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ORIGINAL RESEARCH

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Re-sperm collection with a short abstinence time: a strategy for successful cryopreservation in cryptozoospermia

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Abstract

Background: Recent studies suggest that short-term abstinence may improve semen parameters; however, its impact on cryptozoospermia remains underexplored. This study aimed to evaluate the impact of a 2-hour abstinence interval on sperm cryopreservation outcomes and assisted reproductive success in patients with cryptozoospermia. **Methods**: This retrospective study was conducted at a human sperm bank affiliated with a tertiary hospital from September 2023 to September 2024. A total of 78 patients diagnosed with cryptozoospermia or severe oligozoospermia undergoing fertility preservation were included. Each participant provided two semen samples via masturbation: the first after 2–7 days of abstinence, and the second following a 2-hour abstinence interval. The study compared sperm cryopreservation efficacy and assisted reproductive outcomes between these two collection protocols. **Results**: Comparative analysis revealed a significant reduction in semen volume with short-term abstinence ($p < 0.001$). Notably, in cryptozoospermic patients, the second sample showed a significantly higher motile sperm concentration ($p = 0.003$), increasing cryopreservation success rates from 53.19% (25/47) to 63.83% (30/47). However, no significant difference was observed in post-thaw sperm survival rates between the first and second samples (45.59% $\pm$ 21.16% *vs.* 40.83% $\pm$ 19.82%, $p = 0.626$). Preliminary clinical data from 7 patients utilizing cryopreserved sperm revealed 4 clinical pregnancies, including 2 live births. **Conclusions**: These findings indicate that short-interval sperm collection enhances cryopreservation feasibility for cryptozoospermic patients without compromising post-thaw viability, potentially reducing reliance on invasive testicular sperm extraction. While larger prospective studies are needed to confirm these reproductive outcome benefits, this dual-collection protocol offers a cost-effective strategy to optimize sperm availability for intracytoplasmic sperm injection cycles in cases of severe male factor infertility. These results highlight the clinical value of tailored abstinence protocols in fertility preservation programs.

Keywords

Cryptozoospermia; Extremely severe oligozoospermia; Motile sperm; Sperm cryopreservation; Short abstinence

1. Introduction

Approximately 17.5% of childbearing-age couples are unable to conceive naturally, with 50% of these cases attributable to male factors [1]. A low sperm count is a significant cause of male infertility. Cryptozoospermia is defined as the absence of sperm cells in freshly ejaculated semen, although rare spermatozoa can be detected in the sediment after high-speed centrifugation [2]. In contrast, extremely severe oligozoospermia is characterized by the presence of spermatozoa in some semen samples under direct microscopic examination, but in concentrations too low to be accurately quantified [3].

Intracytoplasmic sperm injection (ICSI) is an effective treatment for both cryptozoospermia and extremely severe oligozoospermia. However, not all patients with cryptozoospermia can find sufficient viable sperm on the day of egg retrieval, necessitating testicular sperm extraction (TESE) [4]. Patients undergoing TESE may experience pain, hematoma and inflammation, and they also face a risk of androgen deficiency due to testicular failure [5]. Although a study by K. Alrabeeah *et al.* [4] demonstrated that micro-TESE enabled successful sperm retrieval in 92% (22/24) of cryptozoospermic patients, it must be emphasized that there remains a subset of these individuals for whom surgical sperm retrieval is unattainable. Freezing

sperm in advance at a sperm bank can increase their chances of achieving pregnancy using their own sperm [4, 6].

Several studies have indicated that the duration of abstinence is linked to semen parameters [7–9]. The World Health Organization (WHO) recommends an optimal abstinence period of 2 to 7 days for men, while the European Society of Human Reproduction and Embryology (ESHRE) suggests a shorter interval of 3 to 4 days [10]. The optimal abstinence time remains controversial, particularly for men with low fertility, where a 2 to 7-day abstinence period may be less beneficial [7]. Furthermore, some studies have reported that ejaculating shortly after a standard abstinence period (*e.g.*, 2–7 days) results in a second semen sample with higher sperm concentration, improved morphology, increased motility, and reduced sperm DNA damage compared to the first sample [11]. Notably, sperm DNA damage has been shown to adversely affect post-implantation development and increase the risk of spontaneous abortion [12]. These findings may explain why short-term abstinence (*e.g.*, <2 days) before ejaculation has been associated with improved outcomes in assisted reproduction techniques [13, 14].

