Asian Journal of Andrology (2022) 24, 533–539
www.asiaandro.com; www.ajandrology.com

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ORIGINAL ARTICLE

Assisted Reproduction Technology

Clinical benefits of a modified Cryopiece system for cryopreservation of rare ejaculated and testicular spermatozoa for ICSI

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Cryopreservation of rare testicular-retrieved spermatozoa for intracytoplasmic sperm injection (ICSI) in patients with severe oligozoospermia and azoospermia remains a major challenge in clinical practice. This study evaluated the Cryopiece system as a potential technique to cryopreserve rare human spermatozoa for ICSI. Small numbers of ejaculated (24 patients) and testicular (13 patients) spermatozoa were cryopreserved using the Cryopiece system. The total number of recovered spermatozoa and motility were assessed after thawing. Thirty-seven couples underwent ICSI using spermatozoa cryopreserved by the Cryopiece system, and ICSI outcomes (rates of fertilization, embryo cleavage, and clinical pregnancy) were evaluated. The average sperm post-thaw retrieval rate was 79.1%, and motility was 29.7%. Ejaculated spermatozoa had a higher post-thaw motility (32.5%) than testicular spermatozoa (21.8%; *P* = 0.005). ICSI achieved a fertilization rate of 61.9%, embryo cleavage rate of 84.6%, and clinical pregnancy rate of 43.3%. The ICSI outcomes in the ejaculated and testicular frozen-thawed spermatozoa were similar. Assisted oocyte activation (AOA) after ICSI with motile (72.1%) or immotile (71.9%) spermatozoa resulted in a significantly higher fertilization rate than that when using motile spermatozoa without AOA (52.0%; *P* = 0.005). However, AOA did not enhance the clinical pregnancy rate (55.6% or 40.0% *vs* 35.3%; *P* = 0.703). The Cryopiece system is simple and useful for the cryopreservation of small numbers of ejaculated or testicular spermatozoa for ICSI in patients with severe oligozoospermia or nonobstructive azoospermia.

Asian Journal of Andrology (2022) **24**, 533–539; doi: 10.4103/aja2021101; published online: 21 January 2022

Keywords: Cryopiece system; intracytoplasmic sperm injection outcome; rare sperm cryopreservation

INTRODUCTION

Cryopreservation of human spermatozoa plays an important role in clinical assisted reproduction technology (ART), especially in patients with severe oligozoospermia (SOZ) or for testicular spermatozoa in patients with nonobstructive azoospermia (NOA). Cryopreservation of such a small number of spermatozoa is clinically important, as it could help avoid repeated surgical procedures for sperm extraction[1] and mitigate the risk of not finding spermatozoa in the next ejaculate in patients with SOZ. However, conventional sperm cryopreservation methods are inadequate for small numbers of spermatozoa from such patients. Many methods have been reported, including empty zona pellucida,[2] Cryoloop,[3] Cryotop,[4] Cell Sleeper,[5] and sperm vitrification device (VD).[6] Although a successful pregnancy had been reported following intracytoplasmic sperm injection (ICSI) with empty zona pellucida cryopreserved spermatozoa,[7] this method is not practical for routine use. Several other studies reported achieving live births following ICSI with spermatozoa cryopreserved with various other carriers.[6,8]

We have recently developed the Cryopiece system (Gao Hong Biotechnology Technology Co., Ltd., Shanghai, China) for cryopreservation of small numbers of spermatozoa from patients with SOZ or NOA and achieved three deliveries after ICSI.[9] This study aimed to evaluate the efficiency of the Cryopiece system for cryopreservation of small numbers of spermatozoa for ICSI, testing it in 37 infertile couples.

PARTICIPANTS AND METHODS

Ethical approval

The clinical information was obtained from the Department of Andrology and Department of ART, Shanghai General Hospital, Shanghai Jiao Tong University (Shanghai, China). The Research and Ethics Committee of Shanghai Jiao Tong University School of Medicine approved this study (license number: 2016KY196). All participating patients signed informed consent.

Patients and sperm cryopreservation with the Cryopiece system

Thirty-nine ICSI cycles for 37 couples were performed with frozen-thaw spermatozoa using the Cryopiece system (**Figure 1**) from

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Received: 04 June 2021; Accepted: 11 November 2021

January 2016 to December 2019. The couples had no indication of female factors infertility. Females aged >38 years were excluded from the study. All male partners had a very small number of spermatozoa in their ejaculates or the testicular sperm extraction (TESE) biopsies.

