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A novel solution for freezing individual spermatozoa using a right angular cryopiece embedded in a grooved petri dish

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Abstract

Herein, we introduced a novel individual sperm freezing device named SpermCD, which consists of a right angular cryopiece (RA-Cryopiece, or "C") and a grooved petri dish ("D"). SpermCD allows embryologists to transfer sperm and perform ICSI on the same focal plane. Thirty-five patients underwent single sperm cryopreservation using SpermCD, including four patients with non-obstructive azoospermia (NOA), 14 patients with virtual azoospermia and 17 patients with cryptozoospermia. One hundred and twenty-five cryopreserved spermatozoa from nine patients were thawed on the day of the oocyte retrieval and 121 spermatozoa were found, with a sperm recovery rate of 97.1 ± 4.6%. Sixty-five MII oocytes from their spouse were injected with thawed sperm. Normal fertilization and high-quality embryo rates were 68.0% ± 33.2% and 24.4% ± 22.2%. Nineteen transplantable embryos were formed after fertilization with frozen sperm, eight of which were transplanted in five couples, resulting in four successful deliveries. SpermCD is a simple and practical individual sperm freezing device.

KEYWORDS
azoospermia, cryopiece, intracytoplasmic sperm injection, rare spermatozoa, testicular sperm

1 | INTRODUCTION

It's known that cryptozoospermia means spermatozoa are absent from fresh preparations but are always observed in a centrifuged pellet in the semen sample (World Health Organization, 2010). A more extreme condition, virtual azoospermia, refers to the occasional presence of spermatozoa, after an extended search, in the ejaculate of men diagnosed with azoospermia (Bendikson et al., 2008; Miller et al., 2017). With the development of microdissection testicular sperm extraction (micro-TESE), small spermatogenic foci are found in the testis of some patients with NOA (Corona et al., 2019). Freezing these rare and precious sperm from patients with cryptozoospermia, virtual azoospermia or NOA in advance can avoid repeated testicular surgery, oocyte freezing and the use of donated sperm (Abdelhafez et al., 2009; Liu & Li, 2020). Individual sperm freezing technique with high-sperm recovery rate is suitable for freezing these rare sperm.

The basic process of freezing individual sperm is that the embryologist transfers spermatozoa one by one from the sperm deposit to the carrier using micromanipulation instruments prior to oocyte retrieval. The thawed sperm will be injected directly into the MII oocytes, without washing procedure.

At present, the freezing technology for ordinary sperm has been well established. The main technical difficulty is the freezing of rare sperm especially individual sperm. Early carriers for freezing rare sperm such as Zona Pellucida (Cohen et al., 1997) and spherical Volvox globator algae (Just et al., 2004) have little clinical application value due to material sources and ethical issues. Later carriers such as Cryoloop (Desai et al., 2004), Culture Dish (Sereni et al., 2008), Microstraw (Huang et al., 2020; Ziarati et al., 2019), Cryoplus (Wang et al., 2018), Cryolock (Stein et al., 2015) have made great improvements, but they are more suitable for freezing small numbers of sperm than single sperm. Carriers that can freeze single sperm and have

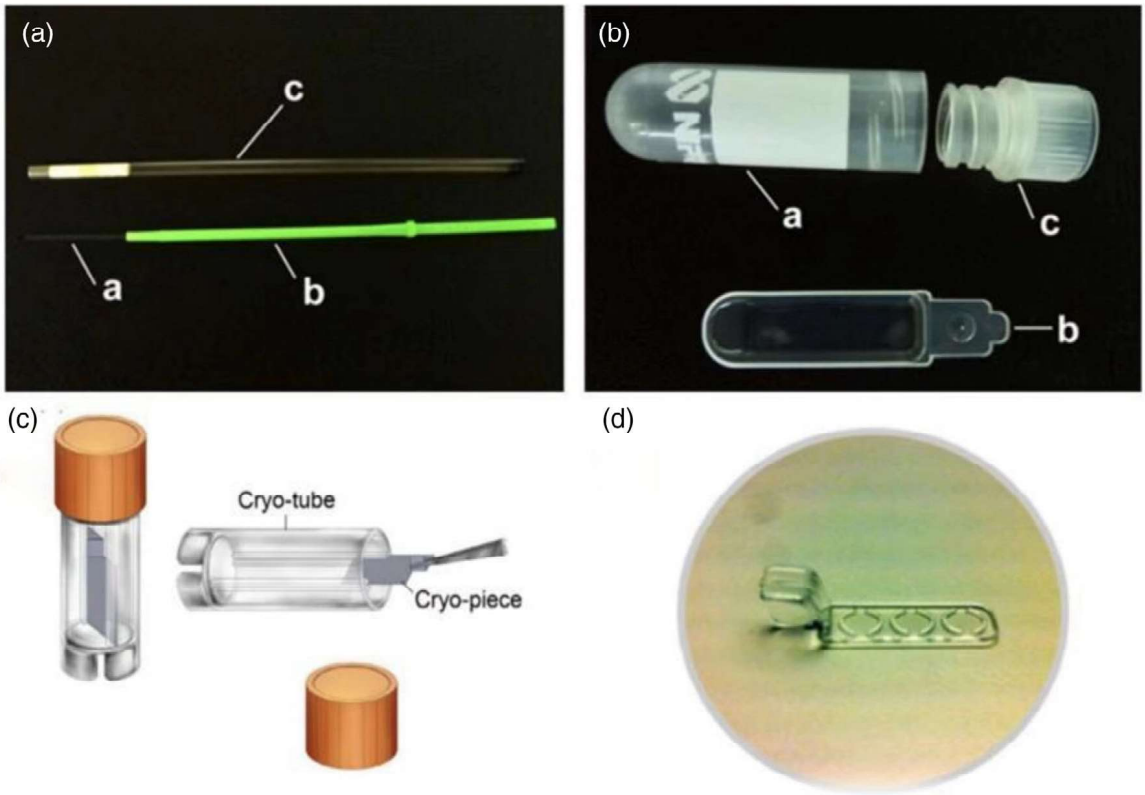

FIGURE 1 The carriers that can freeze individual spermatozoa and have accomplished successful pregnancies include Cryotop, Cell Sleeper, Cryopiece and SpermVD. (a) Cryotop (Adapted from Endo, Fujii, Kurotsuchi, et al., 2012). (b) Cell Sleeper (Adapted from Endo, Fujii, Kurotsuchi, et al., 2012). (c) Cryopiece (Adapted from Sun et al., 2017). (d) SpermVD (Adapted from Berkovitz et al., 2018).

