

Using Fresh and Frozen Testicular Sperm Samples in Couples Undergoing ICSI-MicroTESE Treatment

Safak Tavukcuoglu ^{1*}, Tahani AL-Azawi ¹, Safaa AL-Hasani ¹, Amir Afshin Khaki ¹, Arash Khaki ¹, Seval Tasdemir ²

1- Reproductive Medicine Unit, University of Schleswig-Holstein, Luebeck, Germany

2- Fertijin IVF Center, Istanbul, Turkey

Abstract

Background: We performed this study to evaluate use of fresh and frozen sperm samples in non-obstructive azoospermia microdissection testicular sperm extraction (micro-TESE-ICSI) treatment.

Methods: We performed a total of 82 consecutive in vitro fertilization (IVF) cycles at Fertijin IVF Center in Istanbul, Turkey from January 2010 to March 2012. In 43 participants we used fresh sperm and frozen sperm in the remaining 39 cases. We used fresh and frozen thawed micro surgical testicular sperm extraction (micro TESE) sperm for ICSI with metaphase II (MII) oocytes.

Results: Frozen microTESE sperm was used in 39 cycles, while 43 ICSI cycles were performed using fresh microTESE. Neither the age of male partners (38.33 ± 5.93 and 38.13 ± 8.28) nor that of the female participants (33.16 ± 6.38 and 33.33 ± 6.97) showed significant difference between fresh versus the microTESE and frozen treatment groups, respectively. FSH concentrations were (14.66 ± 13.93 mIU/ml) in fresh TESE group and (17.91 ± 16.29 mIU/ml) in frozen group with no correlations or differences between the two groups. The average number of mature oocytes injected with sperm was 9.23 ± 3.77 , versus 9.26 ± 5.26 in cycles using fresh and frozen microTESE sperm, respectively. Fertilization rate was not significantly different in the fresh microTESE (44.79%) than frozen TESE sperm group (46.76%). The average number of transferred embryos was 1.60 ± 0.49 in fresh sperm group and 1.59 ± 0.50 in frozen sperm group. All embryo transfers were performed on day 3.

Conclusion: Cryopreservation of testicular sperm tissues is more suitable and of great benefite if carried out before ovulation induction and not after, especially in cases with non-obstructive azoospermia.

Keywords: ICSI, *In vitro* fertilization, Microsurgical testicular sperm extraction, Sperm retrieval, Sperm.

To cite this article: Tavukcuoglu S, AL-Azawi T, AL-Hasani S, Khaki AA, Khaki A, Tasdemir S. Using Fresh and Frozen Testicular Sperm Samples in couples Undergoing ICSI-MicroTESE Treatment. *J Reprod Infertil.* 2013;14(2):79-84.

Introduction

Surgical techniques for testicular sperm retrieval, such as testicular sperm extraction (TESE) and microsurgical testicular sperm extraction (mTESE), are prerequisites for treating infertile men (1). Azoospermia due to spermatogenic failure may be classified as primary or secondary. Primary disorders may be of genetic ori-

gin, such as microdeletions in Y-chromosome, or of congenital origin as in cryptorchidism (2). Secondary causes may be due to chemo- or radiotherapy or endocrine dysfunctions (3). Testicular histology generally demonstrates maturation arrest or complete absence of germ cells (4). Male factor infertility represents almost one-third of infertility

* Corresponding Author:
Safak Tavukcuoglu, Reproductive Medicine Unit, University of Schleswig-Holstein, Ratzeburger Alle 160, 23538 Luebeck, Germany
E-mail: saftv@hotmail.com

Received: Oct. 30, 2012

Accepted: Jan. 29, 2013

cases, out of which azoospermia may comprise up to 10% (5). Azoospermia can be either obstructive azoospermia (OA) or non-obstructive azoospermia (NOA). In OA, there is a blockage in the transfer pathway of sperm. In all OA and most of NOA cases, sperm can be retrieved from the testis via microTESE (6, 7). In severe cases, there may be only scattered foci of sperm production and more extensive biopsy may be needed to recover tubules with mature sperm.

