. 2003). Eggs were incubated after ICSI according to standard procedures described elsewhere (Hewitson et al. 2002). Cryoprotectant was donated by the Shanghai Human Sperm Bank (China); it contained 12% (*v/v*) glycerol and 20% (*v/v*) egg yolk in 0.1 M citrate buffer (pH 7.2; Xiang et al. 2005).

Preparation of wet box and cryoleaf. A new transparent polystyrene cryoleaf was laboratory-made specifically for the cryopreservation of spermatozoa in a droplet of cryoprotectant with the aid of micromanipulation. A

laboratory-made closed humidity chamber was used for injection of spermatozoa into the droplet via an ICSI needle (Fig. 1). This maintained the humidity at room temperature until the casing covering the cryoleaf was in place. In addition, moist cotton wool was placed at the end of the casing to maintain the humidity within the tube.

The cryoleaf was made of polystyrene with a Falcon Petri dish (Falcon 353001, BD Biosciences) as source. A dish cover was cut into small squares, and one square was drawn out into a sheet by heating (Fig. 2*A*). The sheet was trimmed to 20.0 mm long and 3.0 mm wide to form a cryoleaf. The cryoleaf was bound to a cryoleaf handle, and then this was protected in a casing containing cotton wool at the bottom (Fig. 2*B*). The cryoleaf handle and the protective casing were made from parts provided by a human embryo transfer tube package (Laboratoire CCD, Paris, France; see Fig. 2*C*). The cryoleaf preparation is shown in Fig. 2*D*.

Source of oocytes. A total of 29 patients donated 103 oocytes after undergoing egg retrieval, in vitro fertilization (IVF), and ICSI; the patients gave informed consent for oocyte donation, fertilization, and embryo development. These oocytes included 62 1-d-old mature (MII) oocytes and 41 germinal vesicle (GV) or immature (MI) oocytes that developed spontaneously into in vitro-matured (IVM) oocytes. The IVM procedure proceeded as follows. Denudation from the cumulus oophorus was performed by a exposure for 15 s to 40 IU/mL of hyaluronidase (Lee Pharmacy, Ft. Smith, AR) in Early Cleavage Media™ (ECM®, Irvine Scientific), followed by mechanical removal of the granulosa cells with the use of a glass pipette at noon on the first day. The GV or MI immature oocytes were then cultured overnight in 10% (*v/v*) SSS-containing ECM droplets in a 37°C, humidity-saturated, 5% (*v/v*) CO_2 incubator (Themo Electron, New York, NY). Twenty hours after retrieval, the oocytes that presented second polar bodies were assumed to be spontaneously maturing IVM eggs.

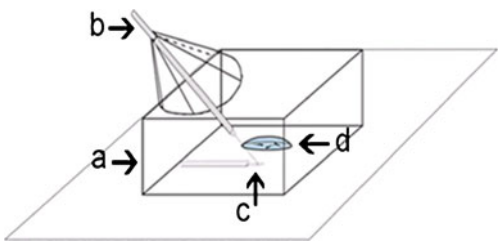

Figure 1. Each spermatozoon was captured by the ICSI needle and injected into a droplet of medium loaded on the cryoleaf. The entire process was performed in a closed humid chamber: (*a*) humid chamber, (*b*) ICSI needle, (*c*) cryoleaf, (*d*) a droplet of medium containing.

Source and preparation of spermatozoa. Sperms were harvested in two batches. In the first batch, 92 sperm samples for cryopreservation were obtained from discarded semen specimens of nine fertile men undergoing a semen analysis, which included 14 sperm samples originating from percutaneous epididymal sperm aspiration (PESA) patients, and sperm counting was strictly performed before freezing and after thawing. In the second batch, sperm samples for cryopreservation were obtained from discarded semen specimens of three fertile men undergoing a semen analysis, and sperm counting was not performed during cryopreservation and thawing.