accomplished successful pregnancies include Cryotop (Endo, Fujii, Kurotsuchi, et al., 2012), Cell Sleeper (Endo, Fujii, Shintani, et al., 2012), Cryopiece (Chen et al., 2022; Sun et al., 2017) and SpermVD (Berkovitz et al., 2018) (Figure 1).

Although the clinical application value of these carriers for freezing single sperm is significantly higher than previous carriers, in practice, these flakelike carriers still have some drawbacks. These carriers except for SpermVD are usually placed directly on the bottom of the petri dish, which inevitably creates a height difference between the carrier surface and the bottom of the dish. During sperm transfer, embryologists must adjust the height of ICSI needle frequently to accommodate this height difference. Meanwhile, the light and thin carriers may also move or even float due to the movement of the platform. All these drawbacks can cause operational difficulties for embryologists. Therefore, it can be considered that an ideal carrier designed for small numbers of sperm especially single sperm needs to be further explored (Abdelhafez et al., 2009; Liu & Li, 2020).

Here, we introduced an individual sperm freezing device named SpermCD, which consists of a RA-Cryopiece and a grooved petri dish. The thickness of RA-Cryopiece's flat sheet is the same as the depth of the groove. After placing RA-Cryopiece into the groove, the surface of the carrier and the petri dish will be on the same focal plane, which allowing the embryologists perform sperm transfer in one plane. RA-Cryopiece also has many advantages such as easy to mark and not easy to move or float.

So far, in our center, nine cases have thawed sperm using SpermCD. This device achieved a sperm recovery rate of 97.1 ± 4.6%. Normal fertilization and high-quality embryo rates were 68.0 ± 33.2% and 24.4 ± 22.2%. Four successful deliveries were achieved after embryos transfer in five patients using frozen sperm. This article will introduce the detailed operation procedure of this new device and the treatment course of nine cases.

2 | MATERIALS AND METHODS

2.1 | Carrier composition

SpermCD is a functional unit consisting of a RA-Cryopiece and a grooved petri dish (PinZhi Ltd.). This functional unit can perform sperm freezing and ICSI. RA-Cryopiece consists of a flat sheet and a vertical sheet. The flat sheet is used for loading freezing droplets and the vertical sheet is used for labeling and handling. The height of the flat sheet is consistent with the depth of the groove, ensuring that the surface of the flat sheet is in the same plane as the bottom of the dish (Figure 2). The outer cannula is a 1.8 ml Cryogenic Vial.

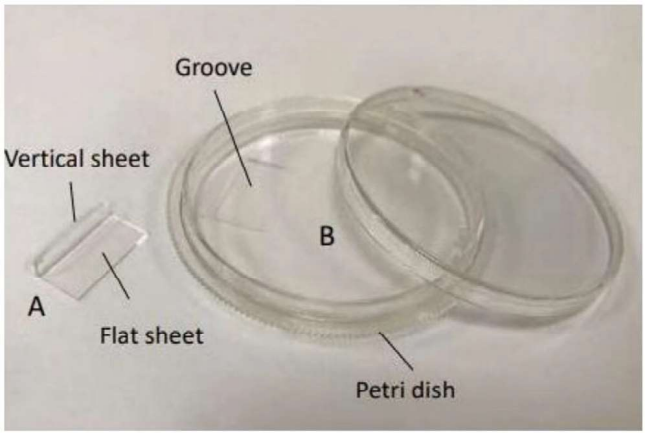

FIGURE 2 Composition of SpermCD. (a) RA-Cryopiece consists of a flat sheet and a vertical sheet. (b) A grooved petri dish.

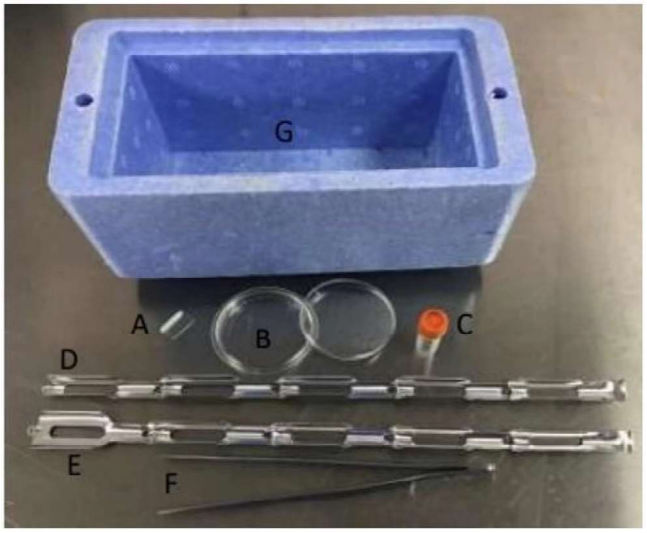

FIGURE 3 Consumables required for single sperm freezing. (a) and (b) are the same as those in Figure 2. (c) 1.8 ml Cryogenic Vial. (d) CryoCane. (e) CryoCane with a flat end. (f) Tweezers. (g) Foam box.

2.2 | Patients

Patients who meet the following conditions will be considered as candidates for single sperm freezing: (1) virtual azoospermia; (2) cryptozoospermia; (3) undergoing micro-TESE.

