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Original Research

Impact of Spermatozoa Cryopreservation on Clinical Outcomes of Intracytoplasmic Sperm Injection in Patients with Azoospermia: A Retrospective Cross-Sectional Study

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Abstract

Background: This study was undertaken to evaluate the effect of cryopreservation on the clinical outcomes of intracytoplasmic sperm injection (ICSI) cycles using sperm retrieved from testicular sperm extraction (TESE) in patients with azoospermia. **Methods**: This retrospective analysis included the clinical data of 56 pairs of fresh and frozen sperm injection cycles from 56 couples after TESE from January 2019 to December 2021 at the Reproductive Medicine Center of the First Affiliated Hospital of Sun Yat-sen University, of which 42 pairs were ICSI cycles using fresh and frozen sperm from the same TESE procedure. We compared the embryological and laboratory characteristics and pregnancy outcomes of the subsequent first embryo transfer (ET) cycles between the fresh and frozen groups. **Results**: There were no significant differences in the fertilization, cleavage, good-quality day 3 embryo, blastocyst formation, and good-quality blastocyst rates between the groups. However, when only paired ICSI cycles of fresh and frozen sperm from the same TESE procedure were analyzed, we observed that the good-quality day 3 embryo rate (44.8% *vs* 33.2%, $p = 0.029$) and blastocyst formation rate (57.5% *vs* 41.3%, $p = 0.028$) in the fresh group were significantly higher than those in the frozen group. Implantation, clinical pregnancy, early miscarriage, and live birth rates of the first ET cycle were not significantly different in either group. **Conclusions**: ICSI using fresh testicular sperm after TESE in patients with azoospermia appears to yield better embryological and laboratory outcomes than ICSI using cryopreserved testicular sperm, but the success rate of the subsequent first ET cycles does not seem to be affected.

Keywords: azoospermia; sperm; cryopreservation; testicular sperm extraction; intracytoplasmic sperm injection; pregnancy

1. Introduction

Azoospermia is the complete absence of sperm from ejaculated semen, which occurs in approximately 1% of the general male population and 10–15% of infertile men [1,2]. Azoospermia can be divided into obstructive (OA) and non-obstructive (NOA) groups. Patients with OA have normal testicular spermatogenic function, but no sperm is present in the ejaculated semen due to bilateral vas deferens obstruction [3]. NOA is caused by testicular spermatogenic dysfunction due to various factors, such as pituitary tumors, infections, genetic abnormalities, gonadotoxic drugs and treatment, and testicular torsion [4]. In 60% of patients with NOA, mature sperm can be retrieved from testes through testicular sperm extraction [5], allowing intracytoplasmic sperm injections (ICSI) with testicular biopsy sperm to be used to obtain genetic offspring. Spermatozoa cryopreservation allows patients with azoospermia to preserve their fertility and can avoid the risk of sperm extraction failure on the next egg-extraction day and reduce physiological consequences on the testicles [6].

However, cryopreservation can have detrimental effects on mammalian sperm by altering its morphology, decreasing its motility, or increasing oxidative stress and DNA damage [7–9]. Whether cryopreservation of testicular sperm affects ICSI outcomes is debatable and a matter of increasing concern [10]. Wood *et al*. [11] showed that the freezing-thawing procedure for testicular sperm reduces oocyte fertilization but not the pregnancy rate of embryo transfer (ET). Meta-analysis by Liu *et al*. [10] suggested that there was no difference in the fertilization rate (based on four different studies) and clinical pregnancy rate (based on seven different studies) between frozen and fresh testicular sperm in men with OA. Two more studies showed that compared with fresh testicular sperm injection, the fertilization and high-quality embryo rates of frozen testicular sperm injection were similar, but the live-birth rate was significantly lower [12,13]. At present, the effects of testicular sperm cryopreservation on fertilization and embryonic developmental potential require further verification.

It is worth noting that, in these previous studies, most of the fresh and frozen sperm injection cycles were from different patients [14–19]; therefore, bias due to differences between patients may exist. Thus, this retrospective study only included paired fresh and frozen sperm injection cycles from the same patient. This permitted better assessment of the impact of spermatozoa cryopreservation on the clinical outcomes of intracytoplasmic sperm microinjection in patients with azoospermia.

2. Materials and Methods

2.1 Patients

This study collected clinical data on ICSI cycles using sperm derived from testicular sperm extraction (TESE) procedures from January 2019 to December 2021 at the Reproductive Medicine Center of the First Affiliated Hospital of Sun Yat-sen University. All clinical data were retrieved from our reproductive center's non-public database. Inclusion criteria were male patients confirmed with azoospermia (OA or NOA) by semen examination in our hospital; male patients who had undergone TESE biopsy at our center; and some or all of the sperm obtained from TESE were frozen and preserved at our center; the patient had undergone at least one ICSI cycle of fresh sperm and at least one ICSI cycle of frozen sperm with sperm obtained from TESE. Exclusion criteria were cycles with no oocyte retrieved; cycle of preimplantation genetic testing; ICSI cycle using donated sperm; loss to follow-up or incomplete data. TESE-ICSI cycles using fresh and frozen sperm from the same patient with azoospermia were included in pairs, regardless of whether fresh or frozen sperm was used from the same TESE procedure.

