Received: 14 June 2021 | Revised: 17 September 2021 | Accepted: 25 October 2021

DOI: 10.1111/and.14318

ORIGINAL ARTICLE

WILEY

Rapid cryopreservation of small quantities of human spermatozoa by a self-prepared cryoprotectant without animal component

Shasha Liu[1,2] | Bo Liu[1,2] | Wenrui Zhao[1,2] | Xiao Liu[1,2] | Yang Xian[1,2] | Qingyuan Cheng[1,2] | Min Jiang[1,2] | Huanxun Yue[1,2] | Fuping Li[1,2]

[1]Human Sperm Bank, West China Second University Hospital, Sichuan University, Chengdu, China

[2]Key Laboratory of Birth Defects and Related Disease of Women and Children (Sichuan University), Ministry of Education, Chengdu, China

Correspondence
Fuping Li, Human Sperm Bank, West China Second University Hospital, Sichuan University, No. 1416, Section 1, Chenglong Avenue, Chengdu, China.
Email: lfpsnake@scu.edu.cn

Funding information
This work was supported by the National Natural Science Foundation of China (grant no. 81974226).

Abstract

Cryopreservation of small quantities of human spermatozoa whilst maintaining adequate post-thawing motility has been found an essential challenge for male fertility preservation. Therefore, the study used an effective, and convenient rapid-freezing method to freeze small amounts of human spermatozoa by adding self-prepared cryoprotectant (SPC) without animal component. In the feasibility experiment, no significant differences in progressive motility, normal sperm morphology, vitality or DNA fragmentation index between the conventional slow freezing and rapid freezing were realised. The present study prospectively analysed the effects of sperm freezing and resuscitation in 175 patients with severe oligozoospermia (sperm concentration $<1 \times 10^6$/ml). We observed the 120 severe oligozoospermia specimens had a mean recovery rate of 60.19% ± 10.43% and a mean cryosurvival rate of 68.0% ± 9.17%. In addition, 55 cryptozoospermia specimens were analysed. The small-volume cryopreservation showed advantages. The total sperm recovery, motility recovery and sperm loss rates were 98.48%, 50.17% and 1.52% respectively. In short, the SPC is safe and effective, and can be used to rapidly freeze severe oligozoospermia specimens. That is useful for successful sperm freezing whilst avoiding the risk of azoospermia in the later stages and promoting comprehensive fertility preservation.

KEYWORDS
conventional slow freezing, rapid freezing, self-prepared cryoprotectant, small quantities of human spermatozoa

1 | INTRODUCTION

Cryopreserved spermatozoa had first been utilised to attain pregnancy in the 1950s (Sherman, 1973). From that time, cryopreservation technologies have become integral in the management of male infertility, as well as one of the leading aspects of assisted reproductive technology. Male infertility currently accounts for about 50% of fertility-related problems (Jackson et al., 2010). Nearly 75% of male patients with infertility have abnormal sperm characteristics, such as oligozoospermia, necrospermia, azoospermia or sperm deformities (Lopushnyan & Walsh, 2012). Intracytoplasmic sperm injection (ICSI) has been an ideal method for treating severe

male infertility (Ohlander et al., 2014; Di Santo et al., 2012; Tsai et al., 2013). Moreover, several investigations have demonstrated that ICSI utilising frozen-thawed motile spermatozoa from an ejaculation had similar clinical outcomes as that using fresh motile spermatozoa (Philippon et al., 2015; Sokmensuer et al., 2015). The seminal parameters of patients with severe oligozoospermia may fluctuate, and spermatozoa may not always be detected. However, timely and effective cryopreservation of oligozoospermia samples can ensure patient fertility. Cryopreservation aims to ensure usable spermatozoa on the day of egg retrieval and reduces the physical and mental suffering and financial burden experienced by patients. Conventional cryopreservation techniques may not be the most suitable option for oligozoospermia samples given their varying degrees of loss and poor in situ motility during the sperm thawing process (AbdelHafez et al., 2009). Slow-freezing methods initiated in the 1960s, as the core technology of sperm banks, have been extensively implemented until now (Sherman, 1963). As such, researchers have constantly been trying to explore methods for freezing rare sperm, including the use of single sperm freezing technology in some extreme cases to preserve a limited number of spermatozoa (Liu & Li, 2020). Currently, the rare sperm-freezing technology primarily aimed to use diluted semen (concentration $<5 \times 10^6$/ml). It is impossible to truly evaluate the freezing effect of severe oligozoospermia and choose and optimise the freezing method. The SPC is used for the routine slow freezing of volunteer spermatozoa in this human sperm bank, and a good freezing effect has been obtained. The clinical pregnancy rate is higher than 20%. But the freezing effect of a small amount of spermatozoa has not been evaluated. The main objective of the present study is to assess an effective, convenient rapid-freezing method and analyse the freezing effect of small quantities of human spermatozoa. The results presented, herein, would be beneficial for patients with oligozoospermia experiencing ICSI treatment cycles with cryopreserved sperm.

2 | MATERIALS AND METHODS

2.1 | Study population

The study was approved by the committee of Medical Ethics of West China Second University Hospital, Sichuan University, and informed consent was obtained from all participants. From June 2019 to January 2021, a total of 20 normal sperm samples were randomly selected and 175 severe oligozoospermia samples were involved in the current study.

