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ORIGINAL ARTICLE

Male Fertility

ICSI treatment of severe male infertility can achieve prospective embryo quality compared with IVF of fertile donor sperm on sibling oocytes

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Azoospermia, cryptozoospermia and necrospermia can markedly decrease the ability of males to achieve pregnancy in fertile females. However, patients with these severe conditions still have the option to be treated by intracytoplasmic sperm injection (ICSI) to become biological fathers. This study analyzed the fertilization ability and the developmental viabilities of the derived embryos after ICSI treatment of the sperm from these patients compared with *in vitro* fertilization (IVF) treatment of the proven-fertile donor sperm on sibling oocytes as a control. On the day of oocyte retrieval, the number of sperm suitable for ICSI collected from two ejaculates or testicular sperm extraction was lower than the oocytes, and therefore, excess sibling oocytes were treated by IVF with donor sperm. From 72 couples (73 cycles), 1117 metaphase II oocytes were divided into 512 for ICSI and 605 for IVF. Compared with the control, husbands' sperm produced a lower fertilization rate in nonobstructive azoospermia (65.4% vs 83.2%; $P < 0.001$), cryptozoospermia (68.8% vs 75.5%; $P < 0.05$) and necrospermia (65.0% vs 85.2%; $P < 0.05$). The zygotes derived in nonobstructive azoospermia had a lower cleavage rate (96.4% vs 99.4%; $P < 0.05$), but the rate of resultant good-quality embryos was not different. Analysis of the rates of cleaved and good-quality embryos in cryptozoospermia and necrospermia did not exhibit a significant difference from the control. In conclusion, although the sperm from severe male infertility reduced the fertilization ability, the derived embryos had potential developmental viabilities that might be predictive for the expected clinical outcomes.

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INTRODUCTION

In humans, it accounts for 40%–50% of infertile couples presenting for assisted reproductive technologies (ARTs).^{1–4} Male infertility is commonly due to deficiencies in the semen, characterized by decreased spermatogenesis, sperm DNA damage, loss of sperm motility and abnormal sperm morphology.^{5,6} Clinically, semen quality is used as a surrogate measure of male fecundity through the analysis of sperm parameters, including the sperm count, morphology, motility, and volume.⁷ These factors commonly direct the classification of the disease diagnosis and the selection of conventional *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment.

Intracytoplasmic sperm injection is highly technical⁸ and its efficiency is affected by many factors.⁹ Although concerned risks probably occur,^{10–13} numerous reports show the expected treatment outcomes of ICSI treatment of male borderline and idiopathic subfertility^{14–21} and ICSI improves fertilization rates and prevents total failed fertilization compared with conventional insemination.¹⁵ In most severe male infertility cases, including azoospermia, cryptozoospermia and necrospermia, because the effects of empirical drug therapy and surgery are limited, ICSI may be the only option to enable a vast

majority of infertile males to become the genetic fathers of their children. ICSI successfully treats nonobstructive azoospermia with testicular sperm extraction (TESE),^{22–25} although the small number of sperm obtained by TESE may produce a lower rate of fertilization²⁶ and biological pregnancy²⁷ compared with obstructive azoospermia. ICSI treats cryptozoospermia with testicular and ejaculated spermatozoa with different outcomes between both sperm origins,^{28,29} whereas the ICSI treatment of necrospermia is associated with decreased a frequency of embryo formation.³⁰ However, relatively few reports have clearly assessed the effects of ICSI on different types of severe male factor infertility with extremely small number of quality sperm on sibling oocytes.

This retrospective study analyzed ICSI outcomes of three classes of severe male factor infertility compared with IVF treatment of proven-fertile donor sperm on the sibling oocytes: nonobstructive azoospermia with a few sperm from TESE; cryptozoospermia with a few sperm found in the centrifuged sample of two ejaculates; and necrospermia with more than 90% of dead sperm and a few swinging sperm. These patients had a lower number of sperm suitable for ICSI treatment compared with the retrieved oocytes. Although the

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subjects preferred an attempt with their own sperm, they agreed to use proven-fertile donor sperm on sibling oocytes to avoid missing the treatment opportunities on the day of oocyte retrieval. This study was designed to treat the patients' sperm by ICSI, and excess sibling oocytes were fertilized with donor sperm by IVF after the couples consented. Without consideration of the influences from female factors, this study supplied a significantly indicative support for the utilization of ICSI technique to treat severe male factor infertility.