2.2 TESE, Cryopreservation, and Warming of Sperm

Testicular spermatozoa were obtained by open testicular biopsy under local anesthesia. If simultaneous ovarian aspiration was performed, the remaining TESE sperm after ICSI was cryopreserved. If simultaneous ovarian aspiration was not performed, all TESE spermatozoa were cryopreserved.

Sperm were cryopreserved on the same day using Quinn's AdvantageTM Sperm Freezing Medium (SAGE, Trumbull, CT, USA). Spermatozoa samples were mixed 1:1 with Quinn's AdvantageTM Sperm Freezing Medium (SAGE, Trumbull, CT, USA). The mixture was loaded into straws and placed on aluminum racks. The aluminum racks with straws were placed in liquid nitrogen vapor for 30 minutes and then stored in liquid nitrogen.

When thawing, the straw was held in the air with tweezers and shaken gently for 30–40 seconds, and then immersed in a 37 ℃ water bath and shaken gently for 3–5 minutes until all the spermatozoa sample in the straw was thawed. The thawed spermatozoa sample was transferred to a 15 mL centrifuge tube, and washed in 4 mL G-IVFTM PLUS culture medium v.03 (Vitrolife, Frolunda, Sweden), followed by centrifugation for 3 min at 400 g twice. 300 μL of G-IVFTM culture medium was left in the centrifuge tube to resuspend the spermatozoa pellet. Spermatozoa with the best morphology and motility were manually selected for ICSI.

2.3 Controlled Ovulation Stimulation, Fertilization, and Embryo Culture

According to female patients' characteristics, controlled ovulation stimulation (COS) was performed using routine protocols, including a long- or short-acting gonadotropin-releasing hormone (GnRH) agonist protocol, a GnRH antagonist protocol or a mild stimulation protocol, as previously described [20,21]. When the dominant follicle diameter was $\geq$18 mm as monitored by B-ultrasound, human chorionic gonadotropin (HCG) was injected intramuscularly, and ultrasound-guided transvaginal ovarian oocyte retrieval was performed 36 hours later. The retrieved oocytes were fertilized using ICSI. After 16–18 hours, the presence of two pronuclei and two polar bodies signified normal fertilization. Normal fertilized oocytes were cultured in cleavage medium until the third day after ovulation to facilitate cleavages. Our center usually transfers or freezes two cleavage embryos on Day 3 after fertilization for each patient. Among the remaining embryos, the Day 3 embryos from normal fertilized oocytes and with $\geq$4 blastomeres were chosen for the blastocyst culture. Therefore, not all Day 3 embryos were cultured to the blastocyst stage in this study. The protocol for embryo culture was the same for all inclusion cycles.

In clinical practice at our reproductive center, cleavage embryos and blastocysts are scored according to the criteria described below. The criteria for embryo scoring per cleavage stage are based on the embryo cleavage stage scoring system of the Society for Assisted Reproductive Technology [22]. Good-quality day 3 embryos were defined as being at the 7–9-blastomere cleavage stage, cell fragmentation rate score of grade $\geq$2 ($\geq$11%), and symmetry score of grade $\geq$2 (perfect or moderately asymmetric). A blastocyst grading system introduced by Gardner and Schoolcraft in 1999 was used to evaluate the quality of embryos cultured on the fifth day after ICSI [23]. Good-quality blastocysts bore a cell mass and trophoblast grade of $\geq$B, with an expansion grade of $\geq$3. These criteria were decided previously and were used in this study.

2.4 Embryo Transfer

The choice of fresh or frozen-thawed ET (FET) after ICSI is based on the doctor's decision or patient's request. For fresh ET, there are two commonly used luteal support regimens: (1) Crinone vaginal gel (Fleet laboratories Limited, Watford, UK) 90 mg/day vaginally plus Duphaston (Abbott Biologicals B.V., OLST, Netherlands) (2) progesterone (Zhejiang Xianju pharmaceutical Co., Ltd., Taizhou, Zhejiang, China) 40 mg/day intramuscularly plus Duphaston (Abbott Healthcare, USA) 20 mg/day orally. Luteal support was provided on the day of oocyte collection, and 1–2 cleavage-day 3 embryos or day 5–6 blastocysts were transferred to the uterus in the morning 3 or 5 days after oocyte collection. Cryopreservation of the remaining cleavage-embryos or blastocysts was performed using Kitazato products (BioPharma Co. Ltd., Shizuoka, Japan) for subsequent FET. For FET, a natural cycle was conducted for patients with regular menstruation and a normal ovulation history, and hormone replacement therapy cycles were

performed for patients with irregular menstruation or an abnormal ovulation history. Specific protocols for natural cycle and hormone replacement therapy have been previously described in detail [24].

Pregnancy was biochemically confirmed by a positive blood β-HCG test on the 14th day after ET and clinically by the gestational sac and primitive cardiac duct pulsation on the 35th day after ET. Luteal support was gradually reduced after intrauterine clinical pregnancy was determined and discontinued at 10–12 weeks of gestation.

2.5 Outcomes

We compared the embryological and laboratory characteristics of the fresh and frozen sperm injection groups, including their fertilization, normal fertilization, cleavage, normal cleavage, good-quality day 3 embryo, blastocyst formation, and good-quality blastocyst rates. In addition, we compared the pregnancy outcomes in subsequent first ET cycles, including implantation, clinical pregnancy, early abortion, live birth, and birth defect rates between the two groups. We made only simple difference comparisons of these outcomes between the two groups.