Two masturbations with a 2-h interval were recommended to SOZ patients to increase the probability of obtaining spermatozoa in their ejaculates. After liquefaction, the ejaculated semen was centrifuged at 500*g* for 10 min (Eppendorf 5804, Eppendorf, Hamburg, Germany), the pellet was washed with modified human tubal fluid (mHTF; Irvine Scientific, Santa Ana, CA, USA) and centrifuged for another 10 min. Testicular sperm was retrieved by microsurgical TESE (m-TESE). The testicular tissue was dissected using two 1-ml syringe needles, suspended in mHTF, and centrifuged as above. The sperm pellet (100 μl) was spread in a plastic dish (BD Falcon, Durham, NC, USA; **Figure 2a**). The cryoprotectant (CPA) used for the Cryopiece system was a mixture of commercial sperm freezing medium (Origio, Malov, Denmark) and mHTF in equal parts, prepared about 30 min before use. In most NOA cases, less than 20 testicular spermatozoa were found after TESE.

Figure 1: The Cryopiece spermatozoa cryopreservation device.

The Cryopiece system procedure used in this study was slightly modified from our original method, as shown in **Figure 2**. The Cryopiece was firmly attached to the cryotube cap to facilitate smooth cryopreservation and thawing procedures. Two circles on the Cryopiece (**Figure 2a**), labeled M (motile) and I (immotile), marked the positions where CPA microdroplets (1–2 μl) were placed. The sperm pellet (200 μl) was spread on the bottom of a dish (Falcon 353003, BD Falcon), and prewarmed mineral oil (Vitrolife, Göteborg, Sweden) was added to form a thin layer over it. The dish was incubated for 15 min at 37°C. Motile (including tail twitching) spermatozoa with normal head morphology were collected using the ICSI pipettes (Origio) on an inverted microscope and transferred to the CPA M-drop on the Cryopiece (**Figure 2b**). If no motile spermatozoa were found, a small drop of pentoxifylline (3 mmol l^{-1}) was added to the sperm pellet. If pentoxifylline failed to stimulate motility, the hypo-osmotic swelling test (HOST)[10] or the laser method[11] was used to help find live spermatozoa. Live immotile spermatozoa with normal head morphology were collected and placed in the CPA I-drop on the Cryopiece. Residual mineral oil on the Cryopiece was absorbed with a sterile blotting paper before cryopreservation. The cap with the attached Cryopiece was screwed into a Cryotube, incubated at –20°C for 10 min, followed by liquid nitrogen vapors for 5 min, and then stored in liquid nitrogen.

Spermatozoa thawing, ICSI, and assisted oocyte activation (AOA)

Only motile spermatozoa were thawed and used for ICSI in this study. An ICSI dish containing 3 ml of mineral oil was preheated and incubated at 37°C for at least 1 h. The Cryopiece was pulled out of the cryotube, immersed immediately in the prewarmed mineral oil in the ICSI dish, and cultured for 10 min in an incubator (**Figure 2c**). The Cryopiece was observed carefully on the inverted microscope (Ti-U, Nikon, Tokyo, Japan) to determine the location and motility of spermatozoa in the CPA droplet. Spermatozoa were individually selected and placed in a