2.2 | Experimental design

2.2.1 | Feasibility

A frozen analysis of the semen samples obtained from 20 normal sperm samples was conducted to appraise the effectiveness of the rapid cryopreservation by SPC prior to the clinical utilisation in an assisted reproduction process. The average age (mean ± SD), volume (mean ± SD), abstinence duration (mean ± SD), concentration (mean ± SD) and progressive motility (mean ± SD) were 28.13 ± 4.67 years old, 3.88 ± 2.34 ml, 4.13 ± 1.19 days, $123.60 \pm 47.07 \times 10^6$/ml and 63.07% ± 10.56% respectively. Semen from the same ejaculation was categorised into three aliquots: (i) conventional slow freezing—thawing, (ii) rapid freezing—thawing, (iii) without freezing as control. Basic sperm analysis (sperm motility, vitality and morphology) and sperm DNA fragmentation assessment were performed in all three groups.

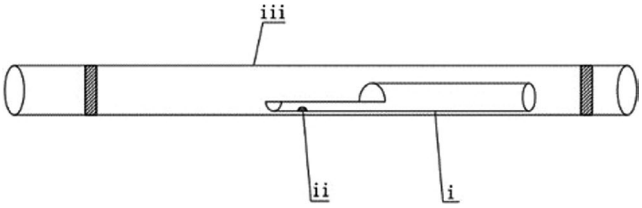

FIGURE 1 Self-designed closed straw carrier system. It equipped with a novel straw (i), cryoprotectant containing sperm (ii), and an outer 0.5-mL straw (iii)

2.2.2 | Verification test

The 175 severe oligozoospermia samples (sperm concentration $<1 \times 10^6$/ml) were considered for a verification test. The average age (mean ± SD), volume (mean ± SD) and abstinence duration (mean ± SD) were 28.42 ± 5.37 years old, 3.34 ± 1.39 ml and 4.90 ± 1.92 days respectively. The number of progressively motile (PR) spermatozoa, sperm vitality and recovery rate were evaluated.

2.3 | SPC

The SPC [sodium chloride, calcium chloride, potassium chloride, magnesium chloride, sodium dihydrogen phosphate, sodium lactate, sodium hydrogen carbonate, HEPES, sucrose, glycine, glucose and glycerol (14% v/v), at pH 7.4] was utilised for cryopreservation (patent no. CN107027741A).

2.4 | Self-designed closed-straw carrier system

Self-designed closed-straw carrier system (Figure 1) equipped with a novel straw, cryoprotectant containing sperm, and an outer 0.5-ml straw (Cryo Bio System, Paris, France). The novel straw is made of 0.25-ml straw (Cryo Bio System, Paris, France). The spermatozoa with normal morphology and motility were captured using the ICSI needles, then loaded on a droplet of freezing medium (1 μl) upon the novel straw. The novel straw was placed in a 0.5-ml straw, which was sealed using an SYMS111 sealer (Cryo Bio System, Paris, France).

2.5 | Sperm preparation

For 120 severe oligozoospermia samples and 15 cryptozoospermia specimens, semen was washed by centrifugation at 800g for 15 min utilizing a double volume of Sydney IVF Gamete Buffer (Cook Medical, Queensland, Australia). After centrifugation, 100 μl spermatozoa suspension was retained and mixed evenly.

For another 40 cryptozoospermia specimens, semen was divided into sterile centrifuge tubes and centrifuged at 800g for 15 min. After centrifugation, seminal plasma was discarded, and 50 μl of the pellet was retained and mixed evenly, then diluted and re-suspended with 500 μl Sydney IVF Sperm Medium. The isolation of motile spermatozoa was carried out by utilizing a density gradient centrifugation technique with Sydney IVF Sperm Gradient, and through centrifugation at 500g for 20 min. Following the final washing step, the supernatant was abandoned; subsequently, the pellet was re-suspended in 0.1 ml Sydney IVF Sperm Medium.

2.6 | Conventional slow freezing and thawing

Sperm specimens were added to SPC (7% glycerol concentration) at a ratio of 1:1. After mixing evenly, the sample (1 ml) was sealed in a 1.8-ml cryotube (Cryo Bio System, Paris, France), which was sealed using an SYMS111 sealer. The cryotubes were equilibrated for 15 min at 4°C and subsequently placed 5 cm above liquid nitrogen (LN_2) for 10 min and finally, plunged and stored for at least 5 days in LN_2 (−196°C). For thawing of sperm, the cryotubes were eliminated from liquid nitrogen and incubated for 10 min at 37°C.

2.7 | Rapid freezing and thawing

For 120 severe oligozoospermia samples and 15 cryptozoospermia specimens, spermatozoa suspension was added to SPC (7% glycerol concentration) at a ratio of 1:1. After mixing evenly, 150 μl of this solution was added to a 0.25-ml straw, which was sealed using an SYMS111 sealer. After a distance of 8 cm from the LN_2 surface was maintained for 10 min, the straw was rapidly immersed into LN_2 (−196°C) for storage at least 5 days. For the thawing procedure, the straw was removed from the LN_2 and incubated in a water bath at 37°C for 2 min. Thereafter, the sealed ends of the straws were cut, and the solution was gently aspirated into a 1.5-ml Eppendorf tube prior to microscopic analysis.