2.6 Statistical Analysis

Sample size calculation was performed using PASS 11.0 software (NCSS, LLC. Kaysville, UT, USA) based on clinical pregnancy rates. The clinical pregnancy rates of the fresh group and the frozen group were set at 50% and 40%, respectively, referring to the clinical pregnancy rate of FET cycles in our center [24]. The sample size of the fresh and frozen groups was 1:1. One-sided test takes $\alpha = 0.05$, power = 90%.

SPSS version 22.0 (IBM Corp., Armonk, NY, USA) (RRID: SCR_016479) was used for statistical analysis. Continuous data are expressed as the mean $\pm$ standard deviation. Student's *t*-test was used for inter-group comparison of continuous data with normal distribution, and the Mann–Whitney U test was used for inter-group comparison of measurement data that did not follow a normal distribution. Categorical data were expressed as case number (%), and comparisons between groups were performed using Pearson's χ^2 test or Fisher's exact test. Statistical significance was set at $p < 0.05$.

3. Results

The sample size calculation showed that each group needed 519 samples, and the two groups needed a total of 1038 samples. However, only 59 pairs of fresh and frozen sperm injection cycles met the inclusion criteria, of which 3 pairs were excluded because of no oocytes retrieved during fresh or frozen sperm injection cycles. Finally, the study included fresh and frozen sperm injection cycles in pairs from 56 patients with azoospermia after TESE. Therefore, the fresh and frozen sperm injection groups in this study each contained 56 cases. Notably, of all 56 patients enrolled in the study, 42 received fresh and frozen sperm ICSI using sperm from the same TESE procedure, 14 used pre-frozen sperm from the TESE procedure for the first ICSI cycle and fresh sperm from a second biopsy for the second ICSI cycle.

There were no significant differences between the fresh and frozen groups in terms of basic characteristics during ICSI such as male age (38.57 $\pm$ 9.32 *vs* 38.86 $\pm$ 9.30, $p = 0.871$), female age (35.09 $\pm$ 5.66 *vs* 35.39 $\pm$ 5.78, $p = 0.779$), female body mass index (21.32 $\pm$ 2.37 *vs* 21.37 $\pm$ 2.33, $p = 0.906$), years of infertility (6.22 $\pm$ 5.16 *vs* 6.40 $\pm$ 5.17, $p = 0.855$), and female basic follicle-stimulating hormone (7.11 $\pm$ 3.63 *vs* 7.30 $\pm$ 3.82 mIU/mL, $p = 0.792$) and luteinizing hormone (3.24 $\pm$ 1.55 *vs* 3.30 $\pm$ 1.55 mIU/mL, $p = 0.843$) (Table 1). The total gonadotrophin dose (2481.70 $\pm$ 942.50 *vs* 2413.84 $\pm$ 912.48 IU, $p = 0.699$) and duration of gonadotropin use (9.57 $\pm$ 2.57 *vs* 9.38 $\pm$ 2.77 days, $p = 0.698$) in COS were similar between the two groups. The two groups had similar numbers of oocytes and meiosis II (MII) oocytes (Table 2). There were no significant differences in embryological and laboratory characteristics between the two groups, such as fertilization (70.9% *vs* 72.1%, $p = 0.718$), cleavage (97.8% *vs* 97.2%, $p = 0.665$), normal cleavage (90.0% *vs* 86.6%, $p = 0.228$), good-quality day 3 embryo (38.9% *vs* 32.5%, $p = 0.138$), blastocyst formation (49.0% *vs* 38.1%, $p = 0.058$), and good-quality blastocyst rates (34.3% *vs* 33.9%, $p = 0.963$) (Table 2).

When analyzing only fresh and frozen sperm injection cycles with sperm from the same TESE procedures, the basic information, COS protocol, gonadotropin dosage, and number of oocytes and MII oocytes retrieved were similar between the two groups (Tables 3,4). There were no statistical differences in the fertilization, cleavage, normal cleavage, and high-quality blastocyst rates (Table 4). However, the good-quality day 3 cleavage (per normal cleavage: 44.8% *vs* 33.2%, $p = 0.029$; per fertilized oocyte: 39.5% *vs* 29.9%, $p = 0.049$) and blastocyst formation rates (57.5% *vs* 41.3%, $p = 0.028$) in the fresh group were significantly higher than those in the frozen sperm injection group (Table 4).

Freeze-all strategy was implemented in some ICSI cycles that were included, when fresh transplanting was not suitable (such as the women were at high risk of ovarian hyperstimulation syndrome, having endometriosis, fever, or vaginitis). However, some patients had not yet returned for frozen embryo transfer at the time of data collection in this study. As we only included the data of the first embryo transfer cycle in each group, the transfer cycle numbers between the treatment groups was different in the paired test. The implantation (13.8% *vs* 17.0, $p = 0.647$), clinical pregnancy (17.9% *vs* 20.8%, $p = 1.000$), early miscarriage (40.0% *vs* 0%, $p = 0.429$), live birth (10.7% *vs* 20.8%, $p = 0.533$), or birth defect rates (0% *vs* 0%) of the subsequent first ET did not differ significantly between the two groups (Table 5). Similar pregnancy outcomes were also observed in the two groups when we analyzed only paired fresh and frozen sperm injection cycles using sperm from the same TESE procedure (Table 6).

Table 1. Basic characteristics of the included patients.

