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Safety evaluation of single-sperm cryopreservation technique applied in intracytoplasmic sperm injection

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Research Article

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Abstract

Intracytoplasmic sperm injection (ICSI) is a technique that directly injects a single sperm into the cytoplasm of mature oocytes. Here, we explored the safety of single-sperm cryopreservation applied in ICSI. This retrospective study enrolled 186 couples undergoing ICSI-assisted pregnancy. Subjects were allocated to the fresh sperm (group A)/single-sperm cryopreservation (group B) groups based on sperm type, with their clinical baseline/pathological data documented. We used ICSI-compliant sperm for subsequent *in vitro* fertilization and followed up on all subjects. The recovery rate/cryosurvival rate/sperm motility of both groups, the pregnancy/outcome of women receiving embryo transfer, and the delivery mode/neonatal-related information of women with successful deliveries were recorded. The clinical pregnancy rate, cumulative clinical pregnancy rate, abortion rate, ectopic pregnancy rate, premature delivery rate, live birth delivery rate, neonatal birth defect rate, and average birth weight were analyzed. The two groups showed no significant differences in age, body mass index, ovulation induction regimen, sex hormone [anti-Müllerian hormone (AMH)/follicle-stimulating hormone (FSH)/luteinizing hormone (LH)] levels, or oocyte retrieval cycles. The sperm recovery rate (51.72%-100.00%) and resuscitation rate (62.09% ± 16.67%) in group B were higher; the sperm motility in the two groups demonstrated no significant difference and met the ICSI requirements. Group B exhibited an increased fertilization rate, decreased abortion rate, and increased safety versus group A. Compared with fresh sperm, the application of single-sperm cryopreservation in ICSI sensibly improved the fertilization rate and reduced the abortion rate, showing higher safety.

Introduction

Infertility is a major health and social issue around the world (Moore and Reijo-Pera, 2000). Currently, male factors are estimated to be responsible for 30% to 50% of infertility cases (Eisenberg *et al.*, 2023). Male infertility is commonly seen as severe oligozoospermia or azoospermia due to spermatogenic dysfunction (Liu and Li, 2020). Notably, intracytoplasmic sperm injection (ICSI) is a useful technique developed on the basis of *in vitro* fertilization-embryo transfer, which assists reproduction by directly injecting sperm into the oocyte cytoplasm and has a promising prospect in male infertility treatment. ICSI offers an opportunity to surmount fertility issues for patients with severe oligozoospermia or azoospermia and those who require testicular sperm retrieval (Liu and Li, 2020). ICSI is currently the most prevalent method of insemination in the world due to its multiple indications and widespread use in clinical practice.

The utilization of testicular spermatozoa with ICSI has yielded ameliorated results in couples with male factor infertility and serious sperm abnormalities [such as severe oligozoospermia, cryptozoospermia, nonobstructive azoospermia (NOA), obstructive azoospermia (OA), and sperm DNA fragmentation] (Zini *et al.*, 2017; Alkandari *et al.*, 2021). Schlegel *et al.* have created an operation known as microdissection testicular sperm extraction (micro-TESE) to obtain the best sperm acquisition rate while minimizing damage to the testicles (Schlegel, 2009). However, patients with NOA have a very low number of viable sperm. Cryopreserving spermatozoa in small amounts using micro-TESE helps male patients with NOA preserve their fertility without requiring them to undergo repetitive surgeries (Park *et al.*, 2003; Miller *et al.*, 2017; Liu and Li, 2020). Conversely, traditional techniques of sperm preservation pose a risk of sperm loss resulting from factors such as the washing process, sperm adhesion to the carrier vessel, and intense centrifugation (Nawroth *et al.*, 2002). Therefore, the traditional techniques pose great

challenges in situations in which there is a low count of sperm (AbdelHafez *et al.*, 2009). In 1997, Cohen *et al.* introduced a novel method for cryopreserving individual spermatozoa using an empty zona pellucida and proposed the principle of single-sperm cryopreservation (Cohen *et al.*, 1997). For decades, researchers have been trying to explore single-sperm cryopreservation methods and carriers, and a large number of cryopreservation techniques have been developed to increase the number of sperm. The safety of single-sperm cryopreservation technology in ICSI application research is becoming increasingly significant as its use expands in the clinic, yet few relevant reports exist in this area. This study mainly discusses the safety of the single-sperm cryopreservation method in ICSI and supplies a theoretical basis for the clinical application of single-sperm cryopreservation technology in ICSI.