MATERIALS AND METHODS

Patients and grouping

Between January 2003 and May 2012, the center treated infertility including 5428 IVF cycles and 5055 ICSI cycles. Of 5055 ICSI-treated cycles, severe male factor infertility included 1974 azoospermia, and 1746 severe oligozoospermia and asthenozoospermia cases.

Of the severe male factor infertility group, 72 couples (73 cycles) consented to attend this study. On the same day of oocyte retrieval, from two ejaculates or TESE, all males had a lower number of sperm that had satisfactory quality and was suitable for microinjection compared with the number of retrieved metaphase II (MII) oocytes. Those who had a high number of quality sperm and even used donor sperm as their requested samples were not included in this study. The average ages of the husbands and wives were 31.42 and 28.47 years, respectively, with an average infertility history of 4.07 years. All female partners had no infertility factors except for nine with salpingitis, and the males had normal karyotypes except for three with a Y chromosome AZFc microdeletion. The subjects were carefully evaluated and classified into three conditions by at least two semen analyses of sperm source, sperm density, motility and number, depending on the standard parameters of semen.³¹ Twenty-eight patients (28 cycles) were diagnosed with nonobstructive azoospermia after carefully excluding obstructive azoospermia and their sperm were retrieved by TESE. Most of the sperms were malformed and immotile. Thirty-four patients (34 cycles) had cryptozoospermia with a dramatically low concentration of sperm in the ejaculated semen that was recorded as $<1 \times 10^4 \text{ ml}^{-1}$, but not as $1 \times 10^5 \text{ ml}^{-1}$ or $1 \times 10^6 \text{ ml}^{-1}$. Twenty-eight patients were found to have no sperm or very few immotile sperms at least once in the 2 times of semen analyses. No more than 10 spermatozoa in each ejaculate were found in 8 cases. As an extremely small number of spermatozoa (dozens of cells) were found in most cases, two ejaculates were required for collecting enough sperm for microinjection into all oocytes. Ten patients (11 cycles) were diagnosed with necrozoospermia. The sperm morphology and membrane integrity were evaluated. The motile sperm were carefully recognized and counted after centrifugation of all sperm samples. The sperm concentration was more than $5 \times 10^6 \text{ ml}^{-1}$ with normal morphology but there were not enough motile sperm found in the semen after two ejaculates for microinjection into all the oocytes. No more than 10 motile sperm per ejaculate were found in 4 cases. For five patients without motile sperm, TESE to retrieve sperm in the semen was performed.

Tropic hyperovulation program

Females received conventional tropic hyperovulation program (THOP) using gonadotropin-releasing hormone/follicle-stimulating hormone/human chorionic gonadotrophin (HCG) program.¹⁴ When the diameter of the dominant follicle was $\geq 18 \text{ mm}$ or at least two follicles $\geq 17 \text{ mm}$, 5000 IU profasi HCG (Serono, Aubonne, Switzerland) was injected. Oocytes were retrieved at 34–36 h after the administration of HCG in Quinn's HEPES-buffered modified human tubal fluid medium (HEPES-modHTF) (SAGE, Pasadena, CA, USA).

Sperm preparation

On the day of oocyte retrieval, the semen were retrieved from two ejaculates and transferred into a conical bottom tube (BD Bioscience, San Jose, CA, USA) containing 2–3 ml HEPES-modHTF medium. After two repeats of washing in HEPES-modHTF medium and centrifugation at 500 g for 8 min, the most volume of supernatant was carefully discarded with a pipette, and a sperm pellet with 0.2–0.5 ml of medium (depending on the size of the pellet) was incubated at room temperature for later use. Extreme care was taken to avoid sperm loss during the above procedures.