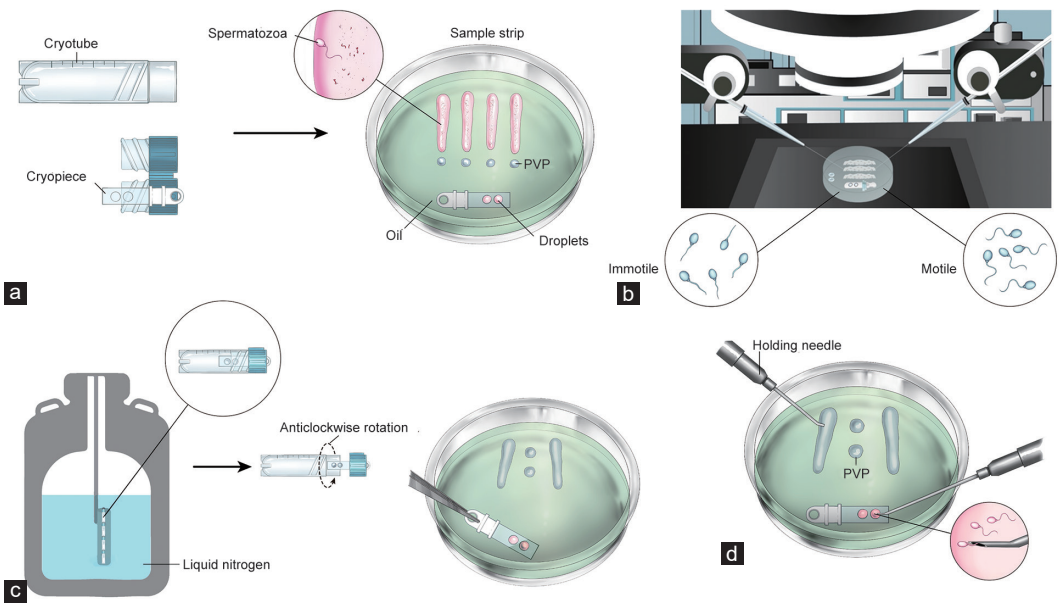

Figure 2: A Cryopiece system model based on the freezing, thawing, and ICSI procedures. (**a**) The cryotube and Cryopiece constitute the Cryopiece system. The Cryopiece with cryoprotectant droplets is placed on the bottom of the dish. Microstrips of semen pellet and polyvinylpyrrolidone (PVP) droplets are also placed directly on the dish, and the entire system is then covered with mineral oil. (**b**) Motile and immotile spermatozoa are collected and loaded into different droplets on the Cryopiece. (**c**) The cryopreserved samples are stored in liquid nitrogen. The Cryopiece with the spermatozoa is placed on the bottom of the dish, covered with the prewarmed mineral oil for thawing. (**d**) The recovered spermatozoa are prepared for ICSI. ICSI: intracytoplasmic sperm injection.

microdroplet of 20 μl containing G-Gamete medium (Vitrolife; **Figure 2d**). Selected motile spermatozoa in the microdroplet were used for ICSI. In some cases, after the patient provided written informed consent, immotile thawed spermatozoa were used for ICSI when no motile spermatozoa or too few motile spermatozoa were found. AOA was performed in some cases by exposing the oocytes to 10 μmol l^{-1} ionomycin (Sigma-Aldrich, St. Louis, MO, USA) for 10 min after ICSI. Briefly, a 4-well round-bottom plate (Thermo Scientific Fisher, San Rafael, CA, USA) was filled with 500 μl IVF-plus (Vitrolife); 5 μl ionomycin (1 mmol l^{-1}) was added to one well to prepare a 10 μmol l^{-1} ionomycin solution. The injected oocytes were transferred to the ionomycin medium 1 h after ICSI and incubated for 10 min at 37°C. The oocytes were then washed sequentially in the other three wells to remove residual ionomycin. The AOA-treated oocytes were cultured in G-1 plus medium (Vitrolife), and fertilization was assessed 16 h to 18 h after ICSI. Fertilized oocytes (two pronuclei, 2PN; one pronucleus, 1PN; and two polar bodies, 2PB) were cultured in G-1 plus medium (Vitrolife) in an incubator at 37°C with 6% CO_2, 5% O_2, and 89% N_2. Embryos were transferred on day 2 or day 3.

Ovarian stimulation and pregnancy outcome

Ovarian stimulation was achieved using a gonadotropin-releasing hormone (GnRH) antagonist (Cetrorelix, Merck Serono, Berlin, Germany), recombinant follicle-stimulating hormone (FSH; Gonal-F, Merck Serono, Berne, Switzerland), and human menopausal gonadotropin (hMG, Livzon Pharmaceutical Group Inc., Zhuhai, China). Ultrasound-guided follicular puncture was performed 36 h after injecting human chorionic gonadotropin (hCG, Livzon Pharmaceutical Group Inc.). Harvested oocytes were denuded enzymatically with 2-hydroxyethyl (HEPES)-buffered medium containing hyaluronidase (Irvine scientific) and mechanically by pipetting with a commercial glass pipette (Origio). Morphologically normal spermatozoa were injected into metaphase-II (MII) mature oocytes.