Materials and methods

Ethics statement

This study was reviewed and approved by the Academic Ethics Committee of Nanchang Reproductive Hospital and complied with the Declaration of Helsinki. All patients and their families were informed of the study's purpose and signed an informed consent.

Study subjects

In total, 233 couples who underwent intracytoplasmic sperm injection (ICSI)-assisted pregnancy and attended Nanchang Reproductive Hospital from January 2018 to January 2022 were selected for this retrospective study. Among them, 31 couples did not match the inclusion criteria, seven couples refused to participate in the study, nine couples provided incomplete information, and 186 couples were finally included as the subjects. Based on the type of sperm they used, all patients were assigned to the fresh sperm group (group A, $n = 134$) and the single-sperm freezing group (group B, $n = 52$). The clinical baseline data of age, body mass index (BMI), disease type, sperm acquisition method of all male patients and age, BMI, ovulation induction regimen, sex hormone levels [anti-Müllerian hormone (AMH), follicle-stimulating hormone (FSH), luteinizing hormone (LH)], oocyte retrieval cycle, and the number of retrieved oocytes of all female subjects receiving embryo transfer were recorded.

Inclusion and exclusion criteria

The inclusion criteria were as follows: conformed to the indications of ICSI-assisted pregnancy and with assisted pregnancy via ICSI with complete data.

The exclusion criteria were as below: with potentially reversible NOA, cryptospermia, and oligozoospermia (recent febrile illness, recent medical illness, or exposure to toxins); cryptorchidism; received micro-TESE in the past; women with diabetes who had endometriosis, uterine fibroids, uterine malformations, ovarian failure, recurrent uterine scar rupture, hereditary diseases, long-term anaemia, pulmonary hypertension, and glycosylated haemoglobin > 10%; with pregnancy contraindications such as severe renal failure (creatinine > 250 mmol/l); with incomplete data.

The primary indication for ICSI was unambiguous, i.e. severe male factor: NOA and OA, following recovery of epididymal/testicular spermatozoa; spermatozoa with rounded heads (globozoospermia); acinesia (such as immotile cilia syndrome); necrozoospermia; and anti-spermatozoa antibodies (Yang *et al.*, 2019). Additional secondary indications included severe cryptozoospermia or oligozoospermia, particularly in cases in which prior ICSI cycles had resulted in unsuccessful outcomes using ejaculate sperm or had exhibited a high incidence of DNA fragmentation in sperm (Colpi *et al.*, 2018).

Diagnostic criteria

As per the standards established by the World Health Organization, severe oligospermia and asthenospermia were diagnosed when the sperm cell count fell below 5 million/ml (Halpern *et al.*, 2018; Tilahun *et al.*, 2022).

Azoospermia was diagnosed on at least two diagnostic semen samples following 1800 *g* centrifugation and investigation of the entire pellet (Verheyen *et al.*, 2004). OA and NOA were further determined according to a physical examination and testicular biopsy. First, the texture, size, and vas deferens of the testes were palpated. If the testes were very small, with the size of a soybean or a fava bean, it suggested azoospermia due to loss of spermatogenesis in the testes, known as NOA. If the size and texture of the testis were normal and the vas deferens were not palpated, it was called congenital unilateral absence of vas deferens or bilateral absence of vas deferens, which was defined as OA caused by obstruction of the duct (Wosnitzer and Goldstein, 2014). If the testes were well developed and textured, the patients would be further examined for the presence of spermatozoa in the testes by a minimally invasive aspiration biopsy of the testes. If there was a high number of spermatozoa in the testes and no spermatozoa in the semen, it was called OA, and the patients were subjected to further examination to define the site of obstruction, while NOA was the medical term for the absence of sperm in the testicles (Wu *et al.*, 2021).