Testicular sperm extraction was performed as previously described.³² In brief, 20–30 mg of testicular tissue was obtained by a small surgical biopsy under local anesthesia. Testicular seminiferous tubules were finely minced and examined for the presence of sperm under an inverted microscope (NIKON, Tokyo, Japan) and then transferred into a 5 ml tube containing 1–2 ml HEPES-modHTF medium. The subsequent procedures to obtain the sperm pellet were the same as the above-described method for the ejaculate.

Donor sperm was obtained from Shanghai Human Sperm Bank, and usage of the donor sperm observed by the Chinese National Regulations for use of Human Donor Sperm in ART. After thawing, the donor sperm was washed in fertilization medium (SAGE, Pasadena, CA, USA) and prepared by density-gradient centrifugation.

In vitro fertilization and intracytoplasmic sperm injection treatments

An ICSI dish set up as one round droplet of 10 μl of 7% (w/v) polyvinylpyrrolidone in HEPES-HTF (SAGE, Pasadena, CA, USA) for ICSI surrounded by 3–4 round droplets of 5 μl HEPES-modHTF medium for oocytes and by 3–4 longer droplets of 20 μl HEPES-modHTF medium for semen. The droplets were overlaid by approximately 2 mm of heavy paraffin oil (SAGE, Pasadena, CA, USA), and the dish was prewarmed on a warm-plate at 37°C for half an hour before ICSI processing. A semen suspension prepared from ejaculated semen was placed at the center of a longer droplet, and motile sperm were selected and removed for ICSI once they swam away from the center. The semen suspension with testicular or immotile sperm was distributed evenly in the droplet, and motile sperm were carefully chosen for ICSI. Husband's sperm was preferably used, and the donor sperm was used only when the number of husband's sperm was not enough or in response to the couple request. Based on the embryologist's evaluation of the sperm quality, the numbers of the donor sperm and husband's sperm was determined by the couples, and a formal agreement was signed. ICSI was performed using an ICSI system (NARISHIGE, Tokyo, Japan) with specific ICSI needles (TPC, Thebarton, Australia).

In vitro fertilization was performed in fertilization medium. Fertilized eggs were cultured in Quinn's embryo culture medium (SAGE, Pasadena, CA, USA) overlaid by approximately 2 mm of heavy paraffin oil. Culture was performed at 37°C with an infrared CO₂ incubator connected to 5% CO₂ in the air.

Evaluation of fertilization and embryo quality

Pronuclei were observed 16–20 h after treatment of ICSI or IVF under an inverted microscope. The fertilization rate was calculated as the percentage of the number of the oocytes with presence of two pronuclei (2PN) divided by the number of mature MII stage oocytes. The cleavage and embryo quality was observed at 48 (Day 2) and 72 (Day 3) hours after treatment, respectively. Those containing 6 or more cells with good morphology and $<20\%$ defragment on Day 3 were considered good-quality embryos. The cleavage rate was the proportion of the embryos having developed to the 2-cell or late stage at Day 2

among all the fertilized eggs. The rate of good-quality embryos was the proportion of good-quality embryos at Day 3 among all embryos that had undergone cleavage.

Embryo transfer and clinical pregnancy

The embryos developed from the husbands' sperm were preferentially transferred. If the embryos derived from husband's sperm were of poor quality the embryos derived from the donor's sperm might be transferred in accordance of the patients' request, embryologists' announcement of the embryo quality and a formal agreement. Normally, two embryos were transferred in each cycle. Three embryos might be transferred only if the female partners were older than 35 years of age. Only the embryos derived from sperm with identical sources were transferred in each cycle. Urinary HCG levels were measured 2 weeks after transfer to diagnose pregnancy. If the gestational sac and fetal cardiac tube pulsations were observed with ultrasonography 4 weeks after transfer, clinical pregnancy was confirmed.

Statistical analysis

The statistical analysis was performed with SPSS for Window (version 21.0, SPSS Inc., Chicago, IL, USA). The rates of fertilization, cleavage embryo and good-quality embryos were assessed by binary logistic regression analysis. The rates depending on the given landmark were set as the dichotomous dependent variable and the patient groups, treatments and sperm sources as covariates. The patient groups were also set as a categorical covariate to contrast the differences between the groups and their interaction with other covariates.