An Olympus IX-73 inverted microscope fitted with the NARISHIGE ON4 micromanipulation system was used for selection of spermatozoa from the semen sample. For another 40 cryptozoospermia specimens, after two-step centrifugation, the novel straw with a droplet of freezing medium (1 μl) was putted into the bottom of a 35-cm dish (Falcon, San Jose, CA, USA), the spermatozoa sample microstrips were directly loaded on the dish, then covered with mineral oil (Vitrolife, Goteborg, Sweden). The cryoprotectant was a 50:50 solution of Sydney IVF Sperm Medium and SPC (glycerol concentration 14%). Individual spermatozoa were transferred into a droplet of freezing medium (1 μl). Self-designed closed-straw carrier system was instantly placed about 5 cm above the LN_2 surface for 10 min and next, immediately plunged and stored for at least 5 days in LN_2 (−196°C). For the thawing procedure, the cryopreserved novel straw was pulled out from the freezing 0.5-ml straw and placed at the bottom of a 35-cm dish, and immediately covered with pre-warmed mineral oil (37°C). Then the dish was cultured in 37°C incubator for 15 min. Finally, the thawed and recovered spermatozoa were examined.

2.8 | Basic sperm analysis

2.8.1 | Sperm motility evaluation

Specimens were permitted to liquefy for up to 30 min prior to the treating. For each evaluation, a 5-μl sperm specimen was placed in a pre-warmed (37°C) Makler counting chamber (Sefi Medical Industries). The sperm motility parameters were objectively analysed both pre-freezing and post-thawing by SSA-II sperm automatic detection and analysis system (SUIJIA SOFTWAAE, Beijing, China). At least 200 spermatozoa per Makler counting chamber were evaluated at ×200 times magnification (Olympus CX41, Tokyo, Japan).

For severe oligozoospermia specimens, a manual counting approach was applied in accordance with WHO guidelines (World Health Organization, 2010). Each semen specimen is evaluated twice. For each evaluation, a 5-μl sperm specimen was placed in a pre-warmed (37°C) Makler counting chamber. Before centrifugation, after centrifugation and after thawing, 35–40 random fields per Makler chamber were counted at ×200 times magnification, after which non-progressive, progressive and immotile sperm counts were recorded, and then the progressive motility and recovery rates were calculated. On the basis of the formula presented through the aforementioned manual, the volume per ×20 times high power field was approximately 8 nl, and the volume of 35–40 random fields per Makler chamber was approximately 0.3 μl. Progressive motility = PR sperm count/total sperm count × 100%, recovery rate = post-thawing progressive motility/pre-freezing progressive motility × 100%.

2.8.2 | Sperm viability assessment

Sperm viability was assessed through vitality testing using eosin alone according to the WHO guidelines (World Health Organization, 2010). Semen samples were fully mixed, after which 5 μl of semen and 5 μl of eosin were placed on a slide and mixed thereon using a pipette tip. A 22 mm × 22 mm coverslip was then used to cover the sample, which was left to stand at room temperature for 30 s.

An optical microscope (Olympus CX33, Tokyo, Japan) was used to examine sperm viability on the glass slide using a ×40 objective lens. Live spermatozoa possess light pink or white heads, whereas dead ones have heads that are stained red or dark pink. The current study also evaluated the cryosurvival rate of the spermatozoa as post-thawing viability/pre-freezing viability × 100%.

2.8.3 | Sperm morphology assessment

The criteria could be applied when assessing the morphological normality of the spermatozoon (Kruger et al., 1987). We use the Papanicolaou staining procedure for sperm morphology. Two hundred spermatozoa per specimen were analysed conforming to WHO protocols (World Health Organization, 2010).

2.9 | Sperm DNA fragmentation assessment

Sperm DNA fragmentation assessment was performed using the sperm chromatin structure assay (SCSA) (The staining kit of sperm DNA fragmentation, ANKEBIO, Anhui, China) according to the manufacturer's instructions. The sperm count was adjusted to $1–2 \times 10^6$/ml. Approximately 5000 sperm cells per sample were evaluated by flow cytometer (Beckman Coulter Inc., Brea, USA). Acridine orange was intercalated to the normal double-stranded DNA fluoresced green and associated with the abnormal single-stranded DNA fluoresced red. The DNA fragmentation index (DFI) was expressed as the ratio of the fluorescent intensity of red to total (red plus green) fluorescence intensities. The data were analysed by Flowjo, a specific dedicated software.

2.10 | Statistical analysis

The achieved outcomes were analysed by taking advantage of the Statistical Package for the Social Sciences (SPSS) version 19.0 (IBM, New York, USA). Results were expressed as mean ± standard deviation or median and interquartile range. p-value < 0.05 was considered to be statistically significant. Data sets were analysed through ANOVA and t-test, utilizing the GraphPad Prism ver.9.0 (GraphPad Software).