	Fresh testicular extraction	Frozen testicular extraction	*p*-value
Number of cycles	56	56	
Age of female patients (years)	35.09 ± 5.66	35.39 ± 5.78	0.779
Age of male patients (years)	38.57 ± 9.32	38.86 ± 9.30	0.871
Basal FSH level (mIU/mL)	7.11 ± 3.63	7.30 ± 3.82	0.792
Basal LH level (mIU/mL)	3.24 ± 1.55	3.30 ± 1.55	0.843
Basal T level (mIU/mL)	0.32 ± 0.35	0.33 ± 0.36	0.926
BMI (kg/m^2)	21.32 ± 2.37	21.37 ± 2.33	0.906
Duration of infertility	6.22 ± 5.16	6.40 ± 5.17	0.855
Stimulation protocol			0.450
Long-acting GnRH-a long protocol, n (%)	17 (30.4)	17 (30.4)	
Short-acting GnRH-a long protocol, n (%)	5 (8.9)	4 (7.1)	
GnRH antagonist protocol, n (%)	28 (50)	23 (41.1)	
Mild stimulation protocol, n (%)	6 (10.7)	12 (21.4)	
Total gonadotrophin dose (IU)	2481.70 ± 942.50	2413.84 ± 912.48	0.699
Duration of gonadotropin use (days)	9.57 ± 2.57	9.38 ± 2.77	0.698

FSH, follicle stimulating hormone; LH, luteinizing hormone; T, testosterone; IU, international units; BMI, body mass index; GnRH, gonadotropin-releasing hormone; GnRH-a, gonadotropin-releasing hormone agonist.

Table 2. Embryological/laboratory patients' characteristics.

	Total			OA			NOA		
	Fresh sperm injection	Frozen sperm injection	*p*-value	Fresh sperm injection	Frozen sperm injection	*p*-value	Fresh sperm injection	Frozen sperm injection	*p*-value
Number of cycles	56	56		40	40		16	16	
Total number of retrieved oocytes	493	495		283	342		210	153	
Mean number of retrieved oocytes	8.80 ± 7.09	8.84 ± 7.23	0.979	7.08 ± 5.10	8.55 ± 7.45	0.304	13.13 ± 9.44	9.56 ± 6.82	0.231
Total number of retrieved MII oocytes	382	398		214	286		168	112	
Mean number of retrieved MII oocytes	6.82 ± 5.39	7.11 ± 5.68	0.785	5.35 ± 3.69	7.15 ± 6.11	0.115	10.50 ± 7.15	7.00 ± 4.59	0.110
Fertilization rate, n (%)	271/382 (70.9)	287/398 (72.1)	0.718	157/214 (73.4)	214/286 (74.8)	0.711	117/168 (69.6)	73/112 (65.2)	0.433
Two-pronuclear zygote rate, n (%)	254/382 (66.5)	255/398 (64.1)	0.478	143/214 (66.8)	189/286 (66.1)	0.862	111/168 (66.1)	66/112 (58.9)	0.224
Cleavage rate, n (%)	265/271 (97.8)	279/287 (97.2)	0.665	152/157 (96.8)	208/214 (97.2)	0.923	113/117 (96.6)	71/73 (97.3)	0.868
Normal cleavage rate, n (%)	244/271 (90.0)	249/287 (86.6)	0.228	139/157 (88.5)	186/214 (86.9)	0.640	105/117 (89.7)	63/73 (86.3)	0.470
Good-quality day 3 embryos/Normal cleavage embryos, n (%)	95/244 (38.9)	81/249 (32.5)	0.138	55/139 (39.6)	58/186 (31.2)	0.116	40/105 (38.1)	23/63 (36.5)	0.837
Good-quality day 3 embryos/fertilized oocytes, n (%)	95/271 (35.1)	81/287 (28.2)	0.083	55/157 (35.0)	58/214 (27.1)	0.101	40/117 (34.2)	23/73 (31.5)	0.703
Blastocyst formation/Day 3 embryos from normal fertilized oocytes and with ≥4 blastomeres, n (%)	70/143 (49.0)	59/155 (38.1)	0.058	32/66 (48.5)	41/111 (36.9)	0.131	38/77 (49.4)	18/26 (69.2)	0.370
Good-quality blastocyst rate, n (%)	24/70 (34.3)	20/59 (33.9)	0.963	6/32 (18.8)	13/41 (31.7)	0.211	18/38 (47.4)	7/18 (38.9)	0.551

OA, obstructive azoospermia; NOA, nonobstructive azoospermia; MII, meiosis II.

Table 3. Basic characteristics of patients included in paired fresh and frozen sperm injection cycles using sperm retrieved from the same TESE procedures.

	Fresh sperm injection	Frozen sperm injection	*p* value
Number of cycles	42	42	
Age of female patients (years)	36.38 ± 5.31	36.62 ± 5.41	0.839
Age of male patients (years)	39.98 ± 9.58	39.95 ± 8.84	0.991
Basal FSH level (mIU/mL)	7.57 ± 4.04	7.60 ± 4.29	0.968
Basal LH level (mIU/mL)	3.17 ± 1.54	3.23 ± 1.56	0.859
Basal T level (mIU/mL)	0.32 ± 0.40	0.35 ± 0.41	0.754
BMI (kg/m^2)	21.19 ± 2.06	21.41 ± 2.17	0.632
Duration of infertility	7.02 ± 5.53	7.31 ± 5.46	0.812
Stimulation protocol			0.102
Long-acting GnRH-a long protocol, n (%)	15/42 (35.7)	10/42 (23.8)	
Short-acting GnRH-a long protocol, n (%)	5/42 (11.9)	1/42 (2.4)	
GnRH antagonist protocol, n (%)	15/42 (35.7)	19/42 (45.2)	
Mild stimulation protocol, n (%)	6/42 (14.3)	12/42 (28.6)	
Total gonadotrophin dose (IU)	2469.64 ± 1002.78	2323.81 ± 844.29	0.473
Duration of gonadotropin use (days)	9.60 ± 2.78	9.02 ± 2.62	0.335

TESE, testicular sperm extraction; FSH, follicle stimulating hormone; LH, luteinizing hormone; T, testosterone; IU, international units, BMI, body mass index; GnRH, gonadotropin-releasing hormone.