Rare motile sperm in the semen were frozen using SpermCD prior to oocyte pick-up. Testicular sperm found were also frozen regardless of motility. On the day of oocyte pick-up, frozen testicular sperm would be thawed directly. Patients with virtual azoospermia or cryptozoospermia attempted to ejaculate by masturbation first. If motile sperm was not found in fresh semen or there were not enough motile sperm, the previously frozen sperm would be thawed.

After thawing, the motile sperm was preferentially chosen for ICSI. If the motile sperm were not enough for the oocytes, chose immotile sperm with better shape. Whether to transplant fresh embryos depended on the patient's condition and treatment plan.

2.3 | Sperm specimen collection and processing

2.3.1 | Ejaculated sperm

Semen samples were collected by masturbation after 3–7 days abstinence. After liquefaction, semen was mixed with same volume of G-MOPS PLUS (Vitrolife Sweden AB.) and the mixture was centrifuged at 3000 g for 15 min. Removing the supernatant, 100 μl of floccule deposit was diluted and resuspended with 3 ml G-MOPS PLUS. After centrifugation at 3000 g for 5 min, the supernatant was carefully removed, leaving about 30 μl of medium above the pellet. Finally, the resuspended deposit was loaded on the microstrips in the grooved petri dish and left for 1 h for spermatozoa swimming out.

If the final resuspended deposit was sticky, added 50 μl of 80 IU/ml Hyaluronidase Solution (FUJIFILM Irvine Scientific Inc.). The pipettor was drawn repeatedly until the mixture became flowable. Three ml G-MOPS PLUS was added again, and the mixture was centrifuged at 3000 g for 5 min, leaving about 30 μl of medium above the pellet. Then, the final resuspended deposit was loaded on the microstrips and left for 1 h for spermatozoa swimming out.

2.4 | Testicular specimen

(Esteves & Varghese, 2012) surgically obtained testicular tissue was placed into a 60 × 15 mm IVF Round Dish (Falcon) containing 3 ml G-MOPS PLUS and blood clots were removed using the needled-tuberculin syringes. After initial washing, the seminiferous tubules were transferred to a new round dish containing 3 ml G-MOPS PLUS and then were mechanically minced using the needled-tuberculin syringes. The cell suspensions were examined under the inverted microscope to confirm the presence of sperm. Successful retrieval was defined as the presence of a certain number of sperm no matter the sperm was motile or not. After micro-TESE ended, all the minced seminiferous tubules were transferred into a 15 ml polypropylene conical tube. It took about 5 min for the large tissues to settle down. The supernatant was then transferred into a new conical tube and centrifuged at 3000 g for 15 min. Finally, the resulting 50 μl pellet was loaded on the microstrips for spermatozoa capture.

2.5 | Sperm capture and freezing procedure

Figure 3 shows the consumables required for single sperm freezing, and Figure 4 shows the entire freezing/thawing procedure based on SpermCD. As shown in Figure 4a, three 20 μl G-MOPS PLUS microstrips and three 5 μl PVP droplets were loaded on the bottom of the grooved petri dish, and then covered with mineral oil. Several of such dishes could be made if necessary. The dishes were equilibrated in the incubator without CO2 at 37°C for at least 30 min. Five μl of semen