Biochemical pregnancy was confirmed by positive β-human chorionic gonadotrophin (β-hCG) in the blood or urine 2 weeks after embryo transfer. Clinical pregnancy was confirmed 4–6 weeks after embryo transfer by ultrasonography based on the presence of a gestational sac and fetal heartbeat in the uterine cavity.

Statistical analyses

The PASW Statistics for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Continuous variables are presented as mean ± standard deviation (s.d.), and Student's *t*-test was used to assess differences between the two groups. Analysis of variance (ANOVA) was used to compare among more than two groups. Chi-squared test or Fisher's exact test was used to compare categorical variables, presented as frequency and percentage. $P < 0.05$ was considered statistically significant.

RESULTS

Thirty-nine oocyte retrieval procedures were performed in the 37 couples. Frozen-thawed testicular spermatozoa were used in 13 cycles, and frozen-thawed ejaculated spermatozoa in the remaining 26 cycles. Two cycles were canceled in response to the patient request as no motile spermatozoa were found after thawing the samples on the Cryopiece. The mean age for the females was 29.5 ± 3.3 years and 31.5 ± 4.8 years for the males (**Table 1**).

A total of 757 spermatozoa were cryopreserved on 50 Cryopieces, with a mean of 15 spermatozoa per Cryopiece. We retrieved 599 spermatozoa (79.1%) after thawing, of which 225 (29.7%) were motile (including tail twitching). We obtained in the entire cohort a fertilization rate of 61.9% (156/252) and embryo cleavage rate of 84.6% (132/156), and 58.3% (77/132) of these embryos were available for transfer. After transferring 1–2 embryos on day 2 or 3, the following outcomes were achieved: an implantation rate of 23.4% (15/64), a clinical pregnancy rate of 43.3% (13/30), and a live birth delivery rate of 33.3% (10/30). The miscarriage rate was 23.1%. There were no ectopic pregnancies. The live born babies, five males and seven females, had a normal gestational age (37.0 ± 1.0 weeks for males and 39.0 ± 1.9 weeks for females) and normal birth weight (3442 ± 859 g for males and 3325 ± 450 g for females). Amniocentesis and fetal karyotyping were performed during pregnancy, and all karyotypes were normal.

A comparison between the ejaculated and testicular spermatozoa groups revealed no significant differences in the mean female and male ages, the mean number of spermatozoa per Cryopiece, and the sperm recovery rate. However, the motile spermatozoa recovery rate in the ejaculated spermatozoa group was significantly higher than that in the testicular spermatozoa group (32.5% *vs* 21.8%, $P = 0.005$). Although the fertilization rate achieved by testicular spermatozoa was significantly higher than that by ejaculated spermatozoa (77.4% *vs* 57.8%; $P = 0.009$), the groups were similar for the other clinical outcomes (**Table 2**).

AOA was performed in 11 ICSI cycles with thawed motile spermatozoa and ten cycles with thawed immotile spermatozoa to improve the fertilization rate. The fertilization rate when using AOA after ICSI with motile (MS-AOA group) or immotile (IS-AOA group) spermatozoa was significantly higher than that when motile spermatozoa were used without AOA (MS group; 72.1% and 71.9%, respectively, *vs* 52.0%; $P = 0.005$). These groups were similar for the mean female and male ages, mean number of injected MII oocytes, and the rates of embryo cleavage, available embryos for transfer, and cycle cancellation. The two AOA groups obtained insignificantly higher clinical pregnancy rates than the MS group (55.6% and 40.0%, respectively, *vs* 35.3%; $P = 0.703$). Although the IS-AOA group presented a higher miscarriage rate than the MS group (100.0% *vs* 0), the number of pregnancies and miscarriages in the ICSI with or without AOA groups were too small for statistical analysis. Eight children (two twins and four singletons) were born in the MS group and four (all singletons) in the MS-AOA group. Two clinical first-trimester pregnancies ended in miscarriage in the IS-AOA group (**Table 3**).

DISCUSSION

Several methods have been developed to cryopreserve a single or small number of ejaculated spermatozoa from males with SOZ or testicular spermatozoa obtained by m-TESE from males with NOA. These methods are in limited use in routine clinical ART due to technical difficulties[1] or lack of commercial supply. The Cryopiece system used in the present study is a relatively simple method for cryopreservation of a single or small number of spermatozoa.