Table 4. Embryological and laboratory characteristics of paired fresh and frozen sperm injection cycles using sperm retrieved from the same TESE procedures.

	Total			OA			NOA		
	Fresh sperm injection	Frozen sperm injection	*p*-value	Fresh sperm injection	Frozen sperm injection	*p*-value	Fresh sperm injection	Frozen sperm injection	*p*-value
Number of cycles	42	42		32	32		10	10	
Total number of retrieved oocytes	289	359		203	270		86	89	
Mean number of retrieved oocytes	6.88 ± 5.48	8.55 ± 7.65	0.255	6.34 ± 4.97	8.44 ± 8.07	0.216	8.60 ± 6.90	8.90 ± 6.49	0.921
Total number of retrieved MII oocytes	219	305		152	232		67	73	
Mean number of retrieved MII oocytes	5.21 ± 3.86	7.26 ± 6.28	0.076	4.75 ± 3.36	7.25 ± 6.67	0.063	6.70 ± 5.08	7.30 ± 5.14	0.796
Fertilization rate, n (%)	162/219 (74.0)	221/305 (72.5)	0.700	113/152 (74.3)	180/232 (77.6)	0.465	49/67 (73.1)	41/73 (56.2)	0.036
Two-pronuclear zygote rate, n (%)	148/219 (67.6)	204/305 (66.9)	0.867	100/152 (65.8)	167/232 (72.0)	0.197	48/67 (71.6)	37/73 (50.7)	0.011
Cleavage rate, n (%)	156/162 (96.3)	216/221 (97.7)	0.600	110/113 (97.3)	176/180 (97.8)	0.875	46/49 (93.9)	40/41 (97.6)	0.740
Normal cleavage rate, n (%)	143/162 (88.3)	199/221 (90.0)	0.579	98/113 (86.7)	165/180 (91.7)	0.174	45/49 (91.8)	34/41 (82.9)	0.198
Good-quality day 3 embryos/Normal cleavage embryos, n (%)	64/143 (44.8)	66/199 (33.2)	0.029	44/113 (38.9)	53/180 (29.4)	0.093	20/49 (40.8)	13/41 (31.7)	0.372
Good-quality day 3 embryos/fertilized oocytes, n (%)	64/162 (39.5)	66/221 (29.9)	0.049	44/152 (28.9)	53/232 (22.8)	0.178	20/67 (29.9)	13/73 (17.8)	0.093
Blastocyst formation/Day 3 embryos from normal fertilized oocytes and with ≥4 blastomeres, n (%)	42/73 (57.5)	50/121 (41.3)	0.028	25/45 (55.6)	39/97 (40.2)	0.087	17/28 (60.7)	11/24 (45.8)	0.283
Good-quality blastocyst rate, n (%)	14/42 (33.3)	17/50 (34.0)	0.946	5/45 (11.1)	13/97 (13.4)	0.703	9/28 (32.1)	4/24 (16.7)	0.198

TESE, testicular sperm extraction; OA, obstructive azoospermia; NOA, nonobstructive azoospermia; MII, meiosis II.

Table 5. Pregnancy outcomes in subsequent first ET cycles after ICSI.

	Total			OA			NOA		
	Fresh sperm injection	Frozen sperm injection	*p*-value	Fresh sperm injection	Frozen sperm injection	*p*-value	Fresh sperm injection	Frozen sperm injection	*p*-value
Embryo transfer cycles	28	24		21	19		7	5	
Fresh or frozen embryo transfer			0.494			0.583			1.000
Fresh embryo transfer, n (%)	20/28 (71.4)	15/24 (62.5)		16/21 (76.2)	13/19 (68.4)		4/7 (57.1)	2/5 (40.0)	
Frozen embryo transfer, n (%)	8/28 (28.6)	9/24 (37.5)		5/21 (23.8)	6/19 (31.6)		3/7 (42.9)	2/5 (60.0)	
Number of embryos transferred per cycle	1.82 ± 0.39	1.79 ± 0.51	0.813	1.81 ± 0.40	1.74 ± 0.56	0.474	1.86 ± 0.38	2.00 ± 0.00	0.424
Transfer day			1.000			0.960			1.000
Day 3, n (%)	27/28 (96.4%)	23/24 (95.8)		21/21 (100.0)	18/19 (94.7)		6/7 (85.7)	5/5 (100.0)	
Day 5/6, n (%)	1/28 (3.6)	1/24 (4.2)		0/21 (0.0)	1/19 (5.3)		1/7 (14.3)	0/5 (0.0)	
Embryo quality			0.154			0.007			0.535
Good-quality embryo, n (%)	15/28 (53.6)	7/24 (29.2)		12/21 (57.1)	5/19 (26.3)		3/7 (42.9)	2/5 (40.0)	
Poor-quality embryo, n (%)	3/28 (10.7)	6/24 (25.0)		0/21 (0.0)	5/19 (26.3)		3/7 (42.9)	1/5 (20.0)	
One poor-quality embryo with one good-quality embryo, n (%)	10/28 (35.7)	11/24 (45.8)		9/21 (42.9)	9/19 (47.4)		1/7 (14.3)	2/5 (40.0)	
Implantation rate, n (%)	8/58 (13.8)	8/47 (17.0)	0.647	5/45 (11.1)	8/35 (22.9)	0.158	3/13 (23.1)	0/12 (0.0)	0.247
Clinical pregnancy rate/ET, n (%)	5/28 (17.9)	5/24 (20.8)	1.000	2/21 (9.5)	5/19 (26.3)	0.328	3/7 (42.9)	0/5 (0.0)	0.310
Live birth rate/ET, n (%)	3/28 (10.7)	5/24 (20.8)	0.533	1/21 (4.8)	5/19 (26.3)	0.143	2/7 (28.6)	0/5 (0.0)	0.600
Early miscarriage rate/ET, n (%)	2/5 (40.0)	0/5 (0.0)	0.429	1/2 (50.0)	0/5 (0.0)	0.608	1/3 (33.3)	0	-
Birth defects rate/ET, n (%)	0	0	-	0	0	-	0	0	-