All subjects gave informed consent for the use of donated samples for fertilization and embryo development. A portion of the seminal spermatozoa from each man was used for cryopreservation and thawing. The remaining fresh portion served as control for comparison with thawed spermatozoa from the same semen sample.

Control semen samples were placed in sperm separation medium (Isolate®, Irvine Scientific) and pelleted for 20 min by density gradient centrifugation (300×*g*). The pellets were rinsed with 10% (*v/v*) SSS-containing HTF and then centrifuged (300×*g*, 5 min). The rinse and centrifugation step was repeated. The pellets were then resuspended in 10% (*v/v*) SSS-containing ECM and stored in an embryo incubator for use. Semen (1 mL) from the experimental group was placed in a tube bottom, followed by the slow addition of cryoprotectant (1 mL), with blending at a low speed. A 10-μL aliquot of the mixture was next transferred to a dish bottom to form a droplet, which was then covered with mineral oil (Irvine Scientific). Spermatozoa were equilibrated in the mixtures for 6 min at room temperature.

Sperm cryopreservation. Sperm-free seminal plasma was obtained by filtration of semen through 0.22-μm filters (Millipore, Temecula, CA). A droplet (0.2 μL) consisting of 0.1 μL autologous seminal plasma (the spermatozoa of PESA patients were not placed in autologous seminal plasma but rather in HEPES-buffered 10% (*v/v*) SSS-supplemented HTF instead), and 0.1 μL cryoprotectant was prepared and placed on a cryoleaf at 37°C (Note: Sperm and protectants were mixed at room temperature. During sperm transfer with the needle into droplets, the wet box was placed in a 37°C hot stage, and the cryoleaf was placed inside the box, so we considered that it may also reached 37°C on the cryoleaf. In theory and in practice, cryogenic sperm needs no heating to 37°C. However, we forgot to turn off the hot stage, leading to the heating to 37°C. We had to report 37°C truthfully. In the next clinical application, we will turn off the hot stage.). The equilibrated individual or small numbers of spermatozoa from the experimental group was captured with an ICSI needle with the aid of micromanipulation and transferred into the

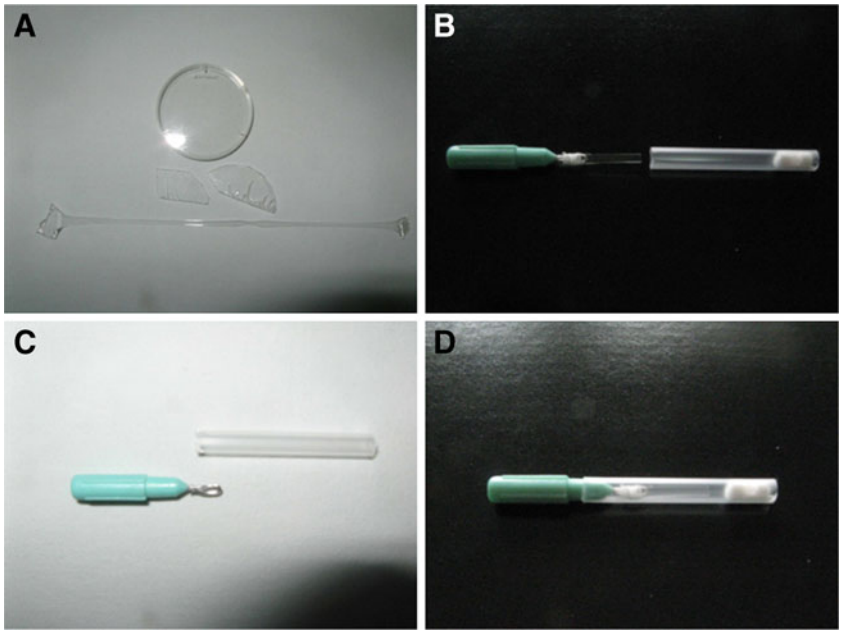

Figure 2. Material source and construction of cryoleaf. (*A*) a dish cover, a trimmed to a square and a heated to form a drawn sheet; (*B*) A cryoleaf bound to a handle by cotton thread is placed to a protective casing containing cotton wool at the bottom; (*C*) the cryoleaf handle and a casing provided by a human embryo transfer tube package; (*D*) a cryoleaf prepared for cryopreservation.