Different studies have shown that the combination of human testicular sperm biopsy and intracytoplasmic sperm injection (ICSI) are efficient methods in the treatment of male infertility with azoospermia (8, 9). The successful use of TESE procedures with ICSI led to the use of cryopreserved-thawed spermatozoa. The addition of sperm cryopreservation is valuable as it may reduce the number of procedures required for pregnancy success, and repeated testicular biopsies can then be avoided (10). It seems that comparable success could be achieved with frozen-thawed and testis spermatozoa (11, 12). However, limited pieces of data exist about different factors connected with the efficacy of ICSI using frozen non-motile testicular spermatozoa. Dafopoulos et al. reported in their study that they used motile spermatozoa for ICSI in 159 cycles while they used immotile spermatozoa in 138 other cycles (13).

The aim of this study was to compare use of fresh and frozen sperm samples in non-obstructive azoospermia through microdissection testicular sperm extraction (microTESE) treatment.

Methods

Patients: A total of 82 consecutive IVF cycles were performed at the Fertijin IVF Center in Istanbul, Turkey from January 2010 to March 2012. This center has been approved by the Ministry of Health of Turkey and; therefore, under its ethical rules. In the study, we used fresh sperm in 43 and frozen sperm in 39 participants with non-obstructive azoospermia. Fresh and frozen thawed m-TESE sperm were used for ICSI with metaphase II (MII) oocytes.

Surgical procedures: Micro-TESE was performed under general anesthesia by removing off testicular tissue through a transversal incision of the albuginea, either equatorially or in the cranial part of the testis. In microTESE, an incision was made on the testes, covering three-quarters of its circumference and oriented to preserve the predominantly transversal subalbugineal vessels. The tes-

ticular pulp, was searched under the operative microscope ($\times 12$ to 24 magnification) for areas with dilated tubules, and several tiny fragments (approx. $2 \times 2 \times 2 \text{ mm}^3$) of the testicular tissue were sampled from the two separate surfaces, at different levels of depth from the albuginea to the hilum. The volume of testicular tissue collected by microTESE for sperm search was roughly the same. At the time of surgery, a testicular biopsy from the subalbugineal pulp was also made. Some of the patients had pathology reports from previous biopsies but it was the first time for most of them. We cryopreserved the rest of fresh m-TESE sperm after ICSI.

Biological search for spermatozoa and freezing: Testicular fragments were washed in Earl's Balanced Salt Solution (EBSS Sigma, USA) medium to remove blood, then they were placed in sterile glass petri dishes with 0.5 ml of Sperm Washing Medium™ (from Vitrolife, Sweden), and finely minced using insulin enjection needles and while being stirred to a homogeneous suspension, they were directly examined under an inverted microscope at $\times 200$ and $\times 400$ magnifications (Diaphot, Nikon, Japan) for the presence of spermatozoa, the search lasting up to 1 hr . Sperm search was performed in all cases by the same operator, and the results were expressed as positive when at least one recoverable sperm was observed (7). The sperm were frozen on the same day with Vitrolife sperm freezing medium (Vitrolife Sweden). Testicular tissues were mixed 1:1 with sperm freezing medium in sperm cryo vial after labeling stayed 20 min at -20°C in deep freeze and then put on liquid nitrogen vapour freezing technique as mentioned by Dafopoulos (14). If we collected oocytes on the same day, we used fresh testicular sperm samples after centrifuging it for 15 min at 2000 rpm (Heraeus Labofuge 400, Germany) (15). Thawing was performed at room temperature for 15 min . The preparation was then separated from the cryoprotectant by washing in culture medium and centrifugation at 2000 rpm for 15 min . The resulting pellet was resuspended in $500 \mu\text{l}$ of culture medium (Vitrolife, Sweden).

Ovarian Stimulation: All female partners were stimulated using gonadotropin releasing hormone agonist or antagonist, in combination with human menopausal gonadotropin, and/ or recombinant human FSH. Human chorionic gonadotropin was administered when optimal follicle development was achieved, as evaluated by serial transvaginal ultrasound (GE Logiq P5, USA). Oocyte retrieval

was performed via a transvaginal approach under sonographic guidance (GE Logiq P5, USA) 36 hr after human chorionic gonadotropin injection (16). Cumulus-oocyte complexes were collected in MOPS medium (Vitrolife, Sweden) and denudation was performed using hyaluronidase (Vitrolife, Sweden).