3 | RESULTS

3.1 | Sperm parameters and characteristics

The evaluation of the influence of conventional slow freezing and rapid freezing on sperm parameters and characteristics showed no statistically significant differences in progressive motility, normal sperm morphology, vitality, or DNA fragmentation index ($p > 0.05$) (Figure 2). Twenty normal semen specimens were analysed for each outcome. Using SPC, we found a rapid freeze–thaw recovery rate of 67.7% ± 15.47% and cryosurvival rate of 71.80% ± 12.17%.

3.2 | Cryopreservation effectiveness of severely oligozoospermic samples

Rapid-freezing results of 120 human sperm specimens are summarised in Table 1. Given the importance of accurately estimating freezing results in those with exceedingly low sperm count, our patients were divided into two sections considering a semen PR sperm count (0.3 μl) of 15 as the boundary. The results indicating 1 ≤ PR sperm count (0.3 μl) <5 indicated poor freezing effects and were categorised into group A alone; 5 ≤ PR sperm count (0.3 μl) <15, into group B; and 15 ≤ PR sperm count (0.3 μl) <30, into group C. Our results showed that groups A, B, and C had recovery rates of 54.81% ± 11.58%, 62.74% ± 9.51%, and 63.03% ± 7.92% respectively. We found significant differences between groups A and B ($p < 0.05$), and groups A and C ($p < 0.05$) but no substantial discrepancies between groups B and C ($p = 0.861$).

Fresh spermatozoa samples exhibited a mean cryosurvival rate of 62.08% ± 11.85%, which decreased to 42.58% ± 11.43% in frozen-thawed spermatozoa samples. Groups A, B and C had cryosurvival

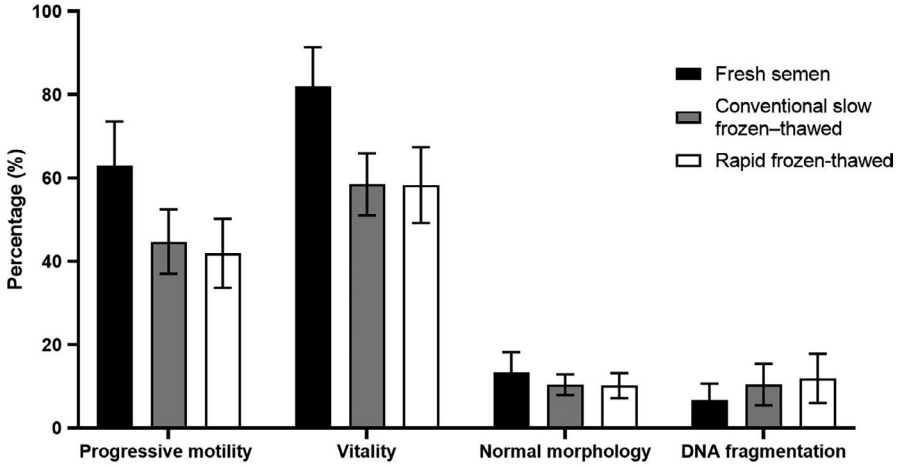

FIGURE 2 Impact on sperm parameters and characteristics of conventional slow-freezing and rapid-freezing protocols

TABLE 1 Rapid-freezing results of 120 severe oligospermia specimens

Group	Before centrifugation		Pre-freezing			Post-thaw			Recovery rate (%)	Cryosurvival rate (%)
	PR sperm count (0.3 μl)	*n*	PR sperm count (0.3 μl)	Progressive motility (%)	Viability (%)	PR sperm count (0.3 μl)	Progressive motility (%)	Viability (%)		
A	3.45 ± 1.08	40	12.23 ± 3.68	33.98 ± 7.60	53.95 ± 8.38	3.88 ± 1.81	18.76 ± 6.12	33.83 ± 9.04	54.81 ± 11.58[a]	62.76 ± 12.72[a]
B	9.80 ± 2.76	40	30.28 ± 11.40	47.33 ± 12.42	63.03 ± 10.71	9.83 ± 3.95	29.61 ± 8.27	44.60 ± 10.07	62.74 ± 9.51[a]	70.26 ± 5.85[a]
C	22.68 ± 4.62	40	73.20 ± 14.64	48.69 ± 12.64	69.25 ± 11.04	21.48 ± 5.67	30.80 ± 9.0	49.30 ± 9.28	63.03 ± 7.92[a]	70.98 ± 4.21[a]
Total	11.98 ± 8.62	120	38.57 ± 27.87	43.33 ± 12.90	62.08 ± 11.85	11.73 ± 8.40	26.39 ± 9.53	42.58 ± 11.43	60.19 ± 10.43	68.0 ± 9.17

Note: Values are mean ± standard deviation (SD).

Abbreviations: 0.3 μl, the volume of 35–40 random fields per Makler chamber; PR, progressively motile.

[a]Statistically significant at $p \leq 0.05$.

rates of 62.76% ± 12.72%, 70.26% ± 5.85% and 70.98% ± 4.21% respectively. We found significant differences between groups A and B ($p < 0.01$), and groups A and C ($p < 0.01$) but no substantial discrepancies between groups B and C ($p = 0.703$).