Erectile dysfunction (ED) refers to the persistent or recurring incapacity to achieve and/or sustain a satisfactory penile erection that is adequate for sexual gratification, encompassing the ability to engage in satisfactory sexual activity (Burnett *et al.*, 2018).

Diagnostic criteria for azoospermia was that all the sperm cells were not viable (Tilahun *et al.*, 2022).

Ejaculation dysfunction consists of premature ejaculation, non-ejaculation, and retrograde ejaculation. Premature ejaculation diagnostic criteria refer to ejaculation after a very short period of sexual intercourse, some before body contact with a woman (McMahon and Porst, 2011). Diagnostic criteria for non-ejaculation were normal libido and erection but no ejaculation or orgasm during sexual intercourse (Abdel-Hamid and Ali, 2018). Diagnostic criteria for retrograde ejaculation included normal orgasm and ejaculation in matrimonial life but no semen outflow, which was confirmed mainly by the combination of medical history and physical examination, as well as the presence of sperm in the first urine after masturbation confirmed by physical examination (Gray *et al.*, 2018).

Sperm acquisition

Different methods of testicular sperm harvesting were chosen depending on the disease type of the male patients. Sperm were obtained by micro-TESE in patients with NOA and necrospermia or by testicular puncture in patients with OA, severe oligozoospermia, ejaculatory disorders, and ED (Lee *et al.*, 2008; Katayama *et al.*, 2020).

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Single-sperm cryopreservation technique

The main indication for the single-sperm cryopreservation technique was for patients with NOA, because they had a very limited number of surviving sperm. Cryopreservation of spermatozoa from patients with NOA by micro-TESE could prominently improve clinical therapy, reduce the pain and cost of repeated testicular puncture, and preclude the adoption of donor sperm or cancellation of insemination due to insufficiency of available sperm on the day of oocyte retrieval (Park *et al.*, 2003; Miller *et al.*, 2017; Liu and Li, 2020).

The single sperm was frozen using the droplet freezing method. In brief, in a single-sperm freezing dish (model: Tiny-D; specifications: C1R3; production batch No.: 3202101), polyvinylpyrrolidone (PVP) microdroplets and several Modified Human Tubal Fluid (m-HTF) culture drops containing 10% serum substitute supplement (SSS) were covered with mineral oil and were allow to balance in a CO_2-free incubator for 2 h. PVP was used for sterilizing the micro-manipulation needle, while culture drops were utilized for introducing treated sperm. Patient information was labelled at the bottom of the dish. Origio sperm freezing solution was diluted with m-HTF containing 10% SSS at a ratio of 1:1. Liquid nitrogen was added to the foam box, and the liquid nitrogen surface was 4 cm away from the upper edge of the foam box. After the processed sperm and the patient information on the frozen dish were checked, the patient information was marked again on the sperm freezing carrier, 0.5 μl frozen liquid was supplemented, and the position of the card slot in the frozen dish was promptly transferred. The sperm was added to the droplets of an ultra-thin frozen carrier made from cryoprotectant using a micromanipulator and an ICSI needle. The number of sperm placed on each frozen section was comprehensively considered according to the total number of sperm and the expected number of eggs obtained by the woman, which were all recorded on the tube side. The frozen carrier was then taken out and fumigated for 3.5–4.5 min until the freezing liquid droplets were frozen, which were later allowed to stand for 30 s and transferred to a 1.0 ml cryopreservation tube in liquid nitrogen for long-term preservation. On the day of oocyte retrieval, the frozen tube was removed from storage, and the patient records were double-checked. The cap of the cryopreservation tube was unscrewed, and the frozen carrier handle was secured using a plastic adornment and then suspended in the air for a duration of 1–2 s, followed by rapid thawing in mineral oil that had been heated to a temperature of 42°C for a water bath for approximately 5 s. Subsequently, the carrier was transferred to a designated dish for single-sperm injection, enabling the observation of sperm activity rate, and spermatozoa that met the requirements of ICSI were used for ICSI (Verheyen *et al.*, 2004). All operations were performed by experienced embryologists.