RESULTS

In total, 1281 oocytes were retrieved from 73 cycles of 72 cases with an average of 17.55 per cycle. Sibling oocytes from each cycle were randomized into two groups treated by ICSI with husband's sperm and by conventional IVF with donor sperm for control. Overall, 512 out of 1117 MII oocytes were subject to microinjection, and 605 were used as controls. The significant reduction in the rate of the oocytes to form 2PN was demonstrated after they were microinjected with the sperm from the patients of nonobstructive azoospermia ($P < 0.001$), cryptozoospermia ($P < 0.05$) and necrospermia ($P < 0.01$) (Table 1). As the fertilized eggs further developed *in vitro*, at Day 2–3 the rate of total embryos that had undergone cell division was lower than the control in nonobstructive azoospermia ($P < 0.05$), but was similar in cryptozoospermia and necrospermia to the control (Table 1). However, the rates of fertilized eggs and cleaved embryos were not significantly different between three conditions of infertility ($P > 0.05$). The key landmark of the embryonic development for embryo transfer was the rate of the achieved embryos with good morphology after 72 h culture *in vitro*. The rate of these good-quality embryos was not affected by the sperm sources and was also not different between the three diseases (Table 1).

The outcomes after embryo transfer were presented separately (Table 2). In total, 57 women (57 cycles) were implanted with their fresh husbands' sperm-derived embryos with an average of 1.93 and led to 21 cases of pregnancy. As 2 cases were naturally aborted at early and mid-term stages, 19 women gave live birth to 13 boys and 9 girls. Seven women (7 cycles) accepted embryo transfer with fresh donor sperm-derived embryos at an average of 2.43, and 3 became pregnant. One case was aborted in early pregnancy, and 2 were successfully pregnant and give live birth to 1 boy and 2 girls.

DISCUSSION

In this study, 72 couples (73 cycles) from a total of 5055 ICSI cycles

Table 1: The mean rates of fertilization and developmental viabilities of the embryos derived from husband's sperm and donor on sibling oocytes

	Azoospermia		Cryptozoospermia		Necrospermia	
	Husband	Donor	Husband	Donor	Husband	Donor
Number of cycles	28	28	34	34	11	11
Number of oocytes	250	255	242	321	67	128
Number of MII oocytes	228	214	224	282	60	108
Fertilization						
Number	149	178	154	213	39	92
Rate (%)	65.4*	83.2	68.8**	75.5	65.0*	85.2
Cleavage						
Number	141	177	150	204	36	90
Rate (%)	94.6**	99.4	97.4	95.8	92.3	97.8
Good-quality embryo						
Number	76	95	70	99	18	44
Rate (%)	53.9	53.7	46.7	48.5	50	48.9

* $P < 0.001$; ** $P < 0.05$; compared to the rate of the corresponding donor control on sibling oocytes. MII: metaphase II

Table 2: Clinical outcomes after embryo transfer

	Azoospermia		Cryptozoospermia		Necrospermia	
	Husband	Donor	Husband	Donor	Husband	Donor
Embryo transfer						
Cycle	25	1	27	3	5	3
Average number	1.96	2.0	1.89	2.33	2.0	2.67
Pregnancy						
Number	9	0	11	2	1	1
Rate (%)	36.0	0	40.7	66.7	20.0	33.3
Delivery						
Number	8	0	10	1	1	1
Rate (%)	32.0	0	37.0	33.3	20.0	33.3

within 10 years at our center were qualified for our study design. These patients were carefully diagnosed and characterized by the following: (1) on the oocyte retrieval day, either the number of motile sperm from two ejaculates or the relatively good-quality sperm from TESE was lower than the number of oocytes and (2) the couples understood the quality of the husbands' sperm and requested and consented to use donor sperm to fertilize excess sibling oocytes by IVF. The retrospective analysis demonstrated a lower rate of successful fertilization and a satisfactory rate of good-quality embryos in all patients with ICSI treatment, and a lower rate of the embryo cleavage in nonobstructive azoospermia. The derived embryos were able to further develop *in vitro* to be qualified for embryo transfer. The finding revealed that a relatively poor fertilization ability of individual sperm appeared in severe male infertility during ICSI treatment, but the transfer with good-quality embryos might also give rise to relatively satisfactory rates of pregnancy and live births (Table 2). Until date, this study is the first report of utilizing donor sperm on sibling oocytes as a control to evaluate and differentiate the reproductive abilities of the sperm quality in nonobstructive azoospermia, cryptozoospermia and necrospermia. We believe these results provide a relatively strong guideline for treating severe male factor infertility.