droplet. The cryoleaf was subsequently sheathed with a casing. The entire process was performed in a humid chamber to avoid evaporation of the droplet (Fig. 1). The spermatozoa were subsequently frozen according to a protocol in which the cryoleaf was slowly lowered (20 cm in 30 s) to 1 cm over liquid nitrogen surface, exposed to the vapor for 2 min, then dropped into the liquid nitrogen and stored at least 48 h.

Thawing, sperm recovery, and ICSI. Mineral oil (6 mL) was kept in a 60-mm-diameter dish and incubated at 37°C before use. The cryoleaf was thawed in a 37°C oil bath for 1 min, where it was gently shaken to help the droplet of spermatozoa thaw more rapidly (Fig. 3*A*). After the droplet had thawed, the top part of the cryoleaf containing the droplet of spermatozoa was cut off and placed onto the bottom of the dish. A small copper weight was used to prevent any movement of the cryoleaf (Fig. 3*B*). With the aid of micromanipulation and an ICSI needle, all of the spermatozoa were then transferred from the cryoleaf into a tiny droplet (5 μL) of HEPES-buffered, SSS-supplemented HTF covered by mineral oil in an ICSI dish. Spermatozoa were counted in an inverted microscope, and the recovery was assessed. Recovered, motile spermatozoa were moved individually by microneedle into a droplet of medium in an ICSI dish and were immediately injected into MII oocytes or IVM oocytes. The ICSI procedure was performed following our center's routine protocols; each spermatozoon was released into a mature oocyte with polar bodies at either the 6 or 12 o'clock positions. Injected oocytes were then washed three times and transferred to SSS-supplemented ECM for incubation. The fresh, normal, motile spermatozoa prepared as controls were also injected into oocytes. The presence of oocyte polar

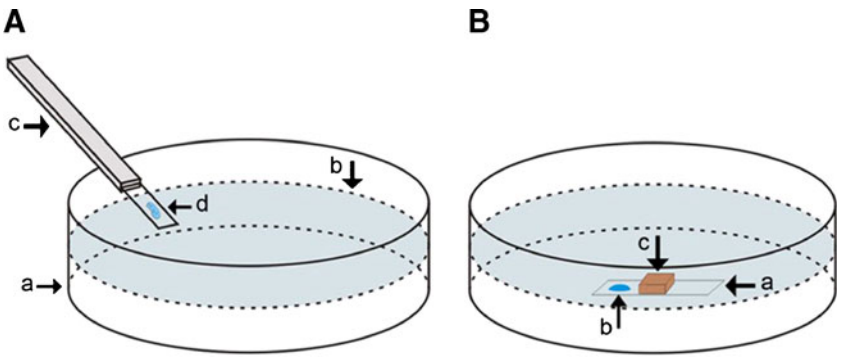

Figure 3. Diagrammatic presentation of sperm thawing. (*A*) Sperm in the droplet was thawed in the 37°C oil bath: *a* 60-mm-diameter dish, *b* mineral oil, *c* cryoleaf, *d* droplet with spermatozoa; (*B*) The cut-off part of the cryoleaf was placed in the bottom of the dish after the droplet had been thawed out, and then a small copper weight was placed onto it to prevent movement of the cryoleaf: *a* the cut-off part of the cryoleaf, *b* the droplet with spermatozoa, *c* small copper weight.

bodies and pronuclei were checked 20 h later. Embryonic cleavage after an additional culture period of 20 to 28 h was observed to determine the success of the method. The fertilization and cleavage rates were assessed.