In patients with cryptozoospermia, the impact of short abstinence periods on sperm quality has been rarely investigated. Notably, one study demonstrated that when patients with cryptozoospermia provided multiple semen samples within 24 hours, sperm concentration did not decrease, while motility significantly improved [15]. The unpredictability of detecting spermatozoa in semen samples constitutes a critical determinant for assisted reproductive technology outcomes, rendering preemptive sperm cryopreservation an essential strategy [6]. Importantly, no studies to date have systematically examined the correlation between short-term abstinence duration and sperm cryopreservation effectiveness.

The primary objectives of this study are to evaluate whether the total number of motile sperm in patients with cryptozoospermia increases after short-term abstinence, and to determine whether the efficacy of sperm cryopreservation in these patients is improved by subsequent sample collection after such abstinence periods. The secondary objectives are to compare the post-freeze-thaw sperm survival rates between semen ejaculated after a normal abstinence period and that ejaculated after short-term abstinence and to preliminarily explore whether there are differences in the assisted reproductive outcomes of sperm from the two different sources.

2. Materials and methods

2.1 Patients

A retrospective analysis was conducted on 78 male infertility patients diagnosed with cryptozoospermia or extremely severe oligospermia, where spermatozoa could be found in some semen samples under direct microscopic examination but in concentrations too low to count. These patients consulted about sperm cryopreservation at the Human Sperm Bank, West China Second University Hospital, Sichuan University (Chengdu, China), from September 2023 to September 2024. The inclusion criteria for this study were having undergone at least one semen analysis within six months in which sperm were detected, sperm concentration less than 10,000/mL, and consent to provide a second semen sample via masturbation two hours after the first sample. Data such as age, body mass index (BMI), testicular volume, hormone levels, and azoospermia factor (AZF) microdeletion results were recorded. This study received approval from the Institutional Review Board of West China Second University Hospital, Sichuan University (Chengdu, China; Approval No. 2023297).

2.2 Semen sample collection and processing

All semen samples were collected by masturbation at the clinic to minimize variables affecting semen parameters. The first semen sample was collected after an abstinence period of 2 to 7 days. A second sample was collected using the same method two hours after the first collection. All samples were incubated at 37 °C for at least 20 minutes to allow liquefaction before analysis. Subsequently, 10 μL of each semen sample was evenly spread on a glass slide, and motile sperm within the entire droplet were counted independently by two experienced embryologists. The average of their counts was recorded.

2.3 Cryopreservation and thawing of sperm

Before cryopreservation, all semen samples underwent centrifugation at 800× g for 30 minutes. Subsequently, the supernatant was carefully aspirated, leaving behind a 100-μL aliquot of the sample, which included the sediment. The sediment was then distributed on a sterile petri dish, and the total count of motile sperm was assessed under an inverted microscope.

Only those samples with more than 10 motile sperm advanced to the subsequent freezing stage. In cases where the motile sperm count was below this threshold, patients were promptly informed that their semen did not satisfy the criteria for freezing.

Next, 25 μL cryoprotectant solution was added to the samples and thoroughly mixed to ensure homogeneous distribution of the cryoprotectant. The resulting mixture was then equally divided and transferred into two straws. To initiate the freezing process, the straws were first exposed to liquid nitrogen vapor for 10 minutes, after which they were fully submerged in liquid nitrogen.

Thawing procedures were conducted on the same day as freezing. If the patient's consent was obtained, we selected one of the two straws in the same batch for thawing to evaluate the sperm survival rate after freezing and thawing. Otherwise, both straws would be retained for subsequent assisted reproductive technology treatments.