The rates of sperm recovery and cryosurvival in male patients of the single frozen sperm group were documented. The calculation formulas were outlined below: recovery rate = (number of recovered sperm/number of thawed sperm) × 100%; cryosurvival rate = (number of cryosurvival sperm/number of recovered sperm) × 100%. The XD-6000X sperm quality detection system (SXZZ20202060156, XINDA, Xuzhou, Jiangsu, China) was used to measure the sperm motility rates of the fresh sperm group and the single-sperm cryopreservation group after recovery and cryosurvival. In short, the semen samples were amplified by the microscope and input into the computer through the electronic camera system. Image processing technology and algorithms were used to detect and analyze the sperm activity rate automatically and quantitatively.

Oocyte retrieval

The female subjects received ovarian stimulation using various protocols, including the luteal-phase stimulation ovarian protocol, ultra-long protocol, mini-stimulation protocol, antagonist protocol, direct stimulation protocol, and modified ultra-long protocol. The procedure of oocyte retrieval was performed 36 h following the injection of human chorionic gonadotropin (hCG; 10000 IU), accomplished with the use of vaginal ultrasound-guided puncture of the ovarian follicles. The removal of the cumulus cells was conducted by subjecting them to an exposure of hyaluronidase (10 IU). The nuclear maturation of oocytes was observed and counted under a stereomicroscope. Only mature oocytes at the metaphase II stage were used for ICSI. All operations were carried out by experienced embryologists.

Follow-up

The study followed up on couples who underwent ICSI treatment, starting from the last menstrual period prior to the initial ICSI procedure and continuing for a minimum of 12 months if no pregnancy ensued. In the event of pregnancy, the follow-up process persisted until the confirmation of an ongoing pregnancy with the use of ultrasound, specifically after the 8th week. The ultrasound image revealed the existence of a gestational sac accompanied by a fetal heartbeat. For pregnancies that resulted in a spontaneous abortion, the follow-up period is extended until the occurrence of a subsequent ongoing pregnancy or, at minimum, for a duration of 12 months. The laboratory indexes of embryos after ICSI [maturation rate, fertilization rate, two pronuclei (2PN) fertilization rate, 2PN cleavage rate, D3 high-quality embryo rate, D5 blastocyst formation rate, and D5 high-quality blastocyst rate] were recorded. The pregnancy and outcome of all women receiving embryo transfers were also recorded. If the delivery was successful, the mode of delivery and neonatal-related information were duly recorded.

The clinical pregnancy rate, cumulative clinical pregnancy rate, abortion rate, ectopic pregnancy rate, premature delivery rate, live birth delivery rate, neonatal birth defect rate, and average birth weight were calculated. The specific calculation formulas were as follows: maturation rate = (number of mature eggs/number of retrieved eggs) × 100%; fertilization rate = (fertilization number/mature egg number) × 100%; 2PN fertilization rate = (2PN fertilization number/mature egg number) × 100%; 2PN cleavage rate = (2PN cleavage number/2PN fertilization number) × 100%; D3 high-quality embryo rate = (D3 high-quality embryo number/2PN cleavage number) × 100%; D5 blastocyst formation rate = (D5 blastocyst formation number/blastocyst culture number) × 100%; D5 high-quality blastocyst rate = (D5 high-quality blastocyst number/D5 blastocyst number) × 100%; clinical pregnancy rate = (number of first transplant pregnancies/total number of transplant cycles) × 100%; cumulative clinical pregnancy rate = (number of all pregnancies/total number of transplant cycles) × 100%; abortion rate = (number of cycles of abortion/number of all clinical pregnancy cycles) × 100%; the ectopic pregnancy rate = (the number of cycles of ectopic pregnancy/the number of all clinical pregnancy cycles) × 100%; preterm birth rate = (the number of preterm birth cycles/the number of all clinical pregnancy cycles) × 100%; live birth delivery

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rate = (number of births with live births/total number of transplant cycles) × 100%; birth defect rate = (number of birth defects/number of births) × 100%; mean birth weight = (sum of all birth weights/number of births) × 100%.