The number of studies on cryopreservation of small numbers of spermatozoa and subsequent ICSI outcomes is still limited. Berkovitz *et al.*[6] used Sperm VD and achieved a sperm recovery rate of 96%, of which 33% were motile. Following ICSI, they achieved a fertilization rate of 59%, clinical pregnancy of 55%, and a live birth delivery rate of 32% (14 newborn). In their report, most cases used ejaculated spermatozoa ($n = 36$). Testicular spermatozoa were collected from eight patients, showing no post-thaw motility. It is known that post-thaw motility of testicular spermatozoa is poorer than ejaculated spermatozoa.[6] Using the Cell Sleeper, researchers reported a high post-thaw motility rate of 56%, a fertilization rate of 66%, and a clinical pregnancy rate of 58%.[12] However, the thickness of the Cell Sleeper tray edge requires the constant adjustment of the ICSI pipette position level, risking pipette breakage. Nakata *et al.*[13] reported the

Table 1: A total number of sperm retrieved and motility recovery in frozen thaw samples and intracytoplasmic sperm injection results for all patients (*n*=37)

Patient number	*Female age (year)*	*Male age (year)*	*Diagnosis*	*Cycle*	*Sperm origin*	*Vitrified motile spermatozoa (n)/piece (n)*	*Thawed motile spermatozoa (n)/retrieved spermatozoa (n)*	*Clinic procedure*	*AOA*	*MII oocyte injected (n)*	*Normal fertilization (2PN), n*	*Embryo cleaved (n)*	*Embryo used (n)/ available embryo (n)*	*Outcome*
1	35	38	NOA	1st	m-TESE	20/2	10/18	ICSI	No	6	5	5	2/4	Delivery (single live birth)
2	30	32	Cryptozoospermia	1st	Ejaculate	40/2	12/30	ICSI	No	11	8	7	2/3	Delivery (twin live birth)
3	26	26	NOA (cryptorchidism)	1st	m-TESE	26/1	10/22	ICSI	No	8	5	4	1/1	No pregnancy
4	28	40	NOA	1st	m-TESE	20/1	6/18	ICSI	No	5	4	3	2/2	Delivery (single live birth)
5	29	25	NOA (non-mosaic KS)	1st	m-TESE	22/1	3/18	ICSI	No	3	2	2	1/1	No pregnancy
6	35	34	NOA	1st	m-TESE	25/1	3/18	ICSI	No	2	1	1	1/1	No pregnancy
7	30	29	Cryptozoospermia	1st	Ejaculate	45/2	12/35	ICSI	No	10	3	2	2/2	No pregnancy
				2nd	Ejaculate	39/2	9/30	ICSI	No	9	3	2	2/2	No pregnancy
8	26	27	Cryptozoospermia	1st	Ejaculate	21/1	10/16	ICSI	No	4	4	4	2/4	Delivery (single live birth)
9	26	30	Cryptozoospermia	1st	Ejaculate	33/2	5/25	ICSI	No	5	3	3	2/2	No pregnancy
10	26	29	NOA (non-mosaic KS)	1st	m-TESE	3/1	0/3	Donor semen	NA	NA	NA	NA	NA	NA