For thawing, the semen samples were immersed in water maintained at 37 °C for 2 minutes. Following thawing, a 10-μL aliquot of the sample was spread evenly on a glass slide to facilitate the counting of motile sperm. The survival rate of the sperm was determined by dividing the number of motile sperm after thawing by the pre-freeze count.

2.4 Statistical analysis

Descriptive statistics were presented as mean ± standard deviation, median (interquartile range) and percentages as appropriate. The Kolmogorov-Smirnov test was used to assess data normality. For data that met parametric test assumptions, the Student's *t*-test was applied. Otherwise, the Mann-Whitney U test or Wilcoxon matched-pairs signed-ranks test was utilized. Categorical variables were analyzed using the chi-square test. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (version 27, IBM Corporation, Somers, NY, USA).

3. Results

From September 2023 to September 2024, a total of 78 male infertility patients met the inclusion criteria based on their semen parameters. Among them, 10 patients declined to provide a second semen sample for various reasons: 4 due to time constraints, 3 due to physical discomfort, 1 because sufficient sperm had already been frozen, and 2 for other unspecified reasons. Consequently, 68 patients were included in the final analysis, with 47 (69.12%) diagnosed with cryptozoospermia and 21 (30.88%) diagnosed with severe oligoasthenospermia. There was no significant difference in baseline characteristics and hormone levels between the two groups (Table 1).

3.1 Effect of abstinence interval on the quality of semen samples

All 68 patients provided their first semen samples by masturbation after a standard abstinence period of 4.93 ± 1.75 days, with the second samples collected two hours later. Significant differences were observed between the two samples in terms of semen volume and total number of motile sperm. However, there was no significant difference in motile sperm concentration between the first and second collections (Table 2).

Both the motility sperm concentration and total motile sperm represent the characteristics of the raw semen without centrifugation treatment.

Among the 47 patients diagnosed with cryptozoospermia, 14 patients (29.79%) showed no sperm in either the first or second semen samples. In contrast, sperm was detected in at least one of the samples for the remaining 33 patients (70.21%). The volume of the first semen sample was greater than that of the second ($p < 0.001$), while the second sample demonstrated a significantly higher motile sperm concentration ($p = 0.003$). However, no significant difference was observed in the total number of motile sperm between the two samples ($p = 0.248$) (Table 3).

In all 21 patients diagnosed with severe oligospermia, sperm were detected in both semen samples. Significant differences were observed between the first and second samples in terms of semen volume, motile sperm concentration, and total number of motile sperm (Table 3).

Both the motility sperm concentration and total motile sperm represent the characteristics of the raw semen without centrifugation treatment.

3.2 Cryopreservation and thawing of semen samples

Overall, 49 patients (72.06%) successfully cryopreserved sperm at the sperm bank. Detailed cryopreservation success rates are presented in Table 4. Concisely, in patients with cryptozoospermia, the contribution of the second semen sample increased the cryopreservation success rate from 25/47 (53.19%) to 30/47 (63.83%). A total of 17 first-collection and 12 second-collection semen samples were thawed. No significant difference was found in the thawed sperm survival rate between the first and second semen samples (45.59% ± 21.16% *vs.* 40.83% ± 19.82%, $p = 0.626$) (Fig. 1). The criteria for successful cryopreservation and the methodology for calculating thawed sperm survival rates are detailed in earlier sections.

TABLE 1. Comparison of the baseline characteristics and hormone levels: cryptozoospermia *vs.* extremely severe oligospermia.

	Total	Cryptozoospermia	Extremely severe oligospermia	*p*
Patients	68	47	21	
Age (yr)	30.78 ± 4.22	30.81 ± 4.60	30.71 ± 3.32	0.933
BMI (kg/m^2)	23.61 (21.24, 26.72)	23.03 (21.22, 26.23)	24.72 (21.83, 27.24)	0.189
Left testicular volume (mL)	8.63 ± 4.82	8.45 ± 4.89	9.03 ± 4.74	0.649
Right testicular volume (mL)	8.54 ± 5.10	8.16 ± 5.20	9.40 ± 4.91	0.358
Total testicular volume (mL)	17.17 ± 9.58	16.60 ± 9.86	18.43 ± 9.02	0.472
FSH (IU/L)	22.66 ± 14.31	21.70 ± 13.11	25.55 ± 17.84	0.447
LH (IU/L)	7.58 ± 4.61	7.45 ± 3.85	7.97 ± 6.53	0.892
E_2 (pg/mL)	26.14 ± 6.84	26.71 ± 7.18	24.43 ± 5.64	0.343
T (ng/mL)	3.44 ± 1.68	3.54 ± 1.84	3.15 ± 1.09	0.518
AZF microdeletions, n (%)	15 (22.06%)	8 (17.21%)	7 (33.33%)	0.134