All protocols used in this study were approved by the local ethics committee.

Statistical analysis. Statistical analysis was performed by using SPSS 12.0 software (SPSS Inc., Chicago, IL). The data are expressed as percentages. Comparisons were performed by hypothesis testing between groups. $P < 0.05$ indicated a statistically significant difference.

Results

Motility recovery and general efficiency. In the first batch of sperms, the recovery of spermatozoa motility was determined by carefully observing the spermatozoa in a tiny droplet (5 μL) immediately following thawing and transfer into HEPES-buffered, SSS-supplemented HTF. Table 1 clearly presented the number of spermatozoa frozen in each case ("1" or ">1"). For each PESA patient, four or ten spermatozoa were frozen. Motility was defined as any motion, including vibration, and the motility recovery rate was defined as the percentage of spermatozoa demonstrating motility after thawing. Spermatozoa that could not be found after thawing were counted as lost spermatozoa. For the spermatozoa coming from PESA, the motility recovery rate and immotility rate were 92.9% (13/14) and 7.1% (1/14), respectively; for ejaculated spermatozoa, the motility recovery rate, the immotility rate, and the loss rate were 61.5% (48/78), 38.5% (30/78), and 1.3% (1/78). These data were also shown in Table 1.

In the second batch, sperm counting was not performed during cryopreservation and thawing, and thus there was no information related to motility recovery and general efficiency.

Fertilizing ability of individually cryopreserved and seminal spermatozoa. A total of 103 oocytes in the first and the second sets of experiments donated by 29 patients were available to test fertilization of the cryopreserved spermatozoa after ICSI. The oocytes were randomly allocated to either the control, fresh spermatozoa group (37 oocytes) or the experimental, frozen–thawed sperm group (66 oocytes). The controls were used to determine the fertilization rate by ICSI of donated spare oocytes for comparison with the fertilization rates of individually frozen–thawed spermatozoa recovered from our novel cryopreservation method.

The fertilization rates for the fresh controls and frozen–thawed spermatozoa group were similar 67.6% (25/37) and 60.6% (40/66), respectively ($P=0.052$). However, both rates were significantly lower than the routine fertilization rate (78.3%, 1078/1376; $P=0.000$ versus controls; $P=0.000$ versus frozen–thawed sperm) in our ICSI program, based on 98 patients who were treated during the same period (unpublished data). Frozen–thawed individual motile spermatozoa were injected into 63 oocytes, of which 40 became fertilized. In contrast, of the three frozen–thawed immotile spermatozoa injected into oocytes (3/66), none became fertilized. The overall embryo cleavage rates (cleavage/fertilized) in the fresh (88%, 22/25) and frozen–thawed groups (85%, 34/40) were not significantly different ($P=0.990$).

A total of 62 MII oocytes were injected with fresh or frozen–thawed spermatozoa; the fertilization rates for these groups were similar at 69.2% (18/26) and 69.4% (25/36), respectively. The cleavage rates between the two groups were also similar at 88.9% (16/18) and 88% (22/25), respectively. A total of 41 IVM oocytes were injected with fresh or frozen–thawed spermatozoa, and the fertilization rates for the two groups were similar at 63.6% (7/11) and 50% (15/30), respectively ($P=0.670$); the cleavage rates were also similar at 85.7% (6/7) and 80% (12/15), respectively. These data are shown in Table 2.

It should here be clarified that the trials were performed in two separate experiments. In the first set of experiments, 16 females donated a total of 53 eggs; 19 of these eggs underwent fresh sperm injection (12 fertilized, 63.2%), and 34 eggs underwent frozen sperm injection (18 fertilized, 52.9%), of which three eggs (3/34) underwent post-thaw with immotile sperm injection and were not fertilized. All of these frozen spermatozoa were derived from the same 92 frozen spermatozoa (Table 1). For the second set of experiments, 13 females donated a total of 50 eggs; these eggs were used with the second batch of sperm, but these sperm were not included in the first set of 92 frozen sperm samples. There were 18 eggs that underwent fresh sperm injection (13 fertilized, 72.2%) and 32 eggs that underwent frozen sperm injection (22 fertilized, 68.8%).