11	33	37	Cryptozoospermia	1st	Ejaculate	8/1	6/8	ICSI	No	6	0	0	0/0	NA
12	27	28	NOA (non-mosaic KS)	1st	m-TESE	20/1	0/12	Oocyte frozen	NA	NA	NA	NA	NA	NA
13	23	25	NOA (YqAZFc microdeletion)	1st	m-TESE	11/1	4/7	ICSI	No	4	3	3	3/3	No pregnancy
14	28	33	Cryptozoospermia (YqAZFC microdeletion)	1st	Ejaculate	27/2	9/21	ICSI	No	9	3	3	2/3	No pregnancy
15	27	27	NOA (YqAZFC microdeletion)	1st	m-TESE	6/1	0/5	ICSI	No	5 (IS)[a]	2	0	0/0	NA
16	27	28	Cryptozoospermia	1st	Ejaculate	21/1	14/21	ICSI	No	14	4	5	2/2	No pregnancy
17	28	31	Cryptozoospermia	1st	Ejaculate	22/3	7/17	ICSI	No	7	1	1	1/1	No pregnancy
18	31	36	Cryptozoospermia	1st	Ejaculate	14/1	10/13	ICSI	No	10	3	3	2/2	No pregnancy
19	28	30	NOA	1st	m-TESE	11/1	6/9	ICSI	Yes	5	4	4	2/3	Delivery (single live birth)
20	27	26	Cryptozoospermia	1st	Ejaculate	20/2	14/20	ICSI	No	8	4	4	2/2	Delivery (single live birth)
21	33	28	Cryptozoospermia	1st	Ejaculate	7/1	1/5	ICSI	No	1	0	0	0/0	NA
22	29	30	Cryptozoospermia	1st	Ejaculate	15/1	7/11	ICSI	No	5	4	4	2/2	Delivery (twin live birth)
23	26	26	Cryptozoospermia	1st	Ejaculate	11/1	3/7	ICSI	Yes	3	3	3	0/0	NA
24	31	30	Cryptozoospermia	1st	Ejaculate	12/1	5/9	ICSI	Yes	3	3	3	2/3	Delivery (single live birth)
25	35	36	Cryptozoospermia	1st	Ejaculate	22/2	10/20	ICSI	Yes	9	6	5	2/3	Delivery (single live birth)
26	29	31	Cryptozoospermia	1st	Ejaculate	47/2	16/38	ICSI	Yes	8	6	6	3/3	No pregnancy
27	37	38	Cryptozoospermia	1st	Ejaculate	12/1	6/9	ICSI	Yes	6	5	3	2/2	No pregnancy
28	28	42	Cryptozoospermia	1st	Ejaculate	24/1	7/22	ICSI	Yes	7	5	5	4/4	No pregnancy
									Yes	6 (IS)[a]	4	3	0/1	No transfer
29	29	31	Cryptozoospermia	1st	Ejaculate	21/1	0/19	ICSI	Yes	19 (IS)[a]	14	16	5/6	No pregnancy
				2nd	Ejaculate	21/1	9/14	ICSI	Yes	9	3	3	2/2	Delivery (single live birth)
30	33	34	Cryptozoospermia	1st	Ejaculate	18/1	0/15	ICSI	Yes	10 (IS)[a]	5	6	5/5	pregnancy (miscarriage)
31	36	43	Cryptozoospermia (mumps orchitis)	1st	Ejaculate	12/1	0/8	ICSI	Yes	5 (IS)[a]	3	3	1/3	Pregnancy (miscarriage)
32	31	28	Cryptozoospermia	1st	Ejaculate	18/1	3/12	ICSI	Yes	3	3	1	2/2	No pregnancy
									Yes	2 (IS)[a]	1	1		
33	28	28	NOA	1st	m-TESE	10/1	0/6	ICSI	Yes	6 (IS)[a]	3	4	0	NA
34	33	37	Cryptozoospermia	1st	Ejaculate	6/1	0/3	ICSI	Yes	3 (IS)[a]	3	0	0	NA
35	29	33	NOA	1st	m-TESE	12/1	1/7	ICSI	Yes	1	0	0	0	NA
									Yes	2 (IS)[a]	0	0	0	NA