BMI: Body mass index; AZF: azoospermia factor; FSH: Follicle - Stimulating Hormone; E_2: Estradiol; T: Testosterone; LH: Luteinizing Hormone.

TABLE 2. Comparison of semen parameters between the first and second semen samples (n = 68).

Semen Parameter	First Sample	Second Sample	*p*
Abstinence time	4.93 ± 1.75 d	2 h	
Volume (mL)	3.18 ± 1.55	1.65 ± 0.85	<0.001
Motility sperm Concentration (sperm/mL)*	16.50 (0.00, 537.5)	50.00 (0.00, 475.00)	0.953
Total motile sperm count (sperm)*	64.35 (0.00, 1583.75)	25.00 (0.00, 777.50)	0.007

**: Both the motility sperm concentration and total motile sperm represent the characteristics of the raw semen without centrifugation treatment.*

TABLE 3. Comparison of semen parameters between the first and second semen samples, in patients with cryptozoospermia (n = 33) and patients with extremely severe oligospermia (n = 21).

	Cryptozoospermia (n = 33)		*p*	Extremely severe oligospermia (n = 21)		*p*
Semen Parameter	First Sample	Second Sample		First Sample	Second Sample	
Abstinence time	4.64 ± 2.06 d	2 h		5.05 ± 1.28 d	2 h	
Volume (mL)	3.11 ± 1.70	1.58 ± 0.95	<0.001	3.65 ± 1.42	2.07 ± 0.67	<0.001
Motility sperm Concentration (sperm/mL)*	0.00 (0.00, 150.00)	100.00 (0.00, 700.00)	0.003	666.00 (300.00, 1367.00)	200.00 (25.00, 925.00)	0.005
Total motile sperm count (sperm)*	0.00 (0.00, 462.50)	100.00 (0.00, 830.00)	0.248	2450.00 (1262.70, 5400.45)	380.00 (75.00, 1616.45)	<0.001

**: Both the motility sperm concentration and total motile sperm represent the characteristics of the raw semen without centrifugation treatment.*

TABLE 4. The cryopreservation success rate of patients.

	Total	Cryptozoospermia	Extremely severe oligospermia
Patients	68	47	21
Number of patients with Sample cryopreservation, n (%)	49 (72.06%)	30 (63.83%)	19 (90.48%)
Number of patients with first Sample cryopreservation, n (%)	44 (64.71%)	25 (53.19%)	19 (90.48%)
Number of patients with second Sample cryopreservation, n (%)	40 (58.82%)	23 (48.94%)	17 (80.95%)

3.3 Outcomes of using cryopreserved sperm in assisted reproductive technology

A total of 7 patients utilized their cryopreserved sperm for assisted reproductive technology. Currently, 4 patients have achieved clinical pregnancy. Among these, 2 have resulted in live births, while the outcomes of the other 2 are still unknown (Table 5).