Fertilization ability of MII and IVM oocytes. The overall fertilization rate of ooctyes was 56.6% (65/103). There were a total of 62 MII oocytes and 41 IVM oocytes used for sperm injection in the first set and the second set of experiments. The fertilization rates for MII and IVM oocytes were similar at 69.4% (43/62) and 53.7% (22/41), respectively ($P=0.100$). The cleavage rates for the two groups were 88.4% (38/43) and 81.8% (18/22), respectively ($P=0.650$).

The fertilization rate of fresh spermatozoa was 69.2% (18/26) for MII oocytes and 63.6% (7/11) for IVM oocytes; these values were similar ($P=0.770$). The cleavage rates for these groups were 88.9% (16/18) and 85.7% (6/7), respectively ($P=0.600$). When frozen–thawed spermatozoa

Table 1. Post-thaw motility recovery and thaw efficiency of sperms in the first batch

Patient no.	Experiment no.	Quantities of frozen sperm	No. (%) of recovered motile sperm	No. (%) of recovered immotile sperm	No. (%) of lost sperm
1	1	1	1 (100)	0 (0)	0 (0)
1	2	1	1 (100)	0 (0)	0 (0)
1	3	1	0 (0)	1 (100)	0 (0)
2	4	10	4 (40)	6 (60)	0 (0)
3	5	12	10 (83.3)	1 (8.3)	1 (8.3)
4	6	10	3 (30)	7 (70)	0 (0)
5	7	10	5 (50)	5 (50)	0 (0)
6 (PESA patient)	8	4	4 (100)	0 (0)	0 (0)
7	9	10	7 (70)	3 (30)	0 (0)
7	10	8	6 (75)	2 (25)	0 (0)
7	11	6	4 (66.7)	2 (33.3)	0 (0)
8	12	9	7 (77.8)	2 (22.2)	0 (0)
9 (PESA patient)	13	10	9 (90.0)	1 (10.0)	0 (0)
Total (overall)		92	61 (66.3)	30 (32.6)	1 (1.1)

were injected, the fertilization rates for MII and IVM oocytes were 69.4% (25/36) and 50% (15/30), respectively (P=0.200). The cleavages for these two groups were 88% (22/25) and 80% (12/15), respectively (P=0.053).

Discussion

This paper describes a novel method for individual-sperm cryopreservation. We prepared a 0.2 μl droplet of cryprotectant solution on a homemade cryoleaf under conditions of high humidity. Using an ICSI needle, we then transferred the selected individual or small numbers of spermatozoa into the droplet by micromanipulation. After being covered, samples were frozen in slow steps. To thaw spermatozoa, the cryoleaf was simply plunged directly into a 37°C oil bath.

The polystyrene cryoleaf is of the requisite hardness and transparency for convenient microscopic manipulation. The droplet of medium is prone to evaporate owing to its small volume, so it is necessary to maintain a fully moisture-saturated atmosphere during the entire process of cryopreservation.

Cohen et al. (1997) introduced individual-sperm cryopreservation by using an empty zona pellucida vehicle to slow-freeze sperm. This group reported the individual sperm loss of 3/10, 2/10, 6/20, and 3/30 during and after thawing due to zona digestion by pronase, zona digestion by reduced pH, mechanical removal through microneedle, and mechanical removal of embryonic zona through microneedle, respectively. In the present study, spermatozoa in the first batch were rarely lost, as the overall loss of sperm was only 1.1% during and after thawing. Our ability not to lose spermatozoa was because we did not perform additional operations, with the exception of plunging the cryoleaf into an oil bath. In addition, the survival rate in the first set of 92 spermatozoa was satisfactory (the motility recovery rates were 92.9% (13/14) and 61.5% (48/78) for PESA spermatozoa and ejaculated spermatozoa, respectively). The low number of lost sperm and the high rate of motility recovery after thawing suggest that this freezing method is efficient and reliable when using small quantities of cryopreserved spermatozoa. We want to make it clear that we did not