Contd...

Table 1: A total number of sperm retrieved and motility recovery in frozen thaw samples and intracytoplasmic sperm injection results for all patients (*n*=37)

Patient number	*Female age (year)*	*Male age (year)*	*Diagnosis*	*Cycle*	*Sperm origin*	*Vitrified motile spermatozoa (n)/piece (n)*	*Thawed motile spermatozoa (n)/retrieved spermatozoa (n)*	*Clinic procedure*	*AOA*	*MII oocyte injected (n)*	*Normal fertilization (2PN), n*	*Embryo cleaved (n)*	*Embryo used (n)/ available embryo (n)*	*Outcome*
36	27	31	NOA (non-mosaic KS)	1st	m-TESE	11/1	0/10	ICSI	Yes	6 (IS)[a]	5	6	1/1	No pregnancy
37	29	28	Cryptozoospermia	1st	Ejaculate	24/1	7/18	ICSI	Yes	7	3	4	2/2	Pregnancy (miscarriage)
Total				39		757/50	225/599			252	141[b]	132	64/77	

[a]IS: injected with immotile spermatozoa as no motile sperm found; [b]the total number of fertilization zygote is 156; of which 141 were with 2PN and 15 with 2PB or 1PN. Patient number 10 used donor sperm for ICSI and patient number 12 had all oocyte cryopreserved as no motile sperm found in frozen thaw samples; ICSI results of these two patients were not included in this study. Both of motile and immotile thawed spermatozoa were used in one ICSI cycle of the patient number 28, 32, and 35. NA: not available; ICSI: intracytoplasmic sperm injection; m-TESE: microsurgical testicular sperm extraction; NOA: nonobstructive azoospermia; 2PN: two pronuclei; 1PN: one pronucleus; 2PB: two polar bodies; AOA: assisted oocyte activation; KS: Klinefelter syndrome; MII: metaphase-II; YqAZFC: Y chromosome AZFc

Table 2: Comparison of sperm recovery rate after frozen-thaw and intracytoplasmic sperm injection outcomes between ejaculate and testicular spermatozoa

Group	*Patient (n)/ cycle*	*Male age (year), mean*	*Female age (year), mean*	*Spermatozoa (n)/Cryopiece (n)*	*Retrieved (n)/frozen spermatozoa (n), %*	*Motile (n)/frozen spermatozoa (n), %*	*Injected MII oocyte (n)*	*Fertilization, n/ total (%)*	*Embryo cleaved, n/total (%)*	*Available embryos for transfer, n/ total (%)*	*Embryo implantation rate, n/total (%)*	*Clinical pregnancy, n/ total (%)*	*Miscarriage, n/total (%)*	*Cycle cancellation, n/total (%)*	*Total deliveries (singleton + twins)*
Ejaculate	24/26	32	30	560/36	446/560 (79.6)	182/560 (32.5)[a]	199	115/199 (57.8)[a]	100/115 (87.0)	60/100 (60.0)	12/51 (23.5)	10/22 (45.5)	3/10 (30.0)	4/26 (15.4)	9 (5+2)
m-TESE	13/13	30	28	197/14	153/197 (77.7)	43/197 (21.8)[b]	53	41/53 (77.4)[b]	32/41 (78.0)	17/32 (53.1)	3/13 (23.1)	3/8 (37.5)	0/3 (0)	5/13 (38.5)	3 (3+0)
Total	37/39			757/50	599/757 (79.1)	225/757 (29.7)	252	156/252 (61.9)	132/156 (84.6)	77/132 (58.3)	15/64 (23.4)	13/30 (43.3)	3/13 (23.1)	9/39 (23.1)	12 (8+2)

$P<0.05$ compared between [a] and [b]. m-TESE: microsurgical testicular sperm extraction; MII: metaphase-II

Table 3: Comparison of intracytoplasmic sperm injection outcomes between frozen-thaw spermatozoa with and without assisted oocyte activation

Group	*Treatment cycle*[a]	*Male age (year), mean*	*Female age (year), mean*	*Injected MII oocyte (n)*	*Fertilization, n/total (%)*	*Embryo cleaved, n/total (%)*	*Available embryos for transfer, n/total (%)*	*Embryo implantation rate, n/total (%)*	*Clinical pregnancy, n/total (%)*	*Miscarriage, n/total (%)*	*Cycle cancellation, n/total (%)*	*Total deliveries (singleton + twins)*
MS	19	32	29	127	66/127 (52.0)[b]	56/66 (84.8)	37/56 (66.1)	8/31 (25.8)	6/17 (35.3)	0/6 (0)	2/19 (10.5)	8 (4+2)
MS-AOA	11	31	30	61	44/61 (72.1)[c]	37/44 (84.1)	23/37 (62.2)	5/20 (25.0)	5/9 (55.6)	1/5 (20.0)	2/11 (18.1)	4 (4+0)
IS-AOA	10	33	30	64	46/64 (71.9)[c]	39/46 (84.8)	17/39 (43.6)	2/13 (15.4)	2/5 (40.0)	2/2 (100.0)	5/10 (50.0)	0
Total	40			252	156/252 (61.9)	132/156 (84.6)	77/132 (58.3)	15/64 (23.4)	13/30 (43.3)[d]	3/13 (23.1)	9/40 (22.5)	12 (8+2)

