Original Article

Comparison of pregnancy and neonatal outcomes of intracytoplasmic sperm injection performed with frozen versus fresh testicular sperm

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Background: It remains controversial whether there is a difference in the prognosis of intracytoplasmic sperm injection (ICSI) using frozen or fresh testicular sperm in patients with obstructive azoospermia (OA). Moreover, in the available studies, few have tracked neonatal outcomes. This study aimed to compare the pregnancy and neonatal outcomes of ICSI using cryopreserved sperm versus fresh sperm collected by testicular sperm aspiration (TESA).

Methods: A total of 317 OA patients treated with ICSI in a university affiliated hospital from January 2016 to December 2020 were included in this study. The participants were divided into two groups according to the type of sperm used for ICSI: frozen sperm group (n=154) and fresh sperm group (n=163). The pregnancy and neonatal outcomes of the two groups were compared.

Results: The data produced by this study showed no significant statistical difference in the 2 pronuclei (2PN) fertilization rate, 2PN cleavage rate, high-quality blastocyst rate, and the average number of transferred embryos in the frozen and fresh sperm groups. Similarly, no difference was found in implantation rate, clinical pregnancy rate, multiple pregnancy rate, miscarriage rate, premature delivery rate, live birth rate, and gender ratio at birth (P>0.05). The average newborn birth weight was similar in both groups (2,932.61±728.40 *vs.* 3,100.32±515.64 g, respectively) (P>0.05). A higher incidence of low birthweight (LBW) newborns was found in the frozen sperm group (20.91% *vs.* 8.49%) (P<0.05). Multiple logistic regression analysis showed that LBW is related to single or twin pregnancies (P<0.01), but not sperm (frozen or fresh) (P>0.05). We further analyzed the twin and single pregnancies in the two groups separately, and found that the incidences of LBW were both similar (P>0.05). There was no difference in the Apgar scores at 1 min and 5 min after birth between the two groups (P>0.05).

Conclusions: The use of frozen testicular sperm by TESA was efficient for men with OA. There were similar pregnancy and neonatal outcomes following TESA-ICSI using frozen or fresh sperm in this retrospective study. Prospective investigations are needed for further validation.

Keywords: Testicular sperm aspiration (TESA); obstructive azoospermia (OA); intracytoplasmic sperm injection (ICSI); pregnancy; newborn birth weight

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Introduction

Infertility has become a global medical and social problem. In recent years, the prevalence of infertility has continued to rise in both developed and developing countries. Statistics have indicated that half of infertility cases are caused by male infertility. Azoospermia has been identified in 5% of infertile men and can be classified as obstructive azoospermia (OA) or non-obstructive azoospermia (NOA) (1,2). In reproductive andrology, OA is a common disease, accounting for 40% of azoospermia. It results from obstruction of the excurrent duct at any location between the rete testis and the ejaculatory duct (3). Previously, OA was a significant problem in male infertility, and sperm donation was the only option, leaving infertile couples with little hope of raising mutually biological children. In 1998, Gorgy *et al.* (4) reported that intracytoplasmic sperm injection (ICSI) using testis sperm by testicular sperm aspiration (TESA) for assisted reproduction is a viable treatment for OA. Since then, ICSI with testis sperm has been widely used, especially for patients with OA, with the advantages of good repeatability, low incidence of complications (bleeding or infections), and excellent success rate of sperm retrieval (96–100%) (5). However, due to the failure rate of in vitro fertilization (IVF), OA patients usually require a second or third puncture. To minimize the damage caused by repeated puncture, many institutions have attempted to freeze the sperm obtained by testicular puncture, which has raised the question of whether there any difference between frozen or fresh testicular sperm. The pregnancy outcomes of ICSI using frozen or fresh testicular sperm remain controversial. Hauser *et al.* suggested that the use of fresh testicular sperm results in better pregnancy outcomes including higher fertilization rate and clinical pregnancy rate (6). However, due to the failure of IVF, repeated punctures may be required, which can cause damage to the testicles. On the contrary, other studies had indicated that pregnancy outcomes are similar after ICSI using frozen or fresh testicular sperm, and moreover using frozen testicular sperm can avoid TESA failure on the day of oocyte collection (7-9). In addition, previous studies rarely talked about neonatal outcome. In this study, frozen and fresh testicular sperm for ICSI were compared in terms of pregnancy and birth outcomes in OA patients. We present the following article in accordance with the STROBE reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-22-125/rc).