ET, embryo transfer; ICSI, intracytoplasmic sperm injection; OA, obstructive azoospermia; NOA, nonobstructive azoospermia; MII, meiosis II.

Table 6. Pregnancy outcomes in subsequent first ET cycles of paired fresh and frozen sperm injection cycles using sperm retrieved from the same TESE procedures.

	Total			OA			NOA		
	Fresh sperm injection	Frozen sperm injection	*p*-value	Fresh sperm injection	Frozen sperm injection	*p*-value	Fresh sperm injection	Frozen sperm injection	*p*-value
Embryo transfer cycles	20	17		16	14		4	3	
Fresh or frozen embryo transfer			0.295			0.526			1.000
Fresh embryo transfer, n (%)	15/20 (75.0)	10/17 (58.8)		13/16 (81.3)	9/14 (64.3)		2/4 (50.0)	1/3 (33.3)	
Frozen embryo transfer, n (%)	5/20 (25.0)	7/17 (41.2)		3/16 (18.8)	5/14 (35.7)		2/4 (50.0)	2/3 (66.7)	
Number of embryos transferred per cycle	1.80 ± 0.41	1.77 ± 0.44	0.802	1.75 ± 0.45	1.71 ± 0.47	0.833	2.00 ± 0.00	2.00 ± 0.00	-
Transfer day			0.934			0.946			-
Day 3, n (%)	20/20 (100.0)	16/17 (94.1)		16/16 (100.0)	13/14 (92.9)		4/4 (100.0)	0	
Day 5/6, n (%)	0/20 (0.0)	1/17 (5.9)		0/16 (0.0)	1/14 (7.1)		3/3 (100.0)	0	
Embryo quality			0.042			0.018			0.382
Good-quality embryo, n (%)	14/20 (70.0)	5/17 (29.4)		10/16 (62.5)	4/14 (28.6)		2/4 (50.0)	1/3 (33.3)	
Poor-quality embryo, n (%)	2/20 (10.0)	5/17 (29.4)		1/16 (0.0)	4/14 (28.6)		2/4 (50.0)	1/3 (33.3)	
One poor-quality embryo with one good-quality embryo, n (%)	4/20 (20)	7/17 (41.2)		6/16 (37.5)	6/14 (42.9)		0/4 (0.0)	1/3 (33.3)	
Implantation rate, n (%)	4/39 (10.3)	8/34 (23.5)	0.127	3/31 (9.7)	8/26 (30.8)	0.094	1/8 (12.5)	0/8 (0.0)	1.000
Clinical pregnancy rate/ET, n (%)	3/20 (15.0)	5/17 (29.4)	0.509	2/16 (12.5)	5/14 (35.7)	0.286	1/4 (25.0)	0/3 (0.0)	1.000
Live birth rate/ET, n (%)	2/20 (10.0)	5/17 (29.4)	0.280	1/16 (6.3)	5/14 (35.7)	0.120	1/4 (25.0)	0/3 (0.0)	1.000
Early miscarriage rate/ET, n (%)	1/3 (33.3)	0/5 (0.0)	0.783	1/2 (50.0)	0/5 (0.0)	0.608	0	0	-
Birth defects rate/ET, n (%)	0	0	-	0	0	-	0	0	-

ET, embryo transfer; TESE, testicular sperm extraction; OA, obstructive azoospermia; NOA, nonobstructive azoospermia; MII, meiosis II.

We performed a stratified analysis of embryo laboratory parameters and pregnancy outcomes according to the type of azoospermia. The results of the stratified analysis are displayed in Tables 2,4,5,6. Our results showed that when all included patients were analyzed, the embryo laboratory parameters and pregnancy outcomes were similar between the fresh and frozen groups in both NOA and OA patients. However, when only paired ICSI cycles of fresh and frozen sperm from the same TESE procedure were analyzed, we observed that in NOA patients, the fertilization rate (56.2% *vs* 73.1%, $p = 0.036$) and normal fertilization rate (50.7% *vs* 71.6%, $p = 0.011$) of the frozen group was significantly lower than the fresh group. Such results were not observed in OA patients.