This study demonstrated that if careful examination of centrifuged semen from two ejaculates under microscope, some cryptozoospermia could be found from the primarily diagnosed nonobstructive azoospermia, and several or dozens of motile sperm and sperm with

good morphology could be found for ICSI. Similarly, such sperm were also identified and isolated in most cases of necrospemia, although necrospemia is considered as one of the worst conditions due to the presence of the large number of dead sperm in the semen.^{16,33} This is clinically significant because TESE procedures and testicular sperm-related fertilization failure may be avoided.

It's notable that the strict study design strongly limited the number of the cycles of individual disease; such a small number was not used to be as a variable factor for statistical analysis in this study. However, 57 cycles preferred to husband sperm-derived embryos for transfer and gave rise to relatively satisfactory rates of pregnancy and live births. The convinced rate of good-quality embryos in this study may also be predictive for clinical outcomes³⁴ and therefore our data indicate that the couples suffering from these diseases have an opportunity to become biological parents with their own embryos.

Numerous reports have exhibited the promising clinical outcomes in the ICSI treatment of severe infertility,^{19,35-38} whereas there has been a concern if ICSI increases a rate of the birth defects in ICSI conceived children, particularly compared to IVF. It's notable a systematic and two large-scale studies do not exhibit a significant difference in birth defects between ICSI and IVF.³⁹⁻⁴¹ In this study, the important purpose is to obtain the pregnancy through searching the qualified sperm for ICSI from the patients who primarily diagnosed as nonobstructive azoospermia and necrospemia. As there is extremely low number of husband sperm with good-quality selected for ICSI, we have paid a close attention to the evaluation of sperm quality and embryonic developmental viability during the clinic, and a long-term follow-up study of the birth defects of infants has initiated. We believe that with development of new technologies, such as next generation sequencing, the genetic and epigenetic bio-information in individual sperm and ICSI-derived embryo can be unveiled so that we can determine the individual sperm quality and select a good-quality sperm for ICSI, and the birth defects originated from the impaired sperm may be prevented. In addition, through blastocyst transfer, disqualified embryos may be eliminated for embryo transfer and the efficiency of pregnancy improved.

AUTHOR CONTRIBUTIONS

JFZ designed the project, and conducted the andrological clinic, ICSI procedures, data recording and preparation. XBC performed the andrological clinical examinations. LWZ performed the experiments in embryo culture and ICSI. MZG conducted the THOP and gynecological clinic. XQQ and JP reviewed and analyzed the data and performed the statistical study. HJS designed, supported and supervised the project. XLJ reviewed and analyzed the data, and wrote the paper.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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REFERENCES

- Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod* 2007; 22: 1506-12.
- de Kretser DM. Male infertility. *Lancet* 1997; 349: 787-90.
- Brugh VM 3rd, Lipshultz LI. Male factor infertility: evaluation and management. *Med Clin North Am* 2004; 88: 367-85.
- Hirsh A. Male subfertility. *BMJ* 2003; 327: 669-72.