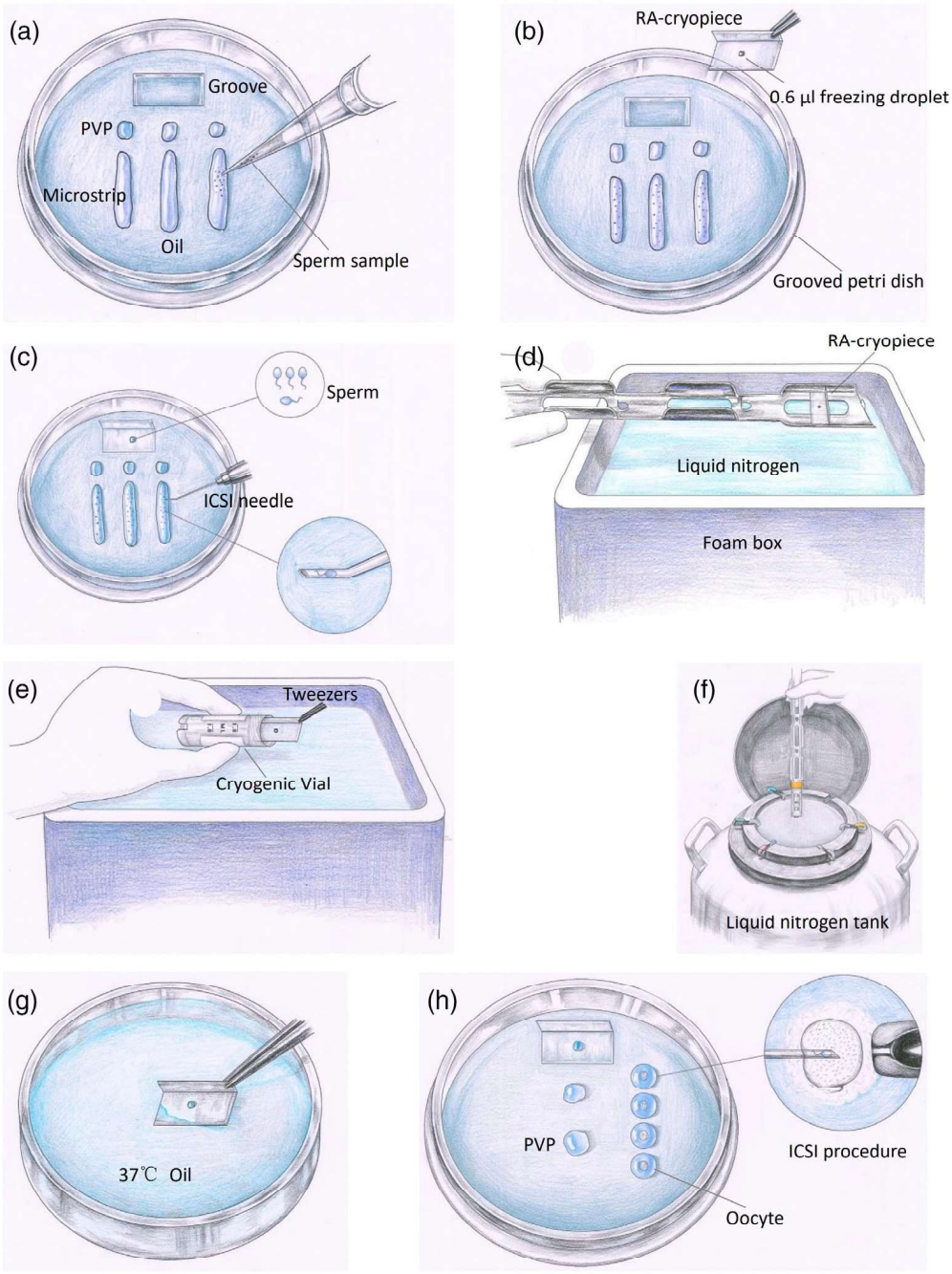

FIGURE 4 The model of SpermCD-based freezing/thawing procedure. (a) PVP and microstrips were loaded on the petri dish and covered with mineral oil. Then added sperm sample to the microstrips. (b) A 0.6 μl freezing droplet was placed onto the flat sheet of labelled RA-cryopiece. RA-cryopiece was then placed into the groove of the petri dish using sterile tweezers. (c) Sperm was transferred to freezing droplet one by one using ICSI needle. (d) RA-cryopiece was placed on CryoCane flattened at the end to enter the cooling steps. (e) Frozen RA-cryopiece was loaded into the labelled 1.8 ml cryogenic vial, and then stored in the liquid nitrogen tank (F). G. RA-cryopiece was transferred to a dish containing 5 ml 37°C mineral oil for 30 s to rewarm. (h) Sperm were selected for ICSI using micromanipulation apparatus.

precipitation or testicular sperm precipitation was added on each microstrip, and the sperm was left for 1 h to swim to the edge of the microstrips.

Freezing droplet was 50/50 v/v mixture of Quinn's Advantage Sperm Freezing Medium (Sage In-Vitro Fertilization Inc.) and G-MOPS PLUS medium. If sperm that met the freezing criteria was found in microstrips, a 0.6 μl of freezing droplet was placed onto RA-Cryopiece's flat sheet. RA-Cryopiece was then placed into the groove of an equilibrated petri dish using sterile tweezers (Figure 4b). Pressed on the flat sheet with the tips of the tweezers and gently moved to ensure the flat sheet was inserted into the groove. Sperm was transferred to freezing droplet one by one using micromanipulation instruments (Figure 4c). If it was semen sperm, only motile sperm was selected. If it was testicular sperm, captured all the sperm found.

After sperm capture ended, RA-Cryopiece was removed from the groove with tweezers and then placed vertically on sterile gauze to absorb the oil. Finally, RA-Cryopiece was placed on CryoCane (Thermo Scientific) flattened at the end and then entered the cooling steps (Figure 4d).

The cooling steps were as follows: first, within about 2.5 min, RA-Cryopiece slowly dropped from 6.5 cm above the liquid nitrogen surface to 4 cm above the liquid nitrogen surface. RA-Cryopiece was then left 4 cm above the liquid nitrogen surface for about 40 s until the 0.6 μl freezing droplet became a white solid. The empty labelled 1.8 ml Cryogenic Vial stayed on the liquid nitrogen surface for a few seconds before RA-Cryopiece was loaded into it (Figure 4e). Tightened the cryovial to prevent liquid nitrogen from entering and then stored it in the liquid nitrogen tank (Figure 4f).

2.6 | Ovarian stimulation and oocyte pick-up

Ovarian stimulation was performed using the routine long protocol of pituitary suppression followed by ovarian stimulation. We administered hCG 5000 IU when at least three follicles were about 18 mm in diameter. Oocyte retrieval was performed by vaginal ultrasound-guided follicular puncture approximately 35–36 h after hCG administration. Forty hours after hCG administration, the oocytes were denuded of cumulus cells and the MII oocytes were selected for injection (Palermo et al., 1992).

2.7 | Sperm thawing and ICSI procedure

As shown in Figure 4h, four 5 μl G-MOPS PLUS droplets and two 5 μl PVP droplets were placed respectively in the grooved petri dish, covered with mineral oil, and balanced at 37°C for at least 30 min.

After checking the patient's information, 1.8 ml Cryogenic Vial was pulled out from the liquid nitrogen tank. Unscrewed the cap of the cryovial using gauze and then RA-Cryopiece was poured into an empty round dish. Since RA-Cryopiece has a three-dimensional design, there is no need to worry about the frozen droplet touching any surface of the dish. RA-Cryopiece was then transferred to a dish containing 5 ml 37°C mineral oil for 30 s to rewarm (Figure 4g). Be careful that the tweezers do not touch the freezing droplet. RA-Cryopiece was subsequently placed into the groove of the petri dish. Pressed on RA-Cryopiece's flat sheet with the tips of the tweezers and gently moved to ensure the flat sheet was inserted into the groove. Sperm were selected for ICSI using micromanipulation apparatus (Figure 4h). After ICSI, all injected oocytes were transferred to G-1 PLUS medium (Vitrolife Sweden AB.) for culture.

The supplemented video shows the procedures including loading sperm deposit, adding freezing droplet, inserting RA-Cryopiece, sperm transfer, freezing–thawing process, and ICSI.

2.8 | Embryo culture, scoring and transfer

Oocytes were examined for fertilization 17–18 h after microinjection.

Morphological evaluation of the embryos at cleavage stage was implemented according to the Istanbul consensus (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). In our center, high-quality embryos are the sum of grade A embryos and grade B embryos. Transplantable embryos are the sum of grade A, B and C embryos.

Embryos were continuously cultured in G-2 PLUS medium (Vitrolife, Sweden AB.) to Day 5/6 for patients accepting preimplantation genetic testing (PGT) or having no high-quality embryo. Blastocyst morphology classification was evaluated using the Gardner scoring system (Gardner et al., 2004). Blastocysts better than grade 3CC were used for transfer, vitrification or biopsy.