4. Discussion

Over recent decades, a steady decline in global male semen quality has been observed, particularly manifested by declining sperm concentration and total sperm count [16]. Factors including obesity, suboptimal dietary patterns, psychological stress, ionizing radiation, abuse of tobacco and alcohol, as well as the COVID-19 pandemic, have collectively led to the decline in male spermatogenic function [17–19]. In cases of cryptozoospermia, testicular tissue may harbor only a limited number of viable spermatogenic foci. However, this residual spermatogenic capacity demonstrates progressive deterioration with disease progression, advancing age, alterations in hormonal profiles, and pathologically elevated inhibin B expression—biochemical changes that may ultimately culminate in complete spermatogenic failure [20, 21]. ICSI has become a cornerstone in assisted reproductive technology for addressing male infertility. However, its clinical efficacy remains critically dependent on successful sperm retrieval, posing significant challenges in cryptozoospermic patients who may face consistent absences of sperm in semen samples. In our study, sperm was absent in the semen of 17 out of 68 patients (25%). This aligns closely with findings by Chong Won Bak *et al.* [22], who reported that 18 out of 65 patients (27.7%) with oligospermia eventually developed azoospermia, particularly those who initially had fewer sperm. This similarity underscores the importance of addressing cryptozoospermia to enhance sperm availability.

Most existing research indicates that shorter abstinence periods lead to reduced semen volume [13, 23–25], similar findings were also observed in our study. Semen volume is a reliable marker of the secretory functions of accessory glands, particularly the seminal vesicles. The reduction in semen volume following short-term abstinence is likely due to insuf-

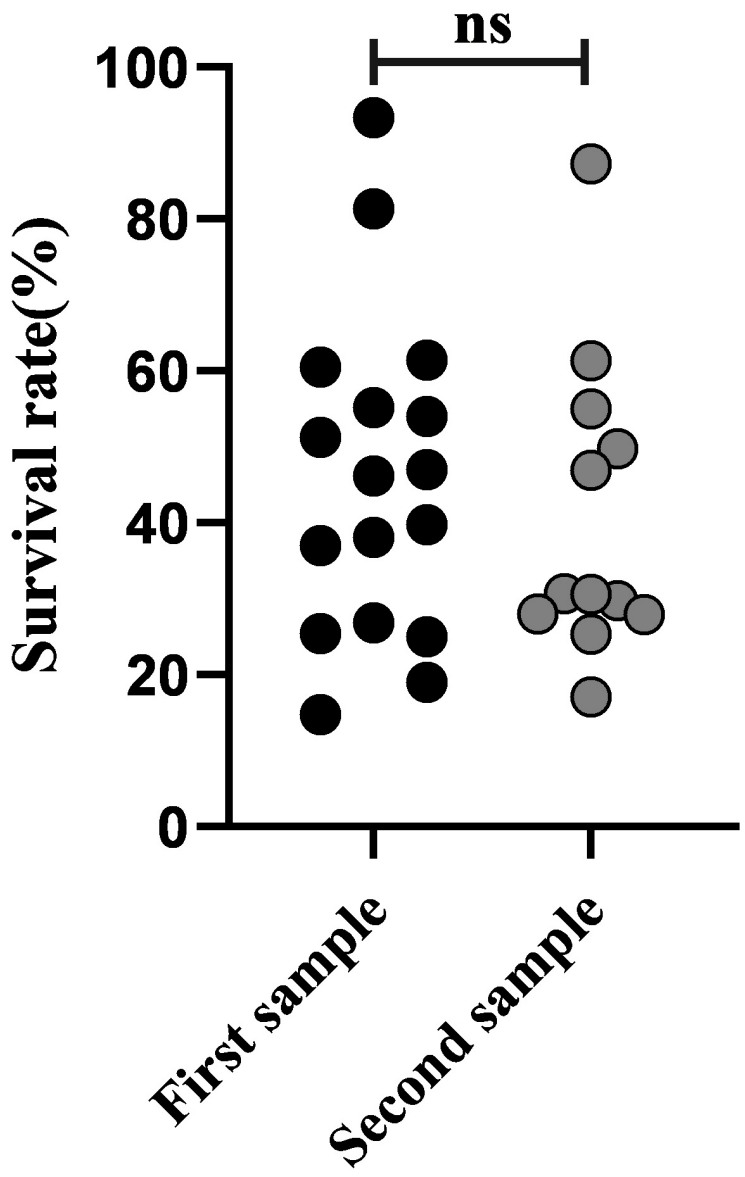

FIGURE 1. Comparison of post-thaw sperm survival rates between the first and second semen samples. ns: no significant difference.

