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

Wei Chen1,*, Chuan Huang1,2,*, Peng Li1, Peng Liu1, Jian Sun1, Zi-Jue Zhu1, Jing Zhai1, Yuan Xu1, Yan Hong1, Jian-Lin Hu1, Yun-Peng Peng1, Zhen-Bo Zhang1, Yu Wu1, Zheng Li1

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.

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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 extraction1 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,3 and sperm vitrification device (VD).5 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

1Department of ART, Department of Andrology, Center for Men’s Health, Institute of Urologic Medical Center, Shanghai General Hospital, Shanghai Key Lab of Reproductive Medicine, Shanghai Jiao Tong University, Shanghai 200080, China; 2Clinical Research Center for Reproduction and Genetics in Hunan Province, Reproductive and Genetic Hospital of CINIT-Xiangya, Changsha 410000, China; 3Department of Urology, Affiliated Hospital of Xuzhou Medical University, Xuzhou 221000, China.

*These authors contributed equally to this work.

Correspondence: Dr. Z Li (lizhengboshi@sjtu.edu.cn) or Dr. Y Wu (wuyu1970@yahoo.com)
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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 500g 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.

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) or the laser method 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...
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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 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% CO₂, 5% O₂, and 89% N₂. 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 (Cetrotrexil, 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.

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 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. 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. 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%. 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. 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 spermatozoa (n/piece) | Thawed motile spermatozoa (n) | Retrieved spermatozoa (n) | Clinic procedure | AOA | MI 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                   |
| 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 (YaAZFc microdeletion) | 1st   | m-TESE       | 11/1                          | 4/7                          | ICSI                      | No              | 4   | 3                      | 3                         | 3/3            | No pregnancy                   |
| 14             | 28                | 33              | Cryptozoospermia (YaAZFC microdeletion) | 1st   | m-TESE       | 27/2                          | 9/21                         | ICSI                      | No              | 9   | 3                      | 3                         | 2/3            | No pregnancy                   |
| 15             | 27                | 27              | NOA (YaAZFC microdeletion) | 1st   | m-TESE       | 6/1                           | 0/5                          | ICSI                      | No              | 5   | (IS)*                  | 2                         | 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                           | 5/5                          | ICSI                      | No              | 3   | 3                      | 3                         | 0/0            | No pregnancy                   |
| 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                         | 3/3            | No pregnancy                   |
| 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                         | 2/2            | 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                   |
| 29             | 29                | 31              | Cryptozoospermia | 1st   | Ejaculate    | 21/1                          | 0/19                         | ICSI                      | Yes             | 19  | (IS)*                  | 14                        | 16             | 5/6                       | No pregnancy |
| 30             | 33                | 34              | Cryptozoospermia | 1st   | Ejaculate    | 18/1                          | 9/14                         | ICSI                      | Yes             | 9   | 3                      | 3                         | 3/3            | Delivery (single live birth)       |
| 31             | 36                | 43              | Cryptozoospermia (mumps orchitis) | 1st   | Ejaculate    | 12/1                          | 0/8                          | ICSI                      | Yes             | 5   | (IS)*                  | 3                         | 3/3            | 1/3                       | Pregnancy (miscarriage) |
| 32             | 31                | 28              | Cryptozoospermia | 1st   | Ejaculate    | 18/1                          | 3/12                         | ICSI                      | Yes             | 3   | 3                      | 3                         | 1/2            | No pregnancy                   |
| 33             | 28                | 28              | NOA       | 1st   | m-TESE       | 10/1                          | 0/6                          | ICSI                      | Yes             | 6   | (IS)*                  | 3                         | 4              | 0                         | NA         |
| 34             | 33                | 37              | Cryptozoospermia | 1st   | Ejaculate    | 6/1                           | 0/3                          | ICSI                      | Yes             | 3   | (IS)*                  | 3                         | 0              | 0                         | NA         |
| 35             | 29                | 33              | NOA       | 1st   | m-TESE       | 12/1                          | 1/7                          | ICSI                      | Yes             | 1   | 0                      | 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) | 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)*               | 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                                                 |                |     |                        |                           |                 | 141*                                  | 64/77               |

*IS: injected with immotile spermatozoa as no motile sperm found; 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 | 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)*                  | 199                       | 115/199 (57.8)*               | 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)*                   | 53                        | 41/53 (77.4)*                 | 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 * and †. m-TESE: microsurgical testicular sperm extraction; 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%. 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. 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 in ejaculated sperm in 13 couples diagnosed with azoospermia or cryptozoospermia. It is possible that the poor quality of ejaculated spermatozoa in males with SOZ is due to an increased risk of oxidative stress or nuclear DNA damage during transit through the male genital tract. It was reported that sperm DNA fragmentation rate was markedly lower in testicular than ejaculated spermatozoa. Furthermore, some reports showed that increased levels of oxidative stress and higher DNA fragmentation were associated with worse fertility outcomes. In addition, testicular spermatozoa DNA fragmentation could increase significantly after cryopreservation in cryotubes, possibly due to the formation of reactive oxygen species that could cause chromatin damage.

In our previous study, we only used motile spermatozoa after thawing. Although some studies reported that spermatozoa immotile after thawing might still lead to normal fertilization, 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. Ebner et al. 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, ZJ, YX, YPF, and YH helped with data collection and interpretation. JZ, ILH, 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.

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

| Group   | Treatment cycle | 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)             | 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)              | 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)              | 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 (81.9)          | 132/156 (84.6)         | 77/132 (58.3)             | 15/64 (23.4)                | 13/30 (43.3)                           | 9/40 (22.5)                          | 12 (8+4)                          |                            |                                |                                  |

Note: Three cycles contained both MS-AOA and IS-AOA treatments; P<0.05 compared between 1 and 2; 3: 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.
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