Fresh embryo transfers were usually performed on day 3. Fresh embryo transfer was cancelled in patients with ovarian hyperstimulation syndrome (OHSS) predisposition or uneven endometrium.

2.9 | Embryo vitrification and thawing

Cryotop device and commercial freezing solutions (Kitazato Corp.) were used for embryos or blastocysts vitrification. Before vitrification, laser treatment was used to induce shrinkage of fully expanded blastocysts (Mukaida et al., 2006). Embryos or blastocysts were firstly equilibrated in equilibration solution for 5–10 min. Subsequently, embryos or blastocysts were put into vitrification medium and loaded onto the surface of the cryotop with a minimum volume within 1 min.

On the day of thawing embryo or blastocyst transfer, embryos or blastocysts were firstly transferred to 37°C thawing solution for 1 min, followed by 3 min in diluent solution and then washed twice in washing solution for 5 min. Warmed embryos or blastocysts were then cultured for at least 2 h prior to further evaluation. Cleavage-stage embryos with more than half the blastomeres surviving or re-expanded blastocysts were judged to be survived. Only survived embryos or blastocysts could be transferred.

2.10 | Pregnancy assessment

All patients underwent routine luteal phase support after transfer. Serum β-hCG assay was performed 12 days after embryo transfer or 10 days after blastocyst transfer. Clinical pregnancy was defined as a visible sac on the fifth gestational week.

2.11 | Ethical issues

The Medical Ethics Committees of Sir Run Run Shaw Hospital approved the study (licence number of ethics statement: SRRSHRMEC2018003), and informed consent was obtained from all participants.

2.12 | Statistical analysis

Descriptive parameters were expressed as mean ± SD. Calculations were performed by IBM SPSS 21.0 software (SPSS Inc.).

3 | RESULTS

3.1 | Sperm freezing

From January 2019 to October 2021, 35 patients underwent single sperm cryopreservation using SpermCD, including 4 patients with NOA, 14 patients with virtual azoospermia and 17 patients with cryptozoospermia.

3.2 | Thawing results

On the day of the oocyte retrieval, frozen testicular spermatozoa were thawed in two patients with NOA. Frozen ejaculated spermatozoa were thawed in seven patients with virtual azoospermia because motile sperm were not found or not enough in fresh semen. One hundred and twenty-five cryopreserved spermatozoa were thawed and 121 were retrieved, with a mean recovery rate of 97.1 ± 4.6%. The recovery of 44 thawed motile spermatozoa accounted for 35.7 ± 19.0% of all retrieved spermatozoa (Table 1).

3.3 | Fertility outcomes of frozen–thawed spermatozoa

Normal fertilization and high-quality embryo rates were 68.0 ± 33.2% and 24.4 ± 22.2% after using frozen sperm. Nineteen transplantable embryos were formed after fertilization, eight of which were transplanted in five couples, resulting in four singleton deliveries. The remaining 11 transplantable embryos were in cryopreservation (Table 2).

3.3.1 | Cases 1

Frozen ejaculated sperm were thawed for ICSI. Two embryos were transferred, and a singleton pregnancy was achieved, leading to the term delivery of a healthy female infant with a birth weight of 4350 g.

3.3.2 | Case 2

PGT was performed due to the microdeletion of azoospermic factor (AZF) c zone. Seven MII oocytes were injected with frozen sperm. One blastocyst was formed but could not be transplanted after examination.

TABLE 1 Sperm recovery rate and motility rate after thawing

Case	Frozen spermatozoa (*n*)	Motile frozen spermatozoa (*n*)	Recovery rate	Post-thaw motility rate
1	12	12	100% (12/12)	41.7% (5/12)
2	20	20	100% (20/20)	40.0% (8/20)
3	15	15	100% (15/15)	46.7% (7/15)
4	15	5	93.3% (14/15)	7.1% (1/14)
5	12	12	100% (12/12)	50.0% (6/12)
6	15	15	93.3% (14/15)	35.7% (5/14)
7	16	16	87.5% (14/16)	50% (7/14)
8	10	10	100% (10/10)	50.0% (5/10)
9	10	4	100% (10/10)	0% (0/10)
Mean ± SD			97.1 ± 4.6%	35.7 ± 19.0%

Note: Recovery rate, number of retrieved spermatozoa/number of frozen spermatozoa %. Post-thaw motility rate, number of retrieved motile spermatozoa/number of retrieved spermatozoa %.

TABLE 2 Fertilization, embryonic quality, and pregnancy outcomes after ICSI using frozen sperm in nine couples

Case	Female age (year)	MII oocytes (*n*)	Fertilization rate	High-quality embryo rate	Transferred embryos (*n*)	Pregnancy	Remaining embryos (*n*)
1	25	7	28.6%	50%	2	Delivery	
2	30	7	71.4%	20%	Cancel		One blastocyst
3	34	6	66.7%	50%	2	Delivery	
4	29	11	81.8%	11.1%	1	Delivery	One embryo
5	35	6	83.3%	0%	Cancel		
6	26	6	0%		Cancel		
7	32	10	80%	50%	1	Delivery	Five embryos
8	30	5	100%	0%			One blastocyst
9	31	7	100%	14.3%	2	No	Four embryos
Mean ± SD			68.0 ± 33.2%	24.4 ± 22.2%			

Note: One blastocyst in case 2 was deemed untransferable after biopsy and testing. No blastocyst was formed in case 5 accepting PGT. All six MII oocytes were unfertilized in case 6. In case 8, successful pregnancy was achieved after transplantation of two embryos from fresh sperm. Case 9 underwent one cycle of frozen embryo transfer and had no pregnancy.

3.3.3 | Case 3

Frozen ejaculated sperm were thawed for ICSI. Two embryos were transferred, and a singleton pregnancy was achieved. A 2950 g healthy male infant was delivered naturally at term.