Statistical analysis

Data analysis and mapping were performed using SPSS 21.0 (IBM Inc., Chicago, IL, USA) and GraphPad Prism 8.01 (GraphPad Software Inc., San Diego, CA, USA). The Shapiro–Wilk test was used to test the normal distribution. Normally distributed measurement data were presented as mean ± standard deviation and analyzed using the independent sample *t*-test. Non-normally distributed measurement data were expressed as median (minimum, maximum) values and analyzed using the Mann–Whitney *U*-test. The categorical variables were expressed as the number of cases (%), followed by the chi-squared test. The value of $p < 0.05$ was considered statistically significant.

Results

Comparisons of clinical baseline data for male patients

The clinical baseline data of male patients are shown in Table 1. There were no marked differences in age and BMI between the two groups (all $p > 0.05$), but there were evident differences in disease type and sperm acquisition mode (all $p < 0.001$). In the single-sperm cryopreservation group, the recovery rate of sperm was 100.00% (51.72–100.00%), the cryosurvival rate was 62.09% ± 16.67%, and the sperm motility was 56.27% (51.08–67.65%). No significant discrepancy in sperm motility was observed between the two groups of male patients ($p > 0.05$), which met the standard of ICSI. These suggested that the single-sperm cryopreservation technology did not affect sperm motility.

Comparisons of clinical baseline data for women undergoing embryo transfer

The clinical baseline characteristics of women receiving embryo transfer are illustrated in Table 2. There were no obvious differences in age, BMI, ovulation promotion regimen, sex hormone levels (AMH, FSH, and LH), or egg retrieval cycle between the two groups (all $p > 0.05$), while the number of eggs obtained in the single-sperm cryopreservation group was significantly lower than that in the fresh sperm group ($p < 0.01$).

Analysis of embryo laboratory indexes

The sperm and eggs of all subjects were fertilized *in vitro* by ICSI. The results of laboratory indicators (Table 3) showed no conspicuous discrepancies in embryo maturation rate, 2PN fertilization rate, 2PN cleavage rate, or D3 high-quality embryo rate between the two groups (all $p > 0.05$). Compared with the fresh sperm group, the fertilization rate of the single-sperm cryopreservation group was pronouncedly raised ($p < 0.05$), while the D5 blastocyst formation rate and D5 high-quality blastocyst rate notably declined (all $p < 0.01$). The above results implied that the application of single-sperm cryopreservation in ICSI could prominently improve the fertilization rate relative to the application of fresh sperm.

Analysis of embryo transfer clinical indexes

The fertilized eggs *in vitro* were transplanted into the female uterus after culture into multicellular blastocysts, and the number of transplantation cycles was counted separately. The total number of transplantation cycles in group A was 182, and that of group B was 57, with no pronounced discrepancies in fresh cycles and thawing cycles between the two groups (Table 4, all $p > 0.05$). The pregnancy status and outcomes of all women receiving embryo transfer and neonatal-related information were recorded, which depicted that no clear differences were discovered in clinical pregnancy rate, cumulative clinical pregnancy rate, ectopic pregnancy rate, premature delivery rate, delivery mode, or live birth rate between the two groups (Table 4, all $p > 0.05$), while the abortion rate of women undergoing embryo transfer in group B was obviously lower than that of women experiencing embryo transfer in group A (Table 4, $p < 0.05$). Beyond that, there was no apparent difference in the average birth weight of newborns between the two groups (Table 4, $p > 0.05$), while the birth defect rate of newborns in group B was slightly lower than that in group A, but the difference did not reach a significant level (Table 4, $p > 0.05$). These foregoing findings demonstrated that applying single-sperm cryopreservation technology in ICSI could signally reduce the abortion rate and possess higher safety in contrast with applying fresh sperm.