Methods

Study design and patient selection

This research was a retrospective cohort study and included OA patients treated with ICSI in Reproductive Center of the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University from January 2016 to December 2020. Patients were followed up by telephone, and relevant data were obtained from outpatient and inpatient medical records and recorded manually. Men who agreed to freeze their sperm by diagnostic TESA for subsequent assisted reproductive techniques (ART) were included in the "frozen sperm group" (n=154), and the remaining 163 cases using fresh sperm (obtained by TESA on the day of oocyte retrieval) were included in the "fresh sperm group". Inclusion criteria: (I) the male partner was azoospermic and sperm was available by TESA; (II) the female partner age ≤40 years old and the number of embryos transferred was 1 to 2 blastocysts; (III) the female partner had normal ovarian function. Exclusion criteria: (I) incomplete or missing patient information; (II) the female partner had factors that seriously affect embryo implantation, such as severe tubal effusion, adenomyosis, uterine adhesions, uterine malformations, etc.; (III) the partner had other diseases that may interfere with pregnancy outcome.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Independent Ethics Committee of The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (No. 2021-K-74-02) and individual consent for this retrospective analysis was waived.

TESA

The testicle was anesthetized locally using 2% lidocaine. A 23-gauge needle connected with a 5-mL syringe containing 5% G-3-(N-morpholino)-propanesulfonic acid (G-MOPS) medium (Vitrolife, San Diego, CA, USA) was used to puncture the testis through the scrotum skin to obtain sufficient testicular tissue. Immediately after collection, testicular tissue was placed in a dish filled with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered medium (G-MOPS) and sent to the laboratory for microscopy. The obtained testicular tissue was minced with a sterile empty needle, and the suspension

was examined for sperm morphological quality and motility under a microscope (×400).

Sperm freezing and thawing

Sperm freezing medium (SAGE; CooperSurgical, Inc., Trumbull, CT, USA) was added to the semen with an equal volume after preheating it to room temperature. The solution was carefully mixed after each addition. Each 0.6–0.8 mL of mixed semen was put into a 1.5-mL cryotube and refrigerated at 4 ℃ for 30 minutes. Each tube was carefully transferred into an aluminum bracket, which was placed in a freezing cylinder. The cylinder was positioned in a liquid nitrogen storage tank for long-term storage.

When performing ICSI, the cryotubes were removed from the liquid nitrogen tank and placed at room temperature for 15–30 minutes. The samples were then transferred to centrifuge tubes and centrifuged to concentrate the sperm cells. Morphological quality and motility of sperms were evaluated, and high-quality sperm was chosen for ICSI.

The ICSI was performed by frozen-thawed testicular sperm or fresh testicular sperm. The 2 pronuclear (2PN) zygote counts and the embryo development stage on days 3, 5, and 6 after ICSI were observed and recorded. After that, high-quality blastocysts were selected for intrauterine embryo transfer according to the Gardner blastocyst grading system, and serum β-human chorionic gonadotropin (β-HCG) was measured after 14 days to determine the pregnancy status. Clinical pregnancy was defined as the presence of an intrauterine gestational sac with a yolk sac, fetal pole, and fetal heart pulsations within about 4 weeks after implantation. Live birth was defined as the birth of a live infant at ≥24 weeks of gestation. The gender, birthweight of newborns, and Apgar scores at 1 and 5 min after birth were collected. Infants weighing under 2,500 g were classified as low birthweight (LBW), while those above 4,000 g were diagnosed with macrosomia.