Oocyte preparation and ICSI: The cumulus and corona radiata cells were removed by exposure to hyase hyaluronidase $\times 10$ (Vitrolife, Sweden) under stereomicroscopy for 30 s. Only MII oocytes were used for ICSI and this was performed as described by Al-Hasani et al. (17). Both kinds of testicular sperm were picked up from testicular tissue droplets to MOPS (Vitrolife, Sweden) drop in ICSI dishes. Spermatozoa were aspirated into the injection pipette and transferred with polyvinyl pyrrolidone (PVP) droplets. The tail of each sperm was crushed with the injection pipette and then aspirated the tail first for microinjection. All procedures were performed on the heated stage of an inverted microscope (Nikon, TE 300, Tokyo, Japan) at $\times 200$ magnification and three-dimensional hydraulic system micromanipulators.

Embryo culture, evaluation and transfer: Fertilization was checked 16 to 18 hr after ICSI. The fertilized oocytes were transferred and cultured in 0.5 ml fresh G1 (Vitrolife, Sweden) droplets in 5-well dishes covered by mineral oil (Vitrolife, Sweden) at 37°C in 6% CO₂, 5% O₂ and 95% humidity. Then, we continued culture in 0.5 ml G2 (Vitrolife, Sweden) at the same condition. Cleavage was assessed after 48 to 72 hr and embryo quality was evaluated prior to transfer. Embryo quality was evaluated on the basis of morphological and developmental stages. Embryos were classified into two good and poor categories (18). Embryos with normal morphology, which was in a developmental stage appropriate for its age, were classified as transferable and the maximum number of transferred embryos was two. Embryo transfer was

performed on day 3 after oocyte retrieval using the Cook catheter (K-Soft 5000, Australia). Pregnancy was determined as a spontaneous rise in a β -hCG concentration on day 10 after embryo transfer. Clinical pregnancy implied the presence of an intrauterine gestational sac and fetal heart beat on an ultrasound performed in the seventh week of gestation.

Data collection and statistical analysis: The primary outcome measures tabulated were patients count, age and sperm count. Serum FSH level for all male participants was measured before testicular sperm extraction, mature oocyte counts, fertilization rate, number of transferred embryos, embryo quality on day 3, clinical pregnancy and birth rates were included. Results are expressed as means \pm SD for numeric variables. Categorical variables were expressed as proportions (%). Distributions of quantitative variables were compared using the Mann-Whitney U test for variables in every two groups. Proportions were compared using the Chi-squared (χ^2) test or the Fisher's exact test, depending on sample size. The p-values less than 0.05 were considered to be statistically significant.

Results

We retrospectively analyzed 82 consecutive IVF cycles performed at Fertijin IVF Center in Istanbul. They included 39 utilized frozen-thawed microTESE sperm and 43 fresh microTESE sperm, which were used for ICSI of mature oocytes (MII). Frozen microTESE sperm was used in 39 cycles; while 43 ICSI cycles were performed using fresh microTESE (Table 1).

Neither the age of male (38.33 \pm 5.93 and 38.13 \pm 8.28 years) nor that of female (33.16 \pm 6.38 and 33.33 \pm 6.97) participants showed significant differences between fresh versus microTESE and frozen groups, respectively. FSH levels were (14.66 \pm 13.93) in fresh mTESE group and (17.91 \pm

Table 1. Patient Characteristics, serum FSH concentrations, fertilization rates and embryo transfer percentages of the two groups (M \pm SD)

	Fresh MicroTESE	Frozen MicroTESE	p-value
Patients count	43	39	>0.05
Age male (years)	38.33 \pm 5.93	38.13 \pm 8.28	>0.05
Age female (years)	33.16 \pm 6.38	33.33 \pm 6.97	>0.05
Sperm count (10 ⁶ /ml)	4.83 \pm 1.14	5.69 \pm 1.19	>0.05
FSH concentration (mIU/ml)	14.6 \pm 13.93	17.91 \pm 16.29	>0.05
Mature oocyte count	9.23 \pm 3.77	9.26 \pm 5.26	>0.05
Fertilization rate (%)	44.79 \pm 18.27	46.76 \pm 20.85	>0.05
Embryo transfer (%)	1.60 \pm 0.49	1.59 \pm 0.50	>0.05