Discussion

Treatments for male infertility often aim to restore fertility so that conception can occur normally (Taitson *et al.*, 2022). ICSI is a successful treatment for male infertility and has emerged as the prevailing technique for fertilization (Vaegter *et al.*, 2017; Dang *et al.*, 2021). It is also noteworthy that sperm cryopreservation is a widely utilized technique within the field of assisted reproductive technology that serves to preserve male viability (O'Neill *et al.*, 2019). Therefore, to address the issue of sperm viability, the potential implementation of the single-sperm cryopreservation technique in ICSI is being contemplated. As reflected by our present findings, compared with fresh sperm, the use of single-sperm cryopreservation in ICSI could substantially increase the fertilization rate and embryo 2PN rate, decrease the abortion rate, and increase safety.

In general, the utilization of the single-sperm freezing method yields favourable results in terms of sperm motility and functionality post-thawing (Cayan *et al.*, 2001; Peng *et al.*, 2011). In the present study, no statistically significant disparity was observed in sperm motility between the two cohorts of male patients, and both groups exhibited sufficient motility levels to meet the criteria for ICSI. This suggests that sperm motility will not be affected by single-sperm cryopreservation technology. In addition, multiple cohort studies have demonstrated a favourable correlation between the number of oocytes obtained during *in vitro* fertilization procedures and the likelihood of a successful live birth outcome (van der Gaast *et al.*, 2006; Ji *et al.*, 2013; Steward *et al.*, 2014). In our study, it was worth noting that the single-sperm cryopreservation group had fewer retrieved oocytes than the fresh sperm group.

The purpose of individual sperm cryopreservation is to keep the sperm alive and able to fertilize an egg even after being frozen (Le *et al.*, 2019). In this experiment, we conducted *in vitro* fertilization

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Table 1. Comparisons of clinical baseline data for male patients

Characteristics	Group A ($n = 134$)	Group B ($n = 52$)	p-value
Age (years)	30.00 (21.00–63.00)	31.00 (22.00–62.00)	0.884
BMI (kg/m^2)	22.74 (19.43–29.32)	22.66 (19.06–29.40)	0.748
Disease type (cases)			< 0.001
Severe oligozoospermia and asthenozoospermia	73 (54.48%)	4 (7.69%)	–
OA	26 (19.40%)	8 (15.38%)	–
NOA	26 (19.40%)	34 (65.38%)	–
Erectile dysfunction	2 (1.49%)	2 (3.85%)	–
Necrospermia	0 (0.00%)	3 (5.77%)	–
Ejaculatory dysfunction	7 (5.22%)	1 (1.92%)	–
Sperm collection method (cases)			< 0.001
Microscopic sperm extraction	25 (18.66%)	37 (71.15%)	–
Puncture	109 (81.34%)	15 (28.85%)	–
Recovery rate (%)	–	100.00 (51.72–100.00)	–
Cryosurvival rate (%)	–	62.09 ± 16.67	–
Sperm activity rate (%)	57.22 (50.41–65.03)	56.27 (51.08–67.65)	0.211

Note: Group A, fresh sperm group; Group B, single-sperm cryopreservation group. BMI, body mass index; OA, objective azoospermia; NOA, nonobstructive azoospermia. Normally distributed measurement data were presented as mean ± standard deviation. Non-normally distributed measurement data were expressed as median (minimum, maximum) values and analyzed using the Mann–Whitney *U*-test. The categorical variables were expressed as the number of cases (%), followed by the chi-squared test. $p < 0.05$ was accepted as indicative of significant differences.