TABLE 5. The outcomes of using cryopreserved sperm in assisted reproductive technology.

Patient	Disease categories	Origin of frozen sperm	Cryopreservation duration (d)	Number of retrieve oocytes	Number of injected sperm	Number of fertilizations	Number of available embryos	Number of transplantations	Pregnancy	Take-home baby
1	ESO	1st	296	4	3	2	2	2	No	-
2	ESO	1st	25	29	16	12	5	1	Yes	Yes
3	ESO	1st	91	24	16	2	2	2	No	-
4	CZ	1st and 2nd	32	18	13	7	3	2	Yes	Yes
5	CZ	1st	40	17	4	2	2	2	Yes	Unknown
6	CZ	2nd	98	5	4	2	1	1	Yes	Unknown
7	CZ	1st and 2nd	186	20	5	2	2	2	No	-

ESO: extremely severe oligozoospermia; CZ: cryptozoospermia; 1st: The first semen sample; 2nd: The second semen sample; "Unknown" means that the outcome cannot be traced or has not occurred yet.

ficient time for these glands, especially the seminal vesicle and prostate gland, to replenish fully [26]. High serum testosterone levels are known to enhance the secretory activities of these glands, suggesting that the observed reduced volume may be associated with limited testosterone exposure during shorter abstinence periods [27].

While ICSI can fertilize an oocyte by injecting immotile sperm, the random use of immotile sperm often leads to lower fertilization rates [28]. The pH of seminal plasma, influenced by the secretions of accessory glands, plays a significant role in sperm motility. Moreover, longer abstinence periods can lead to sperm damage due to prolonged exposure to reactive oxygen species in the epididymal environment, potentially impairing motility [29]. Previous studies on abstinence duration and sperm motility have shown mixed results, but uniformly used the "percentage of motile sperm" as a motility indicator [23, 30, 31]. However, in conditions like cryptozoospermia and severe oligospermia, even minor counting inaccuracies can cause significant deviations in motility percentages. Therefore, the traditional semen parameters such as "Progressive motility" and "Sperm concentration", may not effectively characterize these conditions. Our study therefore included both motile sperm concentration and total motile sperm count to provide a more precise assessment. We observed that shorter abstinence improved motile sperm concentration in cryptozoospermia patients but not in those with severe oligospermia, likely due to longer epididymal transit times in the latter. Moreover, the presence of pyruvate and lactate, which is crucial for sperm mitochondrial function and motility, are more abundant relative to the lower total sperm count in cryptozoospermia, potentially enhancing motility [32]. However, despite these findings, the total motile sperm count showed no significant difference between the two semen samples, which aligns with the findings of Jessica A. Marinaro *et al.* [15]. Total motile sperm count is influenced by both motile sperm concentration and semen volume. Although the motile sperm concentration increased, the semen volume significantly decreased, offsetting the potential improvement in total motile sperm count.

Semen cryopreservation is pivotal for managing male-factor infertility, particularly among male cancer patients on the verge of undergoing chemotherapy and radiotherapy. In severe oligozoospermia and cryptozoospermia, testicular function worsens over time, making early semen freezing and storage advisable [33]. The freezing process can damage sperm by forming ice crystals that disrupt cell membranes and organelles, reducing sperm survival rate and motility [34]. For those with poor semen quality, the sperm motility rate after freezing ranges from 0 to 92% [35]. These variations can be attributed to differences in the pre-freezing semen quality and the freezing methods employed. Typically, poorer semen parameters lead to low post-thaw recovery of motile and viable spermatozoa [36]. In our study, no significant differences were observed in post-thaw sperm survival rates between semen samples collected after different abstinence periods. This represents the first investigation to explore the feasibility of short abstinence intervals for cryopreservation in cryptozoospermia patients, demonstrating that repeated sperm collection with short abstinence is a viable strategy. In the case of cryptozoospermia, freezing, thawing and selecting an adequate number of sperm pose significant challenges, as cryopreserving a limited quantity of human spermatozoa remains a major hurdle for embryologists. Notably, 63.83% (30/47) of cryptozoospermic patients in our cohort successfully cryopreserved sperm, with 16.67% (5/30) achieving this through short abstinence protocols—highlighting a significant advancement for those who might otherwise struggle with sperm preservation. Moreover, male patients with reduced fertility are more likely to experience pronounced anxiety and psychological stress [37], leading them to frequently visit sperm banks to freeze more sperm for ICSI and gain psychological comfort. Thus, obtaining a larger number of motile sperm for cryopreservation is of dual therapeutic and strategic significance for both the fertility outcomes and the psychological well-being of patients with cryptozoospermia.