- Lewis SE, John Aitken R, Conner SJ, Iulius GD, Evenson DP, *et al*. The impact of sperm DNA damage in assisted conception and beyond: recent advances in diagnosis and treatment. *Reprod Biomed Online* 2013; 27: 325-37.
- Lewis C, Ford AT. Infertility in male aquatic invertebrates: a review. *Aquat Toxicol* 2012; 120-121: 79-89.
- Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, *et al*. World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 2010; 16: 231-45.
- Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992; 340: 17-8.
- Van den Bergh M, Emiliani S, Biramane J, Vannin AS, Englert Y. Impact of the introduction of intracytoplasmic sperm injection (ICSI) on the treatment of severe male sterility. *Rev Med Brux* 1999; 20: A453-6.
- Schröder AK, Diedrich K, Ludwig M. Fertilization rate and preimplantation development after intracytoplasmic sperm injection. *Reprod Biomed Online* 2001; 3: 241-9.
- Mansour RT, Aboulghar MA, Serour GI, Tawab NA, Amin Y, *et al*. Successful intracytoplasmic sperm injection without performing cytoplasmic aspiration. *Fertil Steril* 1996; 66: 256-9.
- Aboulghar MA, Mansour RT, Serour GI, Amin Y, Ramzy AM, *et al*. Management of long-standing unexplained infertility: a prospective study. *Am J Obstet Gynecol* 1999; 181: 371-5.
- Chang PL, Sauer MV, Brown S. Y chromosome microdeletion in a father and his four infertile sons. *Hum Reprod* 1999; 14: 2689-94.
- Gao MZ, Zhao XM, Lin Y, Sun ZG, Zhang HQ. Effects of EG-VEGF, VEGF and TGF-β1 on pregnancy outcome in patients undergoing IVF-ET treatment. *J Assist Reprod Genet* 2012; 29: 1091-6.
- Johnson LN, Sasson IE, Sammel MD, Dokras A. Does intracytoplasmic sperm injection improve the fertilization rate and decrease the total fertilization failure rate in couples with well-defined unexplained infertility? A systematic review and meta-analysis. *Fertil Steril* 2013; 100: 704-11.
- Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, *et al*. The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Hum Reprod* 1995; 10: 1123-9.
- Strassburger D, Friedler S, Raziell A, Schachter M, Kasterstein E, *et al*. Very low sperm count affects the result of intracytoplasmic sperm injection. *J Assist Reprod Genet* 2000; 17: 431-6.
- Komsky-Elbaz A, Raziell A, Friedler S, Strassburger D, Kasterstein E, *et al*. Conventional IVF versus ICSI in sibling oocytes from couples with endometriosis and normozoospermic semen. *J Assist Reprod Genet* 2013; 30: 251-7.
- Becker B, Bertrand E, Van Hoec J, Verhaegen G, Rozenberg S, *et al*. Outcome of conventional IVF and ICSI on sibling oocytes in patients suffering from teratozoospermia. *Int J Fertil Womens Med* 2006; 51: 163-9.
- Fan W, Li SW, Li L, Huang Z, Ma Q, *et al*. Outcome of conventional IVF and ICSI on sibling oocytes in the case of isolated teratozoospermia. *J Assist Reprod Genet* 2012; 29: 905-10.
- van der Westerlaken L, Naaktgeboren N, Verburg H, Dieben S, Helmerhorst FM. Conventional *in vitro* fertilization versus intracytoplasmic sperm injection in patients with borderline semen: a randomized study using sibling oocytes. *Fertil Steril* 2006; 85: 395-400.
- Lewin A, Reubinoff B, Porat-Katz A, Weiss D, Eisenberg V, *et al*. Testicular fine needle aspiration: the alternative method for sperm retrieval in non-obstructive azoospermia. *Hum Reprod* 1999; 14: 1785-90.
- Westlander G, Hamberger L, Hanson C, Lundin K, Nilsson L, *et al*. Diagnostic epididymal and testicular sperm recovery and genetic aspects in azoospermic men. *Hum Reprod* 1999; 14: 118-22.
- Tuuri T, Moilanen J, Kaukoranta S, Makinen S, Kotola S, *et al*. Testicular biopsy gun needle biopsy in collecting spermatozoa for intracytoplasmic injection, cryopreservation and histology. *Hum Reprod* 1999; 14: 1274-8.
- Karacan M, Alwaeely F, Erkan S, Çebi Z, Berberoglu M, *et al*. Outcome of intracytoplasmic sperm injection cycles with fresh testicular spermatozoa obtained on the day of or the day before oocyte collection and with cryopreserved testicular sperm in patients with azoospermia. *Fertil Steril* 2013; 100: 975-80.
- De Croo I, Van der Elst J, Everaert K, De Sutter P, Dhont M. Fertilization, pregnancy and embryo implantation rates after ICSI in cases of obstructive and non-obstructive azoospermia. *Hum Reprod* 2000; 15: 1383-8.
- Celikten A, Batioglu S, Gungor AN, Ozdemir E. Intracytoplasmic sperm injection outcomes of obstructive and nonobstructive azoospermic men. *Arch Gynecol Obstet* 2013; 288: 683-6.
- Amirjannati N, Heidari-Vala H, Akhondi MA, Hosseini Jadda SH, Kamali K, *et al*. Comparison of intracytoplasmic sperm injection outcomes between spermatozoa retrieved from testicular biopsy and from ejaculation in cryptozoospermic men. *Andrologia* 2012; 44 Suppl 1: 704-9.
- Ben-Ami I, Raziell A, Strassburger D, Komarovskiy D, Ron-El R, *et al*. Intracytoplasmic sperm injection outcome of ejaculated versus extracted testicular spermatozoa in cryptozoospermic men. *Fertil Steril* 2013; 99: 1867-71.