In order to further explore the effect of sperm freezing, semen specimens PR sperm count (0.3 μl) < 1 is selected, so the cryptozoospermia specimens meet the standard. The rapid-freezing results of 15 cryptozoospermia specimens are summarised in Table 2. There was a risk that specimens would not have PR spermatozoa after thawing. Depending on specific situations, the small-volume (1 μl) cryopreservation on the self-designed closed straw carrier system showed advantages. Spermatozoa can be found within 30 s after thawing. The rates of total sperm recovery, as well as motility recovery, were 98.48% and 50.17% respectively. The average sperm loss rate was 1.52% (Table 3).

4 | DISCUSSION

Semen cryopreservation is essential for treating the infertility of males, especially for patients with severe oligozoospermia and nonobstructive azoospermia. Studies have shown that those with a minimum of one previous semen analysis showing a total sperm count of <100,000 have a 52% risk for future transient azoospermia (Montagut et al., 2015). Currently, ICSI is the most effective treatment procedure for the serious factor of male infertility. A major difference of ICSI with respect to natural fertilisation is that only a few spermatozoa are required for successful fertilisation. Therefore, effective cryopreservation of spermatozoa can ensure protection against the potentially damaging influences of transient azoospermia when ICSI occurs, reducing the psychological and physical stress in many couples.

Cryopreservation of small amounts of spermatozoa remains a major challenge for male fertility. Although years of research have improved the cryopreservability of sperm, substantial sperm loss and decline in motility after thawing still remain a problem. Influencing factors include cryoprotectants, freezing carriers, optimised sperm-processing methods, freezing volumes, freezing procedures, etc. Accordingly, damage during cryopreservation greatly reduces the chance of finding motile spermatozoa after the ICSI procedure following thawing, with some cases showing not even a single motile spermatozoa (Isachenko et al., 2004). ICSI with inanimate spermatozoa leads to difficult fertilisation and pregnancy (O'Neill et al., 2018; Rubino et al., 2016; Yazawa et al., 2009).

Cryoprotectants are low molecular weight and highly permeable chemicals used to protect spermatozoa from freeze damage through ice crystallisation by decreasing the freezing temperature of the specimens (Royere et al., 1996). The SPC used in this study does not contain egg yolk, and there is no risk of infection by pathogenic bacteria. Volunteer spermatozoa cryopreserved are used within the scope permitted by national regulations, and the clinical pregnancy rate is higher than 20%. The traditional slow-freezing method leads to poor performance in freezing samples with unfavourable

TABLE 2 Rapid-freezing results of 15 cryptozoospermia specimens

	Pre-freezing		Post-thaw		
No.	PR sperm count (0.3 μl)	Progressive motility (%)	PR sperm count (0.3 μl)	Progressive motility (%)	Recovery rate (%)
1	4	36.36	1	12.5	34.4
2	2	28.57	0	0	0
3	4	57.14	1	20	35.0
4	5	62.5	2	33.33	53.33
5	9	29.03	2	13.33	45.92
6	4	66.67	0	0	0
7	5	71.43	0	0	0
8	3	75.0	0	0	0
9	8	66.67	2	25.0	37.50
10	7	33.33	2	12.50	37.50
11	2	33.33	0	0	0
12	2	40.0	0	0	0
13	5	55.56	1	14.29	25.72
14	3	37.5	1	16.67	44.45
15	2	25.0	0	0	0
Mean ± SD	4.33 ± 2.23	47.87 ± 17.59	0.80 ± 0.86	9.84 ± 10.87	20.92 ± 21.12
Median	4.0	40.0	1	12.50	25.72
25–75	2.0–5.0	33.33–66.67	0–2.0	0–16.67	0–37.50

Abbreviations: 0.3 μl, the volume of 35–40 random fields per Makler chamber; PR, progressively motile.

quality perhaps due to the increased number of spermatozoa with damaged plasma membranes and internal organelles in these poor-quality specimens, which increases their susceptibility to cryoinjury (Counsel et al., 2004; Degl'Innocenti et al., 2013). Furthermore, studies have found that a higher cooling rate lowers the solid solution effect (Sieme et al., 2016). Thus, in the current research, the straws were directly placed above the LN_2 vapour to rapidly freeze human sperm, the high average freeze–thaw recovery rate, cryosurvival rate and morphological integrity rate, and low DNA fragmentation rate were obtained. Besides, no significant difference in progressive motility, normal sperm morphology, vitality, or DNA fragmentation index between the conventional slow freezing and rapid freezing were realised. These findings confirm the clinical application value of using SPC to rapidly freeze a small amount of spermatozoa and highlight its potential in assisted reproductive technology.

Semen samples utilised, herein, were obtained from spermatozoa collected from patients with severe oligozoospermia through masturbation, which could truly reflect the freezing effect. Owing to the small number of sperm, the sperm samples were optimised by centrifugation to increase the number and reduce the loss of sperm. Therefore, our goal was to recover as many sperm as possible to meet the utilisation of assisted reproduction technology (ART) under the premise of ensuring the freezing effect. The small size of the straw can reduce sperm loss due to carrier adhesion and increase the number of freezing tubes. Optimised semen cryopreservation for fertility preservation frequently involves the cryopreservation of multiple straws to present flexibility in further utilisation. Moreover, multiple investigations have proposed the occurrence of cross-contamination of biological material during LN_2 storage and succeeding cross-infection of patients (Fountain et al., 1997; Tedder et al., 1995). Hence, utilizing more secure storage devices still carries value to prevent the hypothetical occurrence of cross-contamination (Cobo et al., 2010). The straw can also be sealed using a sealing machine to effectively prevent the potential risk of cross-contamination in LN_2 and further improve safety and reliability.