Based on the current assisted reproductive technology outcome data from our study, patients with cryptozoospermia exhibit a higher utilization rate of cryopreserved sperm obtained following short-term abstinence. This trend is primarily attributed to the increased likelihood of insufficient sperm retrieval on the day of oocyte retrieval, necessitating a greater reliance on cryopreserved specimens to fulfill the treatment requirements. Although our sample size remains limited, preliminary observations indicate a potential correlation between the enhanced clinical pregnancy rate in cryptozoospermia patients and the use of sperm samples collected after short-term abstinence. These findings are consistent with those reported by Filomena Scarselli *et al.* [23], who demonstrated that the use of sperm collected after a 1-hour abstinence interval could improve the outcomes of assisted reproductive technology (ART). However, further research with a larger case series is needed to confirm whether cryopreserving sperm after short abstinence periods offers similar benefits.

Given the scarcity of sperm in cryptozoospermia cases, every retrieved spermatozoon is critically preserved for potential ICSI cycles. This conservation strategy necessitates prioritizing cryopreservation over ancillary assessments like DNA fragmentation analysis. Moreover, conventional laboratory assessment methods, such as semen analysis based on the WHO criteria, may not apply to semen samples with such a limited number of spermatozoa, further complicating this research. Due to these limitations, we were unable to collect data on DNA fragmentation rates or sperm morphology, both of which are important factors in assisted reproductive outcomes and represent a limitation of our study. In future research, we aim to focus more on these aspects.

Patients undergoing self-sperm cryopreservation often face prolonged treatment cycles when using frozen sperm for ART, resulting in extended follow-up periods for assessing pregnancy and miscarriage rates. Future research should focus on whether these findings translate into differences in fertility outcomes. Although numerous studies have explored the effects of short abstinence periods on semen parameters, very few have specifically examined patients with severe oligozoospermia and cryptozoospermia. To our knowledge, no studies have compared the post-thaw survival rates of sperm between normal and short abstinence periods. Our study did not find significant differences, suggesting that cryopreservation of sperm after a short abstinence period is feasible.

Nevertheless, for some cryptozoospermic patients who initially lack sufficient motile sperm, performing two consecutive sperm collections increases the chances of successful sperm cryopreservation. This approach also reduces the time and cost associated with multiple visits to the sperm bank.

5. Conclusions

In summary, our research demonstrates that neither normal nor short abstinence periods significantly affect the freeze-thaw survival rate of sperm. Importantly, performing dual semen collections within a short abstinence interval improves the success rate of cryopreservation and increases the number of preserved sperm in patients with cryptozoospermia. These findings support the adoption of short-interval dual semen collection as a standard protocol for fertility preservation in cryptozoospermic patients, potentially reducing their reliance on invasive TESE procedures. This approach may enhance the likelihood of successful outcomes in assisted reproductive technologies.

AVAILABILITY OF DATA AND MATERIALS

Data regarding any of the subjects in the study can be shared after reasonable request to the corresponding author. No identifying data will be provided.

AUTHOR CONTRIBUTIONS

WRZ—participated in the experimental operations; completed the collection; verification and statistical analysis of the data; and wrote the draft. CL—provided drawing assistance and participated in the experimental operation. BL, JYX, XFL and YL—participated in the experimental operation. FPL and YX—put forward experimental ideas and reviewed and revised the paper. All the authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of West China Second University Hospital, Sichuan University (No: 2023297). Informed consent was obtained from all individual participants included in the study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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