Statistical analysis

Data were analyzed using the software SPSS 20.0 (IBM Corp., Chicago, IL, USA), and Student's *t*-test was used to compare continuous variables. The chi-square test was performed for categorical variables. Unexpectedly, we found the incidence of LBW was different between frozen and fresh sperm group. In order to identify the influencing factors of LBW, we used multiple logistic regression to analysis. A Two-sided P value <0.05 was considered statistically significant.

Results

This study included a total of 317 patients, which were divided into two groups according to the sperm types used for ICSI. In the frozen sperm group, the sperm samples from 154 males were cryopreserved after TESA. Freshly aspirated sperm samples from 163 matched azoospermia patients were used in the fresh sperm group (*Figure 1*). The demographic, fertility-related, and ICSI characteristics were similar in both groups (*Table 1*). The two groups showed no significant differences in female age, male age, duration of infertility, preoperative sex hormone test results, body mass index (BMI), and smoking and alcohol drinking rates.

The ICSI outcomes were similar, with no differences in the 2PN fertilization rate, 2PN cleavage rate, and high-quality blastocyst rate (P>0.05). The specific results are shown in *Table 2*.

For the pregnancy outcomes, the clinical pregnancy rate and the live birth rate were 57.28% and 44.17%, respectively, in the frozen group, and 58.05% and 45.37%, respectively, in the fresh group. The average number of transferred embryos was 1.84 and 1.79 in the frozen and fresh groups, respectively. There was no difference in the average number of transferred embryos, implantation rate, clinical pregnancy rate, and live birth rate. The miscarriage rate, multiple pregnancy rate, preterm birth rate, and gender ratio at birth (male) were similar in the two groups (*Table 3*).

By the end of the follow-up date, there were 11 women still in pregnancy in both groups. A total of 110 babies were born in the frozen sperm group, among whom were 54 males and 56 females, including 23 LBW newborns and 5 cases of macrosomia. By contrast, 106 babies were born in the fresh sperm group, among whom were 51 males and 55 females, including 9 LBW newborns, and 4 cases of macrosomia (*Figure 2*). The average birthweight of newborns was similar between the two groups (P>0.05). Notably, a higher incidence of LBW newborns was found in the frozen sperm group (20.91% *vs.* 8.49%) (P<0.05, *Table 3*). The results of multiple logistic regression analysis showed that LBW was only related to single or twin pregnancies (P<0.01), but not sperm (frozen or fresh), or patients' age (P>0.05, *Table 4*). Therefore, we analyzed the cases of twin pregnancy and single pregnancy in the two groups separately, and found that the incidences of LBW were similar (P>0.05, *Table 3*). Similarly, no difference was