Table 2. Embryo quality, pregnancy and birth rates for both groups of mTESE using fresh or frozen testicular sperm

Fresh Micro TESE			Frozen Micro TESE			p-value
Quality	Number of Embryos	% of Embryo	Quality	Number of Embryos	% of Embryos	
Emryo quality						
Poor	18	41.9%	Poor	19	48.7%	>0.05
Good	25	58.1%	Good	20	51.3%	
Pregnancy rate						
Result	Pregnancy count	Pregnancy (%)	Results	Pregnancy count	Pregnancy (%)	>0.05
Negative	24	55.8%	Negative	22	56.4%	
Positive	19	44.2%	Positive	17	43.6%	
Birth rate						
	Birth counts	Birth rate (%)		Birth counts	Birth rate (%)	>0.05
	16	37.2%		12	30.8%	

16.29) in the frozen group with no differences between the two groups.

The average number of mature oocytes injected with sperm was (9.23 ± 3.77 , 9.26 ± 5.26) in cycles using fresh and frozen microTESE sperm, respectively. Fertilization rate did not significantly differ between fresh microTESE cases (44.79%) and frozen TESE sperm cases (46.76%). The average number of transferred embryos was (1.60 ± 0.49) in fresh group and (1.59 ± 0.50) in frozen group.

All embryo transfers were performed on day 3. Embryo quality was evaluated as good or poor depending on the fragmentation percentage, blastomere count and multinucleation absence prior to transfer. In groups using fresh mTESE sperm, 58.1% were of good and 41.9% of poor embryo qualities, and nearly similar results were produced in the frozen sperm group, 51.3% were of good and 48.7% were of poor quality.

Clinical pregnancy was achieved in 19 couples (44.2%) and 17 couples (43.6%), respectively, in groups using fresh and frozen TESE sperm. Delivery rate was similar between groups. Nineteen pregnancies were established using fresh TESE and resulted in the delivery of 12 healthy neonates. In the frozen TESE group, 17 clinical pregnancies were delivered, resulting in the birth of 10 healthy neonates. Two pregnancies resulted in spontaneous abortion in the fresh sperm group and four abortions occurred in the frozen one. No congenital anomalies or major malformations were noted in both groups (Table 2).

Discussion

Several authors have demonstrated that performing ICSI with fresh or frozen spermatozoa will

produce similar results (12, 16, 19, 20) and that freezing does not affect spermatozoa (21). Sperm recovery rate for ICSI treatment was between 60% to 70% in patients with non-obstructive azoospermia (22) but in our study it was between 30% to 40%. In some cases there were no clear relationship evidence parameters and results, for example between serum FSH concentrations and testicular volume measurements to predict success rate especially in patients with sertoli cell only syndrome or maturation arrest (23). Although, FSH concentration showed no significant difference between the two groups, its concentration correlated with sperm count. However, when sperm collection was not possible on the day of oocyte retrieval for ICSI in some cases, we offered the couples to throw or cryopreserve their oocytes. In these cases, cryopreservation of testicular spermatozoa with diagnostic biopsy or TESE attempt for future ICSI cycles, would avoid both unnecessary female stimulation and the need for repetitive biopsies for successive ICSI cycles (24). This approach has been shown to be feasible (19). Testicular sperm could be frozen within testicular tissue with minimal processing of testicular tissue or after being extracted and purified from the tissue. The preferred method may be to freeze the tissue intact as this allows better post-thaw survival and motility rate (25). Our method was very simple, with fast and good result as compared to other studies. The results showed that the combination of human testicular sperm biopsy and intracytoplasmic sperm injection (ICSI) was an efficient method for the treatment of male infertility with azoospermia. Some authors have reported fertilization rates between 44% to 63% (19), em-

bryo cleavage rates between 89% to 92% (26), implantation rates of 8% to 11% (27) and clinical pregnancy rates per embryo transfer of about 27% (26). On the other hand, results for the use of fresh testicular sperm after ICSI has been reported to be of fertilization rates of 17% to 69% (8), embryo cleavage rates of 94% (26), implantation rates of 9% to 11% (28) and clinical pregnancy rates per embryo transfer of 21% to 36% (29).