The present prospective research in a relatively extensive sample scale divulged the recovered rate and cryosurvival rate of severe oligozoospermia samples were higher than 60%. The recovered rate and cryosurvival rate in group A were lower than those in groups B and C. Significant differences were detected between groups A and B, and between groups A and C. Degl'Innocenti et al. (2013) observed a desirable association between pre-freeze progressive motility and post-thaw semen parameters. In case of severe oligozoospermia, it is reasonable to use the PR sperm count for evaluating and predicting the freezing outcome when the sperm concentration cannot be detected. This approach exhibits clinical reference value. If the sperm count is extremely small, the frozen volume is more important. It can avoid the loss of spermatozoa and shorten the time to search for spermatozoa after thaw. Researchers have used more advantageous carriers in recent years to freeze single or small number of spermatozoa such as Cryotop, Cell Sleepers, Sperm VD and cryopiece (Liu & Li, 2020). The spermatozoa with normal morphology

TABLE 3 The small-volume cryopreservation results of 40 cryptozoospermia specimens

No.	No. of frozen PR sperm	No. (%) of spermatozoa recovered after thawing	No. (%) of motile spermatozoa after thawing	No. (%) of immotile spermatozoa after thawing	No. (%) of lost spermatozoa after thawing
1	13	13 (100)	4 (30.77)	9 (69.23)	0 (0)
2	20	20 (100)	10 (50)	10 (50)	0 (0)
3	19	19 (100)	13 (68.42)	6 (31.58)	0 (0)
4	25	24 (96)	13 (52)	11 (44)	1 (4)
5	20	19 (95)	10 (50)	9 (45)	1 (5)
6	20	20 (100)	8 (40)	12 (60)	0 (0)
7	25	25 (100)	16 (64)	9 (36)	0 (0)
8	19	19 (100)	7 (36.84)	12 (63.16)	0 (0)
9	14	14 (100)	7 (50)	7 (50)	0 (0)
10	19	19 (100)	11 (57.89)	8 (42.11)	0 (0)
11	7	7 (100)	3 (42.86)	4 (57.14)	0 (0)
12	14	14 (100)	7 (50)	7 (50)	0 (0)
13	10	10 (100)	4 (40)	6 (60)	0 (0)
14	18	18 (100)	10 (55.56)	8 (44.44)	0 (0)
15	3	3 (100)	1 (33.33)	2 (66.67)	0 (0)
16	16	15 (93.75)	7 (43.75)	8 (50)	1 (6.25)
17	15	15 (100)	5 (33.33)	10 (66.67)	0 (0)
18	9	9 (100)	5 (55.56)	4 (44.44)	0 (0)
19	12	10 (85.71)	6 (50)	4 (33.33)	2 (16.67)
20	6	6 (100)	4 (66.67)	2 (33.33)	0 (0)
21	13	13 (100)	5 (38.46)	8 (61.54)	0 (0)
22	21	20 (95.24)	9 (42.86)	11 (52.38)	1 (4.76)
23	5	5 (100)	3 (60)	2 (40)	0 (0)
24	8	8 (100)	3 (37.5)	5 (62.5)	0 (0)
25	18	18 (100)	10 (55.56)	8 (44.44)	0 (0)
26	11	11 (100)	4 (36.36)	7 (63.64)	0 (0)
27	5	5 (100)	4 (80)	1 (20)	0 (0)
28	14	13 (92.86)	6 (42.86)	7 (50)	1 (7.14)
29	15	15 (100)	6 (40)	9 (60)	0 (0)
30	9	9 (100)	5 (55.56)	4 (44.44)	0 (0)
31	12	12 (100)	4 (33.33)	8 (66.67)	0 (0)
32	14	14 (100)	8 (57.14)	6 (42.86)	0 (0)
33	32	31 (96.88)	18 (56.25)	13 (40.63)	1 (3.12)
34	10	10 (100)	4 (40)	6 (60)	0 (0)
35	13	13 (100)	7 (53.85)	6 (46.15)	0 (0)
36	15	15 (100)	9 (60)	6 (40)	0 (0)
37	39	37 (94.87)	27 (69.23)	9 (23.08)	2 (5.13)
38	14	14 (100)	7 (50)	7 (50)	0 (0)
39	14	14 (100)	6 (42.86)	8 (57.14)	0 (0)
40	6	6 (100)	1 (16.67)	5 (83.33)	0 (0)
Total	592	583 (98.48)	297 (50.17)	285 (48.14)	9 (1.52)

Abbreviation: PR, progressively motile.

and motility were captured using the ICSI needles. Then spermatozoa were cryopreserved by rapid-freezing technique. However, the extensive usage of these carriers in assisted reproduction laboratories has been restricted through their different deficiencies, including high cost, special equipment requirements, low recovery rate or open systems. There are sealed carriers (the two piece High