4. Discussion

In this study, we retrospectively compared the ICSI outcomes of fresh and cryopreserved spermatozoa obtained from TESE in 56 patients with azoospermia. The application of cryopreservation technology to embryos is rapidly increasing [25]. Generally, FET is safe and reliable. A recent meta-analysis showed that even the live birth rate of FET cycles increases significantly in high responders or patients undergoing preimplantation genetic testing for aneuploidy compared with fresh ET cycles [26]. However, the impact of cryopreservation on the reproductive potential of human sperm remains controversial [10–14] due to differences in sample size and cryopreservation methods among studies. In addition, as the injection cycles were mostly from different patients in these studies, certain bias due to patient differences cannot be excluded.

Currently, only Aizer *et al*. [27] and our study compared the differences in ICSI outcomes of fresh and frozen sperm from the same TESE procedure. Both studies showed similar fertilization rates for fresh and frozen testicular sperm but Aizer *et al*. [27] reported no statistically significant difference in the good-quality day 3 embryo rate per fertilized oocytes between the two groups (55.8% *vs* 55%), whereas we showed that this rate decreased significantly in the frozen group (39.5% *vs* 29.9%, $p = 0.049$). This may be due to the different definitions of good-quality day 3 embryos used. In addition, Aizer *et al*. [27] did not report any blastocyst formation rates, whereas we observed that it was significantly decreased in the frozen sperm cycle (57.5% *vs* 41.3%, $p = 0.028$).

Notably, only two-thirds of the patients in our study underwent a fresh sperm ICSI cycle and later received cryopreserved sperm that had remained from the previous fresh TESE procedure. The remaining one-third received frozen sperm injections after the first TESE procedure and fresh sperm injections after the second procedure. Sperm status may vary between sperm biopsy procedures. This may account for the non-significant differences in embryological and laboratory characteristics between the fresh and frozen groups. However, when we analyzed only the paired fresh and frozen cycles using sperm from the same TESE procedure, the frozen group bore significantly lower good-quality day 3 embryo rate (29.9% *vs* 39.5%, $p = 0.049$) and blastocyst formation rate (41.3% *vs* 57.5%, $p = 0.028$).

Contrary to Aizer *et al*. [27], our study supports the theory that the reproductive potential of retrieved testicular sperm from patients with azoospermia may be impaired by cryopreservation. Freezing/thawing induces intracellular ice crystals, osmotic pressure changes, and chemical/thermal stress, thus impairing sperm motility, morphology, and DNA integrity [28]. In addition, cryopreservation may sharply increase ROS release, which would damage sperm proteins and DNA and increases apoptosis [9,29–31]. Lipid peroxidation caused by excessive ROS may compromise sperm cell membrane integrity, thus reducing the sperm reproductive potential after thawing [32,33]. Notably, we observed that in NOA patients, the fertilization rate (56.2% *vs* 73.1%, $p = 0.036$) and normal fertilization rate (50.7% *vs* 71.6%, $p = 0.011$) of the frozen group was significantly lower than the fresh group. Such results were not observed in OA patients. Our results are supported by the study of Moubasher *et al*. [34]. They showed that catalase activity increased in testicular tissue from OA patients which was not observed in the NOA group, suggesting that testicular tissue from OA patients can withstand the oxidative stress caused by freezing more than NOA [7]. These results indicated that spermatozoa after TESE in NOA patients are more likely to be affected by cryopreservation than in OA patients. The decision of TESE sperm cryopreservation in patients with NOA needs to be made cautiously.

While the safety of cryopreservation of testicular sperm remains uncertain, many new cryoprotectants and techniques have been proven beneficial [35–39]. In 2018, Berkovitz *et al*. [35] reported SpermVD, a novel, efficient carrier method for freezing a small number of spermatozoa from patients with NOA. It can freeze sperm in 0.8–1 μL microdroplets, thereby shortening the search time after thawing from a few hours to a few minutes. Sperm using this technique were with a post-thaw recovery rate of 96% and were successfully used for fertilization [35]. Cryopiece is also a novel carrier for freezing individual or a small amount of spermatozoa. Spermatozoa using this technique had a post-thaw recovery rate of 83% [36]. In China, this technology has successfully helped some oligozoospermia or NOA patients to obtain healthy offspring [36]. Recently, several new cryopreservation supplements, such as Alpha antifreeze Protein III, Curcumin, and Canthaxanthin, have been shown to protect human sperm parameters and sperm DNA, and reduce oxidative damage caused by freeze-thaw process [37–39]. In the future, more efforts are needed to make these new cryoprotectants that better serve the clinical practice. Future advances in this technology could allow patients with azoospermia to have their own healthy offspring.

We observed that poor embryo quality was found in the frozen group, but there was no statistical difference in pregnancy outcomes between the two groups. This result is similar to that of Wood *et al*. [11]. This may be because sperm cryopreservation may reduce the number of fertilized oocytes and high-quality embryos, but the reproductive potential of the resulting transferable embryos is not compromised. We note that the pregnancy outcomes of the frozen group were greater than those of the fresh group (although not statistically different), but due to the small sample size of this study, it cannot be ruled out that such outcomes are due to sampling error. This result needs to be further verified in future studies.