- 30 Sherins RJ, Thorsell LP, Dorfmann A, Dennison-Lagos L, Calvo LP, *et al*. Intracytoplasmic sperm injection facilitates fertilization even in the most severe forms of male infertility: pregnancy outcome correlates with maternal age and number of eggs available. *Fertil Steril* 1995; 64: 369–75.
- 31 Organization WHO. WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction: Cambridge University Press; 1999.
- 32 Zheng J, Huang X, Li C. Predictive factors for successful sperm recovery in azoospermia patients. *Zhonghua Wai Ke Za Zhi* 2000; 38: 366–8.
- 33 French DB, Sabanegh ES Jr, Goldfarb J, Desai N. Does severe teratozoospermia affect blastocyst formation, live birth rate, and other clinical outcome parameters in ICSI cycles? *Fertil Steril* 2010; 93: 1097–103.
- 34 Volpes A, Sammartano F, Coffaro F, Mistretta V, Scaglione P, *et al*. Number of good quality embryos on day 3 is predictive for both pregnancy and implantation rates in *in vitro* fertilization/intracytoplasmic sperm injection cycles. *Fertil Steril* 2004; 82: 1330–6.
- 35 Black M, Liu de Y, Bourne H, Baker HW. Comparison of outcomes of conventional intracytoplasmic sperm injection and intracytoplasmic sperm injection using sperm bound to the zona pellucida of immature oocytes. *Fertil Steril* 2010; 93: 672–4.
- 36 Li Z, Lin H, Xiao W, Wang Y. Fertilization of IVF/ICSI using sibling oocytes from couples with subfertile male or unexplained infertility. *J Huazhong Univ Sci Technolog Med Sci* 2004; 24: 365–8, 84.
- 37 Shi XY, Wu FR, Chen SL, Wang QL, Luo C, *et al*. *In vitro* fertilization versus intracytoplasmic sperm injection for primary and secondary infertility using sibling oocytes: clinical analysis of the outcomes. *Nan Fang Yi Ke Da Xue Xue Bao* 2010; 30: 2263–6.
- 38 Shuai HL, Ye Q, Huang YH, Xie BG. Comparison of conventional *in vitro* fertilisation and intracytoplasmic sperm injection outcomes in patients with moderate oligoasthenozoospermia. *Andrologia* 2014. May 9. doi: 10.1111/and.12291. [Epub ahead of print]
- 39 Yan J, Huang G, Sun Y, Zhao X, Chen S, *et al*. Birth defects after assisted reproductive technologies in China: analysis of 15,405 offspring in seven centers (2004 to 2008). *Fertil Steril* 2011; 95: 458–60.
- 40 Wen J, Jiang J, Ding C, Dai J, Liu Y, *et al*. Birth defects in children conceived by *in vitro* fertilization and intracytoplasmic sperm injection: a meta-analysis. *Fertil Steril* 2012; 97: 1331–7.e1.
- 41 Han JL, Chen H, Niu ZH, Sun YJ, Sun XX, *et al*. A 10-year survey on birth defects after *in vitro* fertilization-embryo transfer in Shanghai. *Zhonghua Fu Chan Ke Za Zhi* 2010; 45: 124–7.