Security Straw) for freezing single or small number of spermatozoa (Desai et al., 2012). The same is the use of ICSI needle to capture spermatozoa with normal morphology and motility. The difference is that using a very finely drawn glass micropipette, spermatozoa were drawn into the capillary tube. The sperm recovery rate is relatively unstable (33%–100%). In our study, the 40 cryptozoospermia sperm specimens were rapidly frozen using the self-made freezing carrier. It is inexpensive and easy to manufacture whilst being able to achieve full sealing. Spermatozoa had been found within 30 s, with only eight specimens losing one or two spermatozoa after thawing. The average sperm loss rate was 1.52%, indicating greatly reduced sperm loss.

Some limitations of the current study need to be considered. First, for cryptozoospermia specimens, small-volume cryopreservation is the direction of future development. This study only analysed the freezing effect of 1 μl volume. Later studies can be tested in different volumes to find the best effect. Second, the rapid-freezing technology utilised herein had been developed for quite a short period (i.e. about 1 year). There are already high-quality frozen embryos and healthy babies born. Given the limited number of clinical use cases, data analysis could not be performed. In the future, we expect that this technology would be better developed in clinical applications and that more cases can be considered to further determine the utility of this freezing method.

In conclusion, the current study showed SPC is safe and effective, and can be used to rapidly freeze small quantities of human spermatozoa. Through appropriate freezing methods, successful sperm freezing can be ensured, patients with oligozoospermia can avoid situations wherein they cannot undergo sperm cryopreservation in the later stages, and comprehensive fertility preservation can be achieved.

COFLICT OF INTEREST

None.

AUTHOR CONTRIBUTION

Shasha Liu and Fuping Li conceived the study design. Shasha Liu, Bo Liu, Xiao Liu, Qingyuan Cheng and Yang Xian were involved in the literature collection. Shasha Liu and Bo Liu made substantial contributions to the sample collection, freezing, and thawing. Shasha Liu and Wenrui Zhao analysed and interpreted the data. Shasha Liu, Min Jiang, Huanxun Yue and Fuping Li revised the manuscript critically for important intellectual content. All authors have read and approved the final version of the manuscript and agree with the order of presentation of the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Fuping Li https://orcid.org/0000-0001-5183-4214

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

Cobo, A., Romero, J. L., Pérez, S., de los Santos, M. J., Meseguer, M., & Remohí, J. (2010). Storage of human oocytes in the vapor phase of nitrogen. *Fertility and Sterility*, *94*(5), 1903–1907. https://doi.org/10.1016/j.fertnstert.2009.10.042

Counsel, M., Bellinge, R., & Burton, P. (2004). Vitality of oligozoospermic semen samples is improved by both swim-up and density gradient centrifugation before cryopreservation. *Journal of Assisted Reproduction and Genetics*, *21*(5), 137–142. https://doi.org/10.1023/b:jarg.0000031245.39921.2c

Degl'Innocenti, S., Filimberti, E., Magini, A., Krausz, C., Lombardi, G., Fino, M. G., & Baldi, E. (2013). Semen cryopreservation for men banking for oligospermia, cancers, and other pathologies: prediction of post-thaw outcome using basal semen quality. *Fertility and Sterility*, *100*(6), 1555–1563. e1551–1553. https://doi.org/10.1016/j.fertnstert.2013.08.005

Desai, N., Goldberg, J., Austin, C., Sabanegh, E., & Falcone, T. (2012). Cryopreservation of individually selected sperm: methodology and case report of a clinical pregnancy. *Journal of Assisted Reproduction and Genetics*, *29*(5), 375–379. https://doi.org/10.1007/s10815-012-9733-y

Di Santo, M., Tarozzi, N., Nadalini, M., & Borini, A. (2012). Human sperm cryopreservation: Update on techniques, effect on DNA integrity, and implications for ART. *Advances in Urology*, *2012*, 1–12. https://doi.org/10.1155/2012/854837

Fountain, D., Ralston, M., Higgins, N., Gorlin, J. B., Uhl, L., Wheeler, C., Antin, J. H., Churchill, W. H., & Benjamin, R. J. (1997). Liquid nitrogen freezers: A potential source of microbial contamination of hematopoietic stem cell components. *Transfusion*, *37*(6), 585–591. https://doi.org/10.1046/j.1537-2995.1997.37697335152.x

Isachenko, V., Isachenko, E., Katkov, I. I., Montag, M., Dessole, S., Nawroth, F., & Van Der Ven, H. (2004). Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. *Biology of Reproduction*, *71*(4), 1167–1173. https://doi.org/10.1095/biolreprod.104.028811

Jackson, R. E., Bormann, C. L., Hassun, P. A., Rocha, A. M., Motta, E. L., Serafini, P. C., & Smith, G. D. (2010). Effects of semen storage and separation techniques on sperm DNA fragmentation. *Fertility and Sterility*, *94*(7), 2626–2630. https://doi.org/10.1016/j.fertnstert.2010.04.049