[a]Three cycles contained both MS-AOA and IS-AOA treatments; $P<0.05$ compared between [b] and [c]; [d]patient number 32 transferred with two embryos respectively from MS-AOA and IS-AOA spermatozoa. MS: motile spermatozoa; MS-AOA: MS with assisted oocyte activation; IS-AOA: immotile spermatozoa with assisted oocyte activation; MII: metaphase-II

use of a Carrier for cryopreservation, achieving a sperm recovery rate of 95%. This Carrier is very similar to the Cell Sleeper in being time consuming and relatively complex during ICSI. Using the Cryopiece system, the sperm recovery rate in the current study was 79%, with 30% post-thaw motility. Our results were similar to those of previous reports that showed recovery rates of 59%–100% and post-thaw motility of 0–100%.[1] In our experience, it was difficult to firmly place the Cryopiece at the bottom of the dish under the mineral oil during the thawing procedure because it was very light. Otherwise, the Cryopiece was simple and easy to use. The Cryopiece replaces the tray in the Cell Sleeper, making it possible to avoid the need for constant position level adjustments of the ICSI pipette during the ICSI procedure. The Cryopiece system has no inner rigid tray as in the Sperm VD, making it easy for the ICSI pipette to reach all spermatozoa, and therefore, convenient for use during the ICSI procedure.

The miscarriage rate in this study was 23.1%, much higher than the overall ART spontaneous abortions (10%–15%) reported in China.[14] This high miscarriage rate was probably related to the poor sperm quality of males with SOZ or NOA. Testicular spermatozoa of 13 patients in this study were cryopreserved with the Cryopiece system. The post-thaw motility recovery rate of these spermatozoa was significantly lower than ejaculated spermatozoa, but they achieved a significantly higher fertilization rate. Another study also reported a higher fertilization rate using fresh or cryopreserved testicular spermatozoa than that in ejaculated sperm in 13 couples diagnosed with azoospermia or cryptozoospermia.[15] It is possible that the poor quality of ejaculated spermatozoa in males with SOZ is due to an increased risk of oxidative stress[16] or nuclear DNA damage during transit through the male genital tract.[17] It was reported that sperm DNA fragmentation rate was markedly lower in testicular than ejaculated spermatozoa.[18] Furthermore, some reports showed that increased levels of oxidative stress and higher DNA fragmentation were associated with worse fertility outcomes.[15,19–21] In addition, testicular spermatozoa DNA fragmentation could increase significantly after cryopreservation in cryotubes, possibly due to the formation of reactive oxygen species[22,23] that could cause chromatin damage.[24]

In our previous study,[9] we only used motile spermatozoa after thawing. Although some studies reported that spermatozoa immotile after thawing might still lead to normal fertilization,[6,25] ICSI with immotile spermatozoa after thawing usually presents lower fertilization rates than when using motile spermatozoa. Several studies have reported that the addition of AOA with ionomycin after ICSI could significantly increase the fertilization rate in patients with severe teratozoospermia.[26,27] Ebner *et al*.[28] showed that AOA with a calcium ionophore could enhance fertilization in patients with cryptozoospermia. In our study, we applied AOA in some cases with motile or immotile spermatozoa after thawing, showing significantly enhanced fertilization rates regardless of the motility status. However, similar clinical pregnancy rates were achieved regardless of AOA application. Two patients with AOA after ICSI with immotile spermatozoa had miscarriage.

In conclusion, the Cryopiece system was very useful for cryopreservation of small numbers of spermatozoa from patients with SOZ or NOA. The results also showed that better outcomes could be achieved when performing ICSI with spermatozoa that are motile after thawing.

AUTHOR CONTRIBUTIONS

WC and CH designed the study and played major roles in data collection, statistical analysis, and manuscript preparation. PL, FL, JS, ZJZ, YX, YPP, and YH helped with data collection and interpretation. JZ, JLH, and ZBZ helped with manuscript preparation. ZL and YW designed and supervised the study. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by the National Key R&D Program of China (No. 2017YFC1002003), the National Natural Science Foundation of China (No. 81771637 and No. 81571488), Shanghai Collaborative Innovation Center for Translational Medicine (No. TM202015 to ZJZ), the China Postdoctoral Science Foundation (No. 2019M661521), and the Key Project of Research and Development of Ningxia Hui Autonomous Region of China (No. 2020BFH02002). We really appreciated Dr. De Yi Liu and Miss Alyssa Meng for their critical comments and revision of this manuscript.

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