3.3.4 | Case 4

Frozen testicular sperm was used, and an embryo was transplanted, resulting in a singleton pregnancy. A 4735 g healthy male infant was delivered at term by Caesarean section. One remaining embryo was in cryopreservation.

3.3.5 | Case 5

PGT was performed due to the microdeletion of AZFc zone. 83.3% fertilization rates were achieved after using frozen semen sperm, but no blastocyst was formed.

3.3.6 | Case 6

All six MII oocytes were unfertilized after using frozen semen sperm.

3.3.7 | Case 7

Fifteen oocytes were harvested, 10 of which were mature and inseminated with frozen semen sperm. Fresh embryo transfer was cancelled due to OHSS predisposition, and 6 embryos were in cryopreservation. Three months later, one thawed embryo was transplanted, resulting in a successful pregnancy. A 3830 g healthy male infant was delivered naturally at term.

3.3.8 | Case 8

As fresh ejaculated sperm was not enough, six MII oocytes were injected with fresh sperm and five MII oocytes were injected with frozen sperm. After transplantation of two embryos from fresh sperm, the wife was pregnant and eventually delivered to term. Five grade C embryos from frozen sperm were continuously cultured, forming one transferrable blastocyst which was currently in cryopreservation.

3.3.9 | Case 9

Ten oocytes were harvested, seven of which were mature and inseminated with frozen testicular sperm. Six embryos were frozen because the patient's endometrial condition was deemed unsuitable for fresh embryo transfer. The patient underwent one cycle of frozen embryo transfer and had no pregnancy. Four remaining embryos were currently in cryopreservation.

4 | DISCUSSION

Conventional sperm cryopreservation techniques can result in sperm loss owing to centrifugation, washing procedures and sperm adherence to the carrier vessel (Abdelhafez et al., 2009; Liu & Li, 2020). It is not appropriate to freeze rare sperm using conventional freezing methods.

Rare sperm mainly refer to sperm from patients with virtual azoospermia (Miller et al., 2017) or cryptozoospermia (Herbemont et al., 2021), and sperm from small spermatogenic foci obtained surgically (Corona et al., 2019). Freezing rare sperm in advance ensures that the husband's sperm will be used on the day of oocyte retrieval.

Rare sperm, especially testicular sperm obtained by micro-TESE, is extremely precious and should be fully utilized. Individual sperm freezing technique with high sperm recovery rate ensures that testicular sperm can be frozen in multiple cryopreservation tubes, thus satisfying subsequently multiple ovulation induction treatments (Han et al., 2021; Lin et al., 2022; Chen et al., 2022). Individual sperm freezing technique allows patients to avoid a second testicular surgery to the greatest extent (Tsujimura, 2007).

Except for the earliest spherical carriers, most of the current rare sperm freezing carriers are transparent sheets made of polypropylene or polystyrene materials (Peng et al., 2011; Endo, Fujii, Kurotsuchi, et al., 2012; Endo, Fujii, Shintani, et al., 2012; Stein et al., 2015; Sun et al., 2017; Berkovitz et al., 2018; Wang et al., 2018; Ziarati et al., 2019; Huang et al., 2020; Lin et al., 2021; Zhu et al., 2021; Chen et al., 2022). Freezing sheets can be homemade, commercial or modified on a commercial basis. To ensure a high recovery rate, rare sperm are frozen in a small droplet on the freezing sheet. After thawing, sperm are chosen for ICSI without washing procedure. It's a good idea, but it's not easy in practice. Most flakelike carriers are usually placed directly on the bottom of the petri dish, which inevitably creates a height difference between the carrier surface and the bottom of the dish. Embryologists have to adjust the height of the ICSI needle frequently to transfer sperm between the bottom of the dish and the surface of the carrier (Chen et al., 2022; Endo, Fujii, Kurotsuchi, et al., 2012; Endo, Fujii, Shintani, et al., 2012; Peng et al., 2011; Sun et al., 2017). To date, only SpermCD and SpermVD have eliminated the height difference.

Carriers made of polypropylene or polystyrene materials are very thin and light (Peng et al., 2011; Endo, Fujii, Kurotsuchi, et al., 2012; Endo, Fujii, Shintani, et al., 2012; Sun et al., 2017; Lin et al., 2021; Chen et al., 2022). Based on our experience, the carrier may shift position or even float when moving the platform of an inverted microscope. It takes the embryologist extra time to return the carrier to its original position or press it to the bottom of the dish. The carrier movement can mess up the layout of the petri dish and, in the worst cases, may cause the embryologist fail to perform sperm transfer

during freezing and ICSI procedure. Floating carriers that are not repositioned in time may cause the ICSI needle to break.

When placing RA-Cryopiece into the groove, press this carrier with tweezers and gently move it, which will ensure that RA-Cryopiece is embedded in the groove. The embedded carrier will not move. The weight of RA-Cryopiece itself and the tension between RA-Cryopiece and the groove greatly reduces the probability of the carrier floating.

The vertical plate of RA-Cryopiece can be easily labelled, ensuring that the embryologist can always check the patient's information during any step of micromanipulation. The three-dimensional design of RA-Cryopiece also protects the freezing droplet loaded with sperm, because the droplet never touches any plane, no matter what position the carrier is in. The outer cannula of RA-Cryopiece is a conventional sperm cryovial which is easy to get and relatively sealed.

A total of 125 sperm were thawed in 9 patients, and 121 sperm were found after thawing, with a mean recovery rate of 97.1 ± 4.6%. Normal fertilization and high-quality embryo rates were 68.0 ± 33.2% and 24.4 ± 22.2%, indicating that SpermCD did not cause serious damage to sperm fertilization ability and sperm quality. High recovery rate and four successful deliveries indicate clinical application value of this device. Meanwhile, the thawed ejaculated sperm were all from patients with virtual azoospermia not cryptozoospermia, suggesting that single sperm freezing technology is more suitable for patients with occasional presence of spermatozoa.