Kruger, T. F., Acosta, A. A., Simmons, K. F., Swanson, R. J., Matta, J. F., Veeck, L. L., Morshedi, M., & Brugo, S. (1987). New method of evaluating sperm morphology with predictive value for human in vitro fertilization. *Urology*, *30*(3), 248–251. https://doi.org/10.1016/0090-4295(87)90246-9

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

Lopushnyan, N. A., & Walsh, T. J. (2012). Surgical techniques for the management of male infertility. *Asian Journal of Andrology*, *14*(1), 94–102. https://doi.org/10.1038/aja.2011.62

Montagut, M., Gatimel, N., Bourdet-Loubère, S., Daudin, M., Bujan, L., Mieusset, R., Isus, F., Parinaud, J., & Leandri, R. (2015). Sperm freezing to address the risk of azoospermia on the day of ICSI. *Human Reproduction*, *30*(11), 2486–2492. https://doi.org/10.1093/humrep/dev234

Ohlander, S., Hotaling, J., Kirshenbaum, E., Niederberger, C., & Eisenberg, M. L. (2014). Impact of fresh versus cryopreserved testicular sperm

upon intracytoplasmic sperm injection pregnancy outcomes in men with azoospermia due to spermatogenic dysfunction: a meta-analysis. *Fertility and Sterility*, *101*(2), 344–349. https://doi.org/10.1016/j.fertnstert.2013.10.012

O'Neill, C. L., Chow, S., Rosenwaks, Z., & Palermo, G. D. (2018). Development of ICSI. *Reproduction*, *156*(1), F51–F58. https://doi.org/10.1530/REP-18-0011

Philippon, M., Karsenty, G., Bernuz, B., Courbiere, B., Brue, T., Saias-Magnan, J., & Perrin, J. (2015). Successful pregnancies and healthy live births using frozen-thawed sperm retrieved by a new modified Hotchkiss procedure in males with retrograde ejaculation: first case series. *Basic and Clinical Andrology*, *25*, 5. https://doi.org/10.1186/s12610-015-0021-4

Royere, D., Barthelemy, C., Hamamah, S., & Lansac, J. (1996). Cryopreservation of spermatozoa: A 1996 review. *Human Reproduction Update*, *2*(6), 553–559. https://doi.org/10.1093/humupd/2.6.553

Rubino, P., Vigano, P., Luddi, A., & Piomboni, P. (2016). The ICSI procedure from past to future: A systematic review of the more controversial aspects. *Human Reproduction Update*, *22*(2), 194–227. https://doi.org/10.1093/humupd/dmv050

Sherman, J. K. (1963). Improved methods of preservation of human spermatozoa by freezing and freeze-drying. *Fertility and Sterility*, *14*(1), 49–64. https://doi.org/10.1016/s0015-0282(16)34746-x

Sherman, J. K. (1973). Synopsis of the use of frozen human semen since 1964: State of the art of human semen banking. *Fertility and Sterility*, *24*(5), 397–412. https://doi.org/10.1016/S0015-0282(16)39678-9

Sieme, H., Oldenhof, H., & Wolkers, W. F. (2016). Mode of action of cryoprotectants for sperm preservation. *Animal Reproduction Science*, *169*, 2–5. https://doi.org/10.1016/j.anireprosci.2016.02.004

Sokmensuer, L. K., Bozdag, G., Esinler, I., Sever, A., & Gunalp, S. (2015). Live birth after transfer of vitrified-warmed blastocyst derived from ICSI with frozen-thawed sperm: case report. *Clinical and Experimental Obstetrics and Gynecology*, *42*(2), 251–252.

Tedder, R. S., Zuckerman, M. A., Brink, N. S., Goldstone, A. H., Fielding, A., Blair, S., Patterson, K. G., Hawkins, A. E., Gormon, A. M., Heptonstall, J., & Irwin, D. (1995). Hepatitis B transmission from contaminated cryopreservation tank. *Lancet*, *346*(8968), 137–140. https://doi.org/10.1016/s0140-6736(95)91207-x

Tsai, Y. R., Lan, K. C., Tsai, C. C., Lin, P. Y., Kung, F. T., Liu, Y. C., Huang, F. J. (2013). Pregnancy outcome and neonatal data of children born after intracytoplasmic sperm injection with a different duration of cryopreservation of spermatozoa obtained through testicular sperm extraction. *Taiwanese Journal of Obstetrics & Gynecology*, *52*(3), 329–334. https://doi.org/10.1016/j.tjog.2012.08.003

World Health Organization. (2010). WHO laboratory manual for the examination and processing of human semen.

Yazawa, H., Yanagida, K., Hayashi, S., & Sato, A. (2009). The oocyte activation and Ca2+ oscillation-inducing abilities of mouse and human dead (sonicated) spermatozoa. *Zygote*, *17*(2), 175–184. https://doi.org/10.1017/S0967199408005157

How to cite this article: Liu, S., Liu, B., Zhao, W., Liu, X., Xian, Y., Cheng, Q., Jiang, M., Yue, H., & Li, F. (2022). Rapid cryopreservation of small quantities of human spermatozoa by a self-prepared cryoprotectant without animal component. *Andrologia*, 54, e14318. https://doi.org/10.1111/and.14318