Table 2. Comparisons of clinical baseline data for women undergoing embryo transfer

Characteristics	Group A ($n = 134$)	Group B ($n = 52$)	p-value
Age (years)	29.00 (20.00–46.00)	28.00 (21.00–46.00)	0.920
BMI (kg/m^2)	21.36 (16.65–31.22)	21.24 (16.44–29.05)	0.743
Ovulation induction protocol (cases)			0.104
Ultra-long protocol	7 (5.22%)	1 (1.92%)	–
Modified ultra-long protocol	89 (66.42%)	30 (57.69%)	–
Luteal-phase stimulation ovarian protocol	3 (2.24%)	6 (11.54%)	–
Antagonist protocol	33 (24.63%)	15 (28.85%)	–
Mini-stimulation protocol	1 (0.75%)	0 (0.00%)	–
Direct stimulation protocol	1 (0.75%)	0 (0.00%)	–
AMH (ng/ml)	3.25 (0.09–17.80)	2.47 (0.19–10.15)	0.123
FSH (mIU/ml)	7.22 (1.27–28.71)	7.63 (2.16–33.24)	0.277
LH (mIU/ml)	4.42 (0.28–32.07)	4.18 (0.39–20.67)	0.936
Oocyte retrieval cycle (cases)			0.180
1	121 (90.30%)	48 (92.31%)	–
2	10 (7.46%)	1 (1.92%)	–
3	2 (1.49%)	3 (5.77%)	–
4	1 (0.75%)	0 (0.00%)	–
Number of oocytes obtained (numbers)	14.00 (1.00–40.00)	11.50 (1.00–33.00)	0.006

Note: Group A, fresh sperm group; Group B, single-sperm cryopreservation group. AMH, anti-Müllerian hormone; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone. Non-normally distributed measurement data were expressed as median (minimum, maximum) values and analyzed using the Mann–Whitney *U*-test. The categorical variables were expressed as the number of cases (%), followed by chi-squared test. $p < 0.05$ was accepted as indicative of significant differences.

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Table 3. Analysis of embryo laboratory indexes

Characteristics	Group A ($n = 134$)	Group B ($n = 52$)	p-value
Maturity rate (%)	87.50 (44.00–100.00)	85.71 (40.00–100.00)	0.920
Fertilization rate (%)	78.57 (0.00–100.00)	91.67 (33.33–100.00)	0.010
2PN fertilization rate (%)	68.30 (0.00–100.00)	78.76 (0.00–100.00)	0.112
2PN cleavage rate (%)	100.00 (0.00–100.00)	100.00 (0.00–100.00)	0.447
D3 High-quality embryo rate (%)	50.00 (0.00–100.00)	45.80 (0.00–100.00)	0.126
D5 blastocyst formation rate (%)	62.02 (0.00–100.00)	0.00 (0.00–100.00)	< 0.001
D5 High-quality blastocyst rate (%)	33.33 (0.00–100.00)	0.00 (0.00–100.00)	0.003

Note: Group A, fresh sperm group; Group B, single-sperm cryopreservation group. 2PN, two pronuclei; d3, the third day; d5, the fifth day. Normally distributed measurement data were presented as mean ± standard deviation and analyzed using the independent sample *t*-test. Non-normally distributed measurement data were expressed as median (minimum, maximum) values and analyzed using the Mann–Whitney *U*-test. The categorical variables were expressed as the number of cases (%), followed by the chi-squared test. Statistical significance was defined as $p < 0.05$.

Table 4. Analysis of embryo transfer clinical indexes

Characteristics	Group A ($n = 134$)	Group B ($n = 52$)	p-value
Fresh cycle (cases)			0.940
0	72 (53.73%)	29 (55.77%)	–
1	60 (44.78%)	22 (42.31%)	–
2	2 (1.49%)	1 (1.92%)	–
Thawing cycle (cases)			0.342
0	47 (35.07%)	26 (50.00%)	–
1	64 (47.76%)	21 (40.38%)	–
2	18 (13.43%)	4 (7.69%)	–
3	3 (2.24%)	0 (0.00%)	–
4	1 (0.75%)	1 (1.92%)	–
5	1 (0.75%)	0 (0.00%)	–
Total number of transplantation cycles	182	57	–
Clinical pregnancy rate (%)	38.46%	43.86%	0.467
Cumulative clinical pregnancy rate (%)	51.10%	54.39%	0.665
Abortion rate (%)	20.43%	3.23%	0.024
Ectopic pregnancy rate (%)	1.08%	3.23%	0.410
Preterm birth rate (%)	13.98%	22.58%	0.259
Mode of delivery (cases)			0.463
Caesarean section	69 (85.19%)	23 (79.31%)	–
Vaginal birth	12 (14.81%)	6 (20.69%)	–
Live birth rate (%)	44.51%	50.88%	0.400
Birth defect rate (%)	0.93%	0.00%	0.552
Average birth weight (g)	2883.89 ± 665.28	2900.79 ± 551.51	0.376