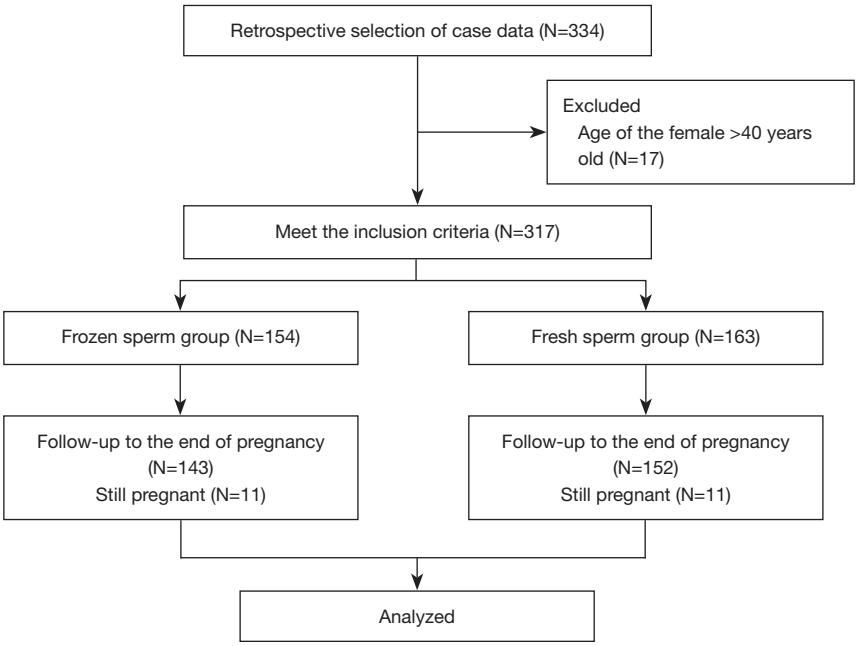

Figure 1 Flow chart showing the design, inclusion, and exclusion criteria of cases in the study.

Table 1 Baseline characteristics of the two groups

Characteristics	Frozen sperm (n=154)	Fresh sperm (n=163)	P value
Female age (years)	29.67±4.80	30.02±5.09	0.523
Male age (years)	32.39±5.23	33.40±6.19	0.117
Infertility (years)	4.01±3.19	4.03±3.29	0.947
LH (IU/L)	4.9±2.3	5.0±2.3	0.929
FSH (IU/L)	8.8±4.7	9.8±5.0	0.070
Prolactin (ng/mL)	9.0±4.3	9.7±4.4	0.162
Estradiol (pg/mL)	27.3±8.1	27.4±8.4	0.912
Testosterone (ng/mL)	5.2±1.8	4.9±1.7	0.139
BMI (kg/m^2)	21.8±1.9	22.1±1.6	0.193
Smoking rate (%)	50.65% (78/154)	54.60% (89/163)	0.496
Alcohol rate (%)	47.40% (73/154)	49.08% (80/163)	0.089

LH, luteinizing hormone; FSH, follicle stimulating hormone; BMI, body mass index.

found in the Apgar scores of the frozen and fresh groups at 1 min (9.38±0.79 *vs.* 9.65±0.48) and 5 min (9.90±0.37 *vs.* 9.96±0.20) (P>0.05, *Table 3*).

Discussion

In females, previous studies have suggested that the use of frozen-thawed rather than fresh embryos results in better pregnancy outcomes in IVF (10-12). In males, whether there is a difference in the outcomes of ICSI using frozen or fresh testicular sperm has remained controversial. This study was designed to explore whether there are differences in embryo quality, pregnancy, and neonatal outcomes of ICSI using frozen or fresh spermatozoa collected by TESA.

Table 2 Comparison of the outcomes of ICSI between the two groups

Outcomes	Frozen sperm (n=154)	Fresh sperm (n=163)	P value
2PN fertilization rate (%)	81.21% (1,297/1,597)	83.30% (1,267/1,521)	0.128
2PN cleavage rate (%)	97.07% (1,259/1,297)	96.92% (1,228/1,267)	0.826
High-quality blastocyst rate (%)	44.62% (232/520)	44.76% (235/525)	0.962

P value by ANOVA, chi-square test. Significance: P<0.05. ICSI, intracytoplasmic sperm injection; 2PN, 2 pronuclei; ANOVA, analysis of variance.

Table 3 Comparison of pregnancy outcomes between the two groups

Outcomes	Frozen sperm (n=154)	Fresh sperm (n=163)	χ^2	P value
Average number of embryos transferred	1.84±0.93	1.79±0.96	–	0.511
Implantation rate (%)	43.28% (145/335)	42.39% (142/335)	0.055	0.815
Clinical pregnancy rate (%)	57.28% (118/206)	58.05% (119/205)	0.025	0.875
Multiple pregnancy rate (%)	24.58% (29/118)	19.33% (23/119)	0.953	0.329
Miscarriage rate (%)	12.71% (15/118)	12.61% (15/119)	0.001	0.980
Preterm birth rate (%)	10.17% (12/118)	5.88% (7/119)	1.477	0.224
Live birth rate (%)	44.17% (91/206)	45.37% (93/205)	0.059	0.808
Gender ratio at birth (%)	96.43% (54/56)	92.73% (51/55)	0.021	0.886
Average newborn birth weight (g)	2,932.61±728.40	3,100.32±515.64	–	0.052
Incidence of LBW newborns (%)	20.91% (23/110)	8.49% (9/106)	6.597	0.010
Twin pregnancy (%)	47.22% (17/36)	26.67% (8/30)	2.938	0.113
Single pregnancy (%)	8.11% (6/24)	1.33% (1/75)	2.511	0.086
Apgar score				
1 min after birth	9.38±0.79	9.65±0.48	–	0.065
5 min after birth	9.90±0.37	9.96±0.20	–	0.389