According to a large retrospective study published in our center (including 3400 FET cycles), the clinical pregnancy rate in FET cycles in our center is about 50%, and the live birth rate is about 44% [24]. However, in this manuscript, the clinical pregnancies in the fresh and frozen groups were 17.9% and 20.8%, respectively, and the live birth rates were 10.7% and 20.8%, respectively, which were significantly lower than the average at our center. The possible reason is that the number of embryo transfer cycles included in this study is extremely limited (28 in the fresh sperm injection group and 27 in the frozen sperm injection group), which is prone to sampling errors. Therefore, the results of the effect of cryopreservation of TESE sperm on pregnancy outcomes obtained by this study need to be interpreted cautiously.

Unfortunately, because not all obtained embryos were transferred in the ICSI cycle, we were unable to compare the cumulative pregnancy rates between the two groups, and compared only the pregnancy outcomes of the subsequent first ET. We found no impairment in pregnancy outcomes in the subsequent first ET in the frozen sperm injection group. Moreover, frozen testicular sperm may lead to a reduction in the number of good-quality day 3 embryos and blastocysts. Thus, it is possible that the cumulative pregnancy rate of frozen sperm injection cycles eventually decreases, rather than becoming better than that of fresh sperm ICSI cycles, as shown by Aizer *et al*. [27]. Future studies are required to further validate the effect of frozen testicular sperm injection on the cumulative pregnancy rate in patients with azoospermia.

The strength of our study is the analysis of ICSI cycle outcomes of fresh and frozen testicular sperm injections with sperm from the same patient or even from the same TESE procedure, which constitutes a rare and valuable study population. Compared with the study of Aizer *et al*. [27], our study contributed the analysis of blastocyst formation and good-quality blastocyst rates.

Nevertheless, our study also has several limitations. First, it is a retrospective study with a small sample size from a single center. Compared to the study by Aizer *et al*. [27], our study included fewer couples (56 cases in our study, 75 cases in the study by Aizer *et al*. [27]), and only two-thirds of the paired fresh and frozen sperm injection cycles used sperm from the same TESE procedure. Moreover, the sample size calculation showed that each group needs 519 samples, and the two groups need a total of 1038 samples. However, there were 56 ICSI cycles in each group in this study, and embryo transfer was not performed in every ICSI cycle. Therefore, the sample size of this study is inappropriate, which greatly limits the reliability of the results of this paper. Second, as the sample size of this study was very small (paired samples, n = 42) from the retrospective dataset of 2-year collection, we were not able to perform multivariate logistic regression analysis to exclude the impact of these confounding variables. However, this study used paired tests. Both fresh and frozen sperm ICSI cycles were from the same patient and partly from the same TESE procedure, which could partially reduce the bias caused by inter-individual heterogeneity. Third, this study lacked assessment data on sperm quality, such as motility, morphology, and DNA fragmentation rate of fresh and frozen-thawed sperm samples. In our center, we did not evaluate the quality or survival ability of thawed TESE sperm before ICSI, and only manually selected spermatozoa with the best morphology and motility. Therefore, we couldn't directly assess the impact of cryopreservation on spermatozoa retrieved from TESE procedures, but could only indirectly infer from the embryological and laboratory characteristics and pregnancy outcomes. Fourth, as some of the included ICSI cycles did not transfer all embryos obtained, we only compared the pregnancy outcomes of the subsequent first ET between the two groups and did not analyze the difference in cumulative pregnancy rates. Fifth, this study did not conduct long-term follow-ups on the live births of the two groups, and, therefore, could not gain insights into the long-term impact of cryopreservation on the safety of the offspring. It is worth noting that the conclusions of this study are based on the patients with azoospermia undergoing TESE, so it may not be appropriate to extend our conclusion to patients with azoospermia who underwent other types of sperm retrieval procedures such as percutaneous epididymal sperm aspiration and testicular sperm aspiration.

5. Conclusions

The results of this study suggest that fresh sperm injections yield better embryological and laboratory outcomes than frozen ones in patients with azoospermia after TESE, but the success rate of the subsequent first-cycle ET is not affected. The impact of cryopreservation on the reproductive potential, cumulative pregnancy rate, and long-term offspring safety of TESE spermatozoa in patients with azoospermia needs to be established in future studies using larger sample sizes.

Data Availability

Repository: Impact of spermatozoa cryopreservation upon clinical outcomes of intracytoplasmic sperm injection in patients with azoospermia: a retrospective cross-sectional study.

https://doi.org/10.6084/m9.figshare.20220165.

This project contains the following underlying data:

Data file 1. (Complete clinical data of 59 azoospermia patients were included in this study).

Abbreviations

COS, controlled ovulation stimulation; ET, embryo transfer; FET, frozen-thawed embryo transfer; GnRH, gonadotropin-releasing hormone; HCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; MII, meiosis II; NOA, non-obstructive azoospermia; OA, obstructive azoospermia; TESE, testicular sperm extraction.

Author Contributions

YW—Conceptualization, Formal Analysis, Project Administration, Resources, Writing – Review & Editing; DC—Data Curation, Investigation, Methodology, Validation, Writing – Original Draft Preparation; BC—Writing – Review & Editing; DH—Data Curation, Formal Analysis; YX—Supervision; CD—Supervision, Writing – Review & Editing. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the First Affiliated Hospital of the Sun Yat-sen University (approval number [2022]480). Informed consent from the involved patients was waived for this study due to its retrospective nature.

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Conflict of Interest

The authors declare no conflict of interest.

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