Note: Group A, fresh sperm group; Group B, single-sperm cryopreservation group. Normally distributed measurement data were presented as mean ± standard deviation and analyzed using the independent sample *t*-test. Non-normally distributed measurement data were expressed as median (minimum, maximum) values and analyzed using the Mann–Whitney *U*-test. The categorical variables were expressed as the number of cases (%), followed by the chi-squared test. Statistical significance was defined as $p < 0.05$.

of all participants' sperm and eggs through ICSI. Some studies have already shown that there are no statistically significant differences between frozen–thawed and fresh spermatozoa in terms of the rates of fertilization, implantation, cleavage, delivery, and clinical pregnancy (Friedler *et al.*, 1997; Gil-Salom *et al.*, 2000; Habermann *et al.*, 2000). However, our findings demonstrated that the single-sperm cryopreservation group exhibited a clear elevation in the fertilization rate in comparison with the fresh sperm group. Conversely, the rates of D5 blastocyst formation and D5 high-quality blastocyst formation were considerably diminished by

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means of the single-sperm cryopreservation technique. Consequently, single-sperm cryopreservation applied in ICSI can substantially boost fertilization rate compared with fresh sperm. Of course, this may also be due to the small sample size included, and the application of single-sperm freezing technology in ICSI still needs to be further explored.

A randomized, controlled trial has revealed that fertilization and pregnancy rates are increased considerably with ICSI when viable sperm are picked from among immotile testicular spermatozoa using the hypo-osmotic swelling test (Sallam *et al.*, 2005). Furthermore, we followed up and recorded the pregnancy and outcome of all women who received embryo transfers and recorded neonatal-related information. Of note, the incidence of spontaneous abortion tends to be higher in pregnancies resulting from ICSI (Teixeira *et al.*, 2020). Our findings manifested that, in the absence of significant differences in clinical pregnancy rate, cumulative clinical pregnancy rate, ectopic pregnancy rate, preterm delivery rate, mode of delivery, and live birth delivery rate between the two groups, the rate of miscarriage was markedly lower in women who underwent embryo transfer in group B than in group A. Overall, these findings highlight that the implementation of the single-sperm cryopreservation technique in ICSI exhibits notable reductions in the abortion rate and enhancements in safety in contrast with using fresh sperm. Similarly, this conclusion is also limited by the sample size.

However, this study encountered some limitations. For one, the study analysis encompassed a limited number of instances, necessitating the need for larger sample sizes and multicentre investigations to enhance the validity of the findings. Moreover, this study examined how sperm cryopreservation affected embryo quality and blastocyst formation during cleavage. Frozen sperm did not influence the quality of cleavage-stage embryos, but it did diminish blastocyst formation and probability. Therefore, future studies should address these limitations and further investigate the safety of single-sperm cryopreservation in ICSI.

In conclusion, this paper demonstrates that the utilization of the single-sperm cryopreservation technique in ICSI yields notable enhancements in the fertilization rate and embryo 2PN rate while concurrently reducing the abortion rate. In addition this approach exhibits a high level of safety.

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

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Competing interestss. The authors have no conflicts of interest to declare.

Ethics statement. This study was reviewed and approved by the Academic Ethics Committee of Nanchang Reproductive Hospital and complied with the Declaration of Helsinki. All patients and their families were informed of the study's purpose and signed an informed consent.

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