Studies have shown that sperm motility and DNA integrity were increased after gradient centrifugation (Fariello et al., 2009; Zhao et al., 2016). However, to minimize the sperm loss, in the present study, the patient's semen was directly centrifuged instead of gradient centrifugation. The centrifuged precipitate may be very sticky, but this question can be solved by adding Hyaluronidase Solution. There are many impurities in the precipitate, which can be diluted by 20 μl microstrips in the petri dish. Meanwhile, the sperm was left for 1 h to swim to the edge of the microstrips. These procedures ensure that sperm can be found in the precipitate as many as possible.

In conclusion, embryologists often spend a long time searching for sperm when sperm counts are extremely low. An ideal carrier should be easy to prepare and use. SpermCD described in this article has been commercialized in China. SpermCD eliminates the height difference, allowing embryologists to transfer sperm and perform ICSI on the same focal plane. RA-Cryopiece is easy to mark and not easy to move or float. High enough recovery rate and 4 successful deliveries in this study confirmed its practicality and effectiveness. SpermCD is a simple and user-friendly device for freezing individual spermatozoa.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at https://figshare.com, reference number 10.6084/m9.figshare.19361279.

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REFERENCES

AbdelHafez, F., Bedaiwy, M., El-Nashar, S. A., Sabanegh, E., & Desai, N. (2009). Techniques for cryopreservation of individual or small numbers of human spermatozoa: A systematic review. *Human Reproduction Update*, *15*(2), 153–164. https://doi.org/10.1093/humupd/dmn061

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. (2011). The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Human Reproduction (Oxford, England)*, *26*(6), 1270–1283. https://doi.org/10.1093/humrep/der037

Bendikson, K. A., Neri, Q. V., Takeuchi, T., Toschi, M., Schlegel, P. N., Rosenwaks, Z., & Palermo, G. D. (2008). The outcome of intracytoplasmic sperm injection using occasional spermatozoa in the ejaculate of men with spermatogenic failure. *The Journal of Urology*, *180*(3), 1060–1064. https://doi.org/10.1016/j.juro.2008.05.025

Berkovitz, A., Miller, N., Silberman, M., Belenky, M., & Itsykson, P. (2018). A novel solution for freezing small numbers of spermatozoa using a sperm vitrification device. *Human Reproduction (Oxford, England)*, *33*(11), 1975–1983. https://doi.org/10.1093/humrep/dey304

Chen, W., Huang, C., Li, P., Liu, F., Sun, J., Zhu, Z. J., Zhai, J., Xu, Y., Hong, Y., Hu, J. L., Peng, Y. P., Zhang, Z. B., Wu, Y., & Li, Z. (2022). Clinical benefits of a modified Cryopiece system for cryopreservation of rare ejaculated and testicular spermatozoa for ICSI. *Asian Journal of Andrology*, *24*, 533–539. https://doi.org/10.4103/aja2021101

Cohen, J., Garrisi, G. J., Congedo-Ferrara, T. A., Kieck, K. A., Schimmel, T. W., & Scott, R. T. (1997). Cryopreservation of single human spermatozoa. *Human Reproduction (Oxford, England)*, *12*(5), 994–1001. https://doi.org/10.1093/humrep/12.5.994

Corona, G., Minhas, S., Giwercman, A., Bettocchi, C., Dinkelman-Smit, M., Dohle, G., Fusco, F., Kadioglou, A., Kliesch, S., Kopa, Z., Krausz, C., Pelliccione, F., Pizzocaro, A., Rassweiler, J., Verze, P., Vignozzi, L., Weidner, W., Maggi, M., & Sofikitis, N. (2019). Sperm recovery and ICSI outcomes in men with non-obstructive azoospermia: A systematic review and meta-analysis. *Human Reproduction Update*, *25*(6), 733–757. https://doi.org/10.1093/humupd/dmz028

Desai, N. N., Blackmon, H., & Goldfarb, J. (2004). Single sperm cryopreservation on cryoloops: An alternative to hamster zona for freezing individual spermatozoa. *Reproductive Biomedicine Online*, *9*(1), 47–53. https://doi.org/10.1016/s1472-6483(10)62109-8

Endo, Y., Fujii, Y., Kurotsuchi, S., Motoyama, H., & Funahashi, H. (2012). Successful delivery derived from vitrified-warmed spermatozoa from a patient with nonobstructive azoospermia. *Fertility and Sterility*, *98*(6), 1423–1427. https://doi.org/10.1016/j.fertnstert.2012.07.1128

Endo, Y., Fujii, Y., Shintani, K., Seo, M., Motoyama, H., & Funahashi, H. (2012). Simple vitrification for small numbers of human spermatozoa. *Reproductive Biomedicine Online*, *24*(3), 301–307. https://doi.org/10.1016/j.rbmo.2011.11.016

Esteves, S. C., & Varghese, A. C. (2012). Laboratory handling of epididymal and testicular spermatozoa: What can be done to improve sperm injections outcome. *Journal of Human Reproductive Sciences*, *5*(3), 233–243. https://doi.org/10.4103/0974-1208.106333

Fariello, R. M., del Giudice, P. T., Spaine, D. M., Fraietta, R., Bertolla, R. P., & Cedenho, A. P. (2009). Effect of leukocytospermia and processing by discontinuous density gradient on sperm nuclear DNA fragmentation and mitochondrial activity. *Journal of Assisted*

Reproduction and Genetics, *26*(2–3), 151–157. https://doi.org/10.1007/s10815-008-9288-0

Gardner, D. K., Surrey, E., Minjarez, D., Leitz, A., Stevens, J., & Schoolcraft, W. B. (2004). Single blastocyst transfer: A prospective randomized trial. *Fertility and Sterility*, *81*(3), 551–555. https://doi.org/10.1016/j.fertnstert.2003.07.023