5 infants with macrosomia were born in the frozen sperm group and 4 infants with macrosomia were born in fresh group. Significance: P<0.05. LBW, low birthweight.

Although great efforts have been made to assist azoospermia patients, 20–30% of them remain unable to obtain sperm suitable for injection. Therefore, most centers have employed repeated punctures to promote sperm acquisition. However, multiple operations may cause damage to the blood supply of the testicular seminiferous tubules, hematoma formation in the testis, testicular tissue fibrosis, and even testicular atrophy, leading to long-term complications such as male autoimmune response, osteoporosis, insulin resistance, and depression (13). In addition, some azoospermia patients may lose their opportunity to be a biological father if TESA fails on the day of oocyte collection. Therefore, if they do not undergo testicular sperm freezing in advance, they are faced with the choices of insemination with donor spermatozoa or oocyte cryopreservation. For this reason, cryopreservation of testicular sperm is important for the preservation of fertility of azoospermia patients. The full and effective use of sperm is key to treating OA. Freezing sperm after TESA is commonly believed to be safe, economical, and effective for OA patients (14), while avoiding the potential damage mentioned above. Nevertheless, Ezzati *et al.* showed that cryopreservation has an adverse effect on sperm, which may be due to structural damage, increased sperm DNA fragmentation, and damage to mitochondrial function (15). Therefore, some researchers have expressed concern about

the pregnancy outcomes of ICSI with frozen testicular sperm and recommend the use of fresh oocytes with fresh testicular sperm as the first choice (6,16). Hauser *et al.* proposed that the use of fresh testicular sperm results in a higher fertilization rate and clinical pregnancy rate than using frozen testicular sperm (6). Therefore, we assert that fresh testicular sperm should be considered first in ICSI for patients with virtual azoospermia or cryptozoospermia because of their superior fertility. In 2015, a retrospective study including 110 cycles of testicular sperm extraction (TESE)-ICSI was conducted by Park *et al.*, and their findings confirmed that statistically significant differences are observed in the pregnancy and implantation rates between fresh and frozen testicular spermatozoa groups, despite similar laboratory outcomes, clinical pregnancy, and delivery (17).

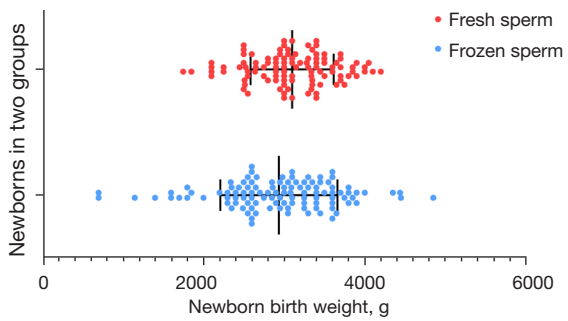

Figure 2 There were 110 babies born in the frozen sperm group, including 23 LBW newborns and 5 cases of macrosomia. There were 106 babies born in the fresh sperm group, including 9 LBW newborns, and 4 cases of macrosomia. The average newborn birth weight (g) in frozen (2,932.61±728.40 g) versus fresh sperm group (3,100.32±515.64 g), P>0.05. A higher incidence of LBW newborns was found in the frozen sperm group (20.91% *vs.* 8.49%) (P<0.05); however, the incidences of LBW newborns in twin pregnancy and single pregnancy were both similar (P>0.05). LBW, low birthweight.