Table 2. Results of fertilization by fresh and frozen–thawed spermatozoa together in the first and the second set of experiments

Sperm treatment	Oocytes	No. of oocytes	No. of fertilized oocytes	No. of cleavage	No. of unfertilized oocytes	No. of dead oocytes
Fresh	MII	26	18	16	6	2
	IVM	11	7	6	4	0
Frozen–thawed	MII	36	25	22	10	1
	IVM	30	15	12	15	0

count the sperm in the second set of experiments before freezing because the first set of 92 spermatozoa is sufficient to demonstrate the parameters including motility recovery rate, and additionally, sperm are motile in droplets and are hard to count, and thus the post-thaw survival rate is not clear. The second batch of sperm is therefore not incorporated into Table 1 (only containing the first batch of 92 frozen sperms).

Previous reports have shown no statistically significant differences between fresh and frozen–thawed spermatozoa in a number of parameters, including fertilization rate, cleavage rate, implantation rate, clinical pregnancy rate, and delivery rate (Friedler et al. 1997; Gil-Salom et al. 2000; Habermann et al. 2000). In this study, no statistically significant differences in the fertilization rates were found between fresh and frozen–thawed spermatozoa, and the cleavage rates were similarly not different; however, the oocyte sample size used in this work was small and of variable quality.

The two kinds of discarded eggs used in this study were donated by females younger than 40 yr old (especially 30–40 yr old), and they would not be employed by most reproductive centers for transplantation (Desai et al. 2004). All of the ooctyes involved in the ICSI experiments are mature MII eggs (MII and IVM oocytes) that present second polar bodies and have a normal ability to be fertilized; however, their potential for developing into a fetus is very poor. It is really likely that the oocytes failed to fertilize during IVF contained already spermatozoa, however, the probability for this is very low (Desai et al. 2004). And we performed a random-control experiment, that is, these eggs were randomly allocated to the experimental groups for frozen–thawed and fresh sperm injection. The randomized treatment would minimize the deviation in the results. We therefore used these discarded eggs in this study to test the ability of sperm to fertilize them, as has previously been done (Desai et al. 2004). Consequently, in this study, the fertilization and cleavage rates together in two sets of experiments for IVM oocytes were slightly lower than for MII oocytes; however, this difference was not statistically significant. In addition, the ICSI success rates reported in this study were smaller than our routine institutional ICSI success rates because the abnormal MII or IVM oocytes used in this study have reduced competence compared with fresh, normal MII eggs used in routine ICSI.

This novel method described here may be used to store small aliquots of spermatozoa or individually selected spermatozoa, such as from the testis or epididymis. Furthermore, this method makes it feasible to perform surgical extractions independent of the time and place of egg retrieval, thus preventing an ICSI failure due to unexpected lack of spermatozoa. Ejaculated spermatozoa from men with severe oligozoospermia may also be selected and effectively cryopreserved. Therefore, the method described in this paper should be ideal for cryopreservation of individual or small numbers of spermatozoa.

To validate this method further, a study on TESE/testicular sperm aspiration samples should be conducted because these immature spermatozoa are more fragile with a more modest freezing–thawing recovery rate. However, we believe that mild freeze–thaw manipulation without washing and centrifugation will minimize the damage to sperm.

Conclusions

The data from this study demonstrate the promising nature of this approach. This cryopreservation procedure is simple to perform and avoids post-thaw dilution and centrifugation. For these reasons, it may have widespread clinical application for preserving ejaculated oligozoospermic sperm.

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Conflict of interests The authors declare that they have no conflict of interests.

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