Han, S., Zhao, L., Yang, C., Xu, J., Yao, C., Huang, C., Zhang, H., Ji, Z., Luo, J., Guo, Y., Hong, Y., Liu, F., Zhou, X., Zhou, Z., Huang, Y., & Li, Z. (2021). Vitrification with microinjection of single seminiferous tubules: An efficient cryopreservation approach for limited testicular tissue. *Reproductive Biomedicine Online*, *43*(4), 687–699. https://doi.org/10.1016/j.rbmo.2021.06.026

Herbemont, C., Mnallah, S., Bennani-Smires, B., Peigne, M., Cedrin-Durnerin, I., Grynberg, M., & Sifer, C. (2021). Cryopreservation of small numbers of human spermatozoa in a stripper tip: Report of the first live-birth worldwide. *Cryobiology*, *99*, 103–105. https://doi.org/10.1016/j.cryobiol.2021.01.005

Huang, C., Gan, R. X., Zhang, H., Zhou, W. J., Huang, Z. H., Jiang, S. H., Ji, X. R., Gong, F., Fan, L. Q., & Zhu, W. B. (2020). Novel micro-straw for freezing small quantities of human spermatozoa. *Fertility and Sterility*, *114*(2), 301–310. https://doi.org/10.1016/j.fertnstert.2020.03.032

Just, A., Gruber, I., Wöber, M., Lahodny, J., Obruca, A., & Strohmer, H. (2004). Novel method for the cryopreservation of testicular sperm and ejaculated spermatozoa from patients with severe oligospermia: A pilot study. *Fertility and Sterility*, *82*(2), 445–447. https://doi.org/10.1016/j.fertnstert.2003.12.050

Lin, F., & Zheng, B. (2022). Cryopreservation of human spermatozoa by freezing testicular seminiferous tubule with novel cryopiece. *Andrologia*, *54*(3), e14356. https://doi.org/10.1111/and.14356

Liu, S., & Li, F. (2020). Cryopreservation of single-sperm: Where are we today? *Reproductive Biology and Endocrinology: RB&E*, *18*(1), 41. https://doi.org/10.1186/s12958-020-00607-x

Miller, N., Biron-Shental, T., Pasternak, Y., Belenky, M., Shefi, S., Itsykson, P., & Berkovitz, A. (2017). Fertility outcomes after extended searches for ejaculated spermatozoa in men with virtual azoospermia. *Fertility and Sterility*, *107*(6), 1305–1311. https://doi.org/10.1016/j.fertnstert.2017.04.005

Mukaida, T., Oka, C., Goto, T., & Takahashi, K. (2006). Artificial shrinkage of blastocoeles using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. *Human Reproduction (Oxford, England)*, *21*(12), 3246–3252. https://doi.org/10.1093/humrep/del285

Palermo, G., Joris, H., Devroey, P., & Van Steirteghem, A. C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet (London, England)*, *340*(8810), 17–18. https://doi.org/10.1016/0140-6736(92)92425-f

Peng, Q. P., Cao, S. F., Lyu, Q. F., Xue, S. G., Jin, W., Liu, X. Y., Zhang, W. J., Nielsen, H. I., & Kuang, Y. P. (2011). A novel method for cryopreservation of individual human spermatozoa. In vitro cellular & developmental biology. *Animal*, *47*(8), 565–572. https://doi.org/10.1007/s11626-011-9428-1

Sereni, E., Bonu, M. A., Fava, L., Sciajno, R., Serrao, L., Preti, S., Distratis, V., & Borini, A. (2008). Freezing spermatozoa obtained by testicular fine needle aspiration: A new technique. *Reproductive Biomedicine Online*, *16*(1), 89–95. https://doi.org/10.1016/s1472-6483(10)60560-3

Stein, A., Shufaro, Y., Hadar, S., Fisch, B., & Pinkas, H. (2015). Successful use of the Cryolock device for cryopreservation of scarce human ejaculate and testicular spermatozoa. *Andrology*, *3*(2), 220–224. https://doi.org/10.1111/andr.12007

Sun, J., Chen, W., Zhou, L., Hu, J., Li, Z., Zhang, Z., & Wu, Y. (2017). Successful delivery derived from cryopreserved rare human spermatozoa with novel cryopiece. *Andrology*, *5*(4), 832–837. https://doi.org/10.1111/andr.12380

Tsujimura, A. (2007). Microdissection testicular sperm extraction: Prediction, outcome, and complications. *International journal of urology: official journal of the Japanese Urological Association*, *14*(10), 883–889. https://doi.org/10.1111/j.1442-2042.2007.01828.x

Wang, M., Wu, Z., Hu, Y., Wang, Y., Tan, Y., Xiang, Y., Wang, L., Jin, L., & Huang, H. (2018). An adapted carrier for the cryopreservation of human testicular spermatozoa. *Reproductive Biomedicine Online*, *37*(5), 590–599. https://doi.org/10.1016/j.rbmo.2018.08.021

World Health Organization. (2010). *WHO Laboratory manual for the examination and processing of human semen* (5th ed.). World Health Organization Press.

Zhao, F., Yang, Q., Shi, S., Luo, X., & Sun, Y. (2016). Semen preparation methods and sperm telomere length: Density gradient centrifugation versus the swim up procedure. *Scientific Reports*, *6*, 39051. https://doi.org/10.1038/srep39051

Zhu, Z. J., Zhai, J., Hu, J. L., Wang, Y. Z., Chen, W., Liu, F., Huang, Y. H., & Li, Z. (2021). Cryopiece, a novel carrier with faster cooling rate, high recovery rate and retrieval rate, for individual sperm cryopreservation. *Translational andrology and urology*, *10*(3), 1121–1132. https://doi.org/10.21037/tau-20-1080

Ziarati, N., Topraggaleh, T. R., Rahimizadeh, P., Montazeri, L., Maroufizadeh, S., Sadighi Gilani, M. A., & Shahverdi, A. (2019). Micro-quantity straw as a carrier for cryopreservation of oligozoospermic semen samples: Effects of storage times and cryoprotectant. *Cryobiology*, *86*, 65–70. https://doi.org/10.1016/j.cryobiol.2018.12.003

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