However, analogous with the results of our study, most studies have suggested similar results in terms of fertilization rate, cleavage rate, and clinical pregnancy rate in ICSI with frozen testicular sperm (7-9). Ou *et al.* (7) found that frozen testicular sperm or fresh testicular sperm extracted from patients with OA had the same 2PN fertilization and pregnancy potential (74.41% *vs.* 76.43% and 46.81% *vs.* 53.39%, respectively). Another retrospective study in 2018 suggested that freezing low-count sperm collected by TESA with a cryoprotectant was an efficient method (18). The clinical pregnancy rates of ICSI with fresh and frozen testicular sperm were 61.7% and 55.1%, respectively. Although the pregnancy rate in the fresh sperm group was higher than that in another group, the difference was not significant. It showed that the two types of sperm were equally reliable for ICSI. Liu *et al.* conducted a meta-analysis and showed that the use of frozen or fresh testicular sperm for OA patients did not affect the fertilization rate and pregnancy rate (19), which is consistent with our results.

To date, there few studies on the effect of frozen testicular sperm on neonatal outcomes following TESA-ICSI. Contrary to our findings, Cai *et al.* evaluated 436 singletons conceived from ICSI cycles with fresh or frozen-thawed epididymal sperm in OA, and found that the birthweight of newborns after ICSI using frozen epididymal sperm was significantly lower than that from fresh epididymal sperm (20). However, in our present study, there was a similar result of gender ratio, average newborn birthweight, and Apgar scores in the two groups. Surprisingly, we found that the incidence of LBW in newborns from the frozen sperm group was higher than that of fresh sperm group. At the same time, we noticed that the rate of multiple pregnancies in the frozen sperm group was 5% higher than that of the fresh group. As LBW is a known complication of multiple pregnancies (21,22), and our study also showed that LBW is significantly related to single or

Table 4 Multiple logistic regression for LBW

Factors	Odds ratio	95% CI low	95% CI high	P value
Sperm (frozen or fresh)	0.405	0.148	1.110	0.079
Female age	0.944	0.790	1.127	0.521
Male age	1.008	0.890	1.141	0.903
Newborns (twin or single)	0.094	0.033	0.262	0.000

Significance: P<0.05. LBW, low birthweight; CI, confidence interval.

twin pregnancies, we investigated the incidence of LBW in twin and single pregnancy separately. Our final results demonstrated that the rates of LBW in twin pregnancy and single pregnancy between the two groups were similar.

The risk of deformity is another problem for newborns after using testicular sperm. No case of fetal or neonatal malformations was found in this study. In a population-based cohort study conducted in Denmark from 1995 to 2009, all children were delivered after TESA and fresh embryo transfer, and the outcome of newborns, including congenital abnormalities after ICSI treatment using epididymal or testicular sperm, showed no difference with that after ICSI/IVF treatment using ejaculate sperm or natural conception (23). A 10-year study in China focusing on the neonatal outcomes of children born after ICSI with epididymal or testicular sperm clearly showed that freezing testicular sperm had no effect on the rate of neonatal deformity (24).

The limitations of this study are the small number of men enrolled, and we did not analyze the morphology and motility of the collected sperm. In addition, longer follow-up is needed to study the long-term effects of different testicular sperm on neonatal development.

Conclusions

For men with OA, the use of frozen testicular sperm by TESA is an efficient treatment. Testicular sperm cryopreservation is the best option available to preserve fertility in patients with azoospermia, which could avoid cancellation of IVF cycles due to the failure of TESA on the day of oocyte collection. There are similar pregnancy and neonatal outcomes of ICSI using frozen or fresh spermatozoa collected by TESA. Prospective investigations are needed for further validation.

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Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-22-125/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-22-125/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-22-125/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Independent Ethics Committee of The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (No. 2021-K-74-02) and individual consent for this retrospective analysis was waived.

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