Considering Van Steirteghem et al.'s experiment (30) which involved comparison of fertilization rates with ICSI for fresh and frozen testicular sperm, frozen testicular sperm had possibly lower fertilization rates than the ejaculated or fresh epididymal sperm (53.4% vs. 64.8%, and 58.5% respectively). However, when we compared fresh and frozen testicular tissues, we achieved similar fertilization rates for fresh and frozen sperm (44.7% and 46.7%, respectively). Moreover, in one study on comparing the pregnancy rate (30), the rates were 33.6% and 44.3% for fresh and the frozen groups, respectively, but our findings showed 44.2% for fresh and 43.6% for frozen sperm.

We achieved 19 pregnancies in fresh mTESE sperm group, although 2 of them were later aborted. Birth rate was 37.2% in this group and 17 pregnancies were achieved in frozen mTESE group, although 4 of which were later aborted. Birth rate was 30.8%. However, our results demonstrated that fresh and frozen-thawed testicular spermatozoa used in ICSI procedure which involved culture and transfer of embryos on day 3 yielded similar results in the two groups. Moreover, there were no significant differences in embryo cleavage rates, quality of embryos, fertilization rate or clinical pregnancy outcomes between the two groups.

Conclusion

Based on our findings, use of frozen TESE seems to be the best way to avoid unnecessary ovulation induction. Regarding the findings of this study, cryopreservation of testicular sperm tissues seems to be more suitable and of great benefit in these cases and good results are expected when ICSI is carried out before and not after ovulation induction, especially in cases with nonobstructive azoospermia.

More evidence is needed through studies with larger sample sizes and different communities to draw a conclusion about the subject in this region using.

Acknowledgement

The authors would like to thank their colleagues at Lab. Tech. Fevzi Akçıl in Istanbul and Ms. Yasemin Özeren (M.Sc.) for their technical support during this study.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Ramasamy R, Yagan N, Schlegel PN. Structural and functional changes to the testis after conventional versus microdissection testicular sperm extraction. *Urology*. 2005;65(6):1190-4.
2. Gordon UD. Assisted conception in the azoospermic male. *Hum Fertil*. 2002;5(Suppl 1):S9-S14.
3. Anniballo R, Ubaldi F, Cobellis L, Sorrentino M, Rienzi L, Greco E, et al. Criteria predicting the absence of spermatozoa in the Sertoli cell-only syndrome can be used to improve success rates of sperm retrieval. *Hum Reprod*. 2000;15(11):2269-77.
4. Cedenho AP, Da Ros CT, Juliano RV. Treatment of non-obstructive azoospermia. *Int Braz J Urol*. 2004;29(Suppl 5):39-41.
5. Chandley AC. The chromosomal basis of human infertility. *Br Med Bull*. 1979;35(2):181-6.
6. Schoysman R, Vanderzwalmen P, Nijs M, Segal-Bertin G, van de Casseye M. Successful fertilization by testicular spermatozoa in an in-vitro fertilization programme. *Hum Reprod*. 1993;8(8):1339-40.
7. Schlegel PN. Testicular sperm extraction: microdissection improves sperm yield with minimal tissue excision. *Hum Reprod*. 1999;14(1):131-5.
8. Schoysman R, Vanderzwalmen P, Nijs M, Segal L, Segal-Bertin G, Geerts L, et al. Pregnancy after fertilisation with human testicular spermatozoa. *Lancet*. 1993;342(8881):1237.
9. Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H, Devroey P. High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. *Hum Reprod*. 1995;10(1):148-52.
10. Gianaroli L, Magli MC, Selman HA, Colpi G, Belgrano E, Trombetta C, et al. Diagnostic testicular biopsy and cryopreservation of testicular tissue as an alternative to repeated surgical openings in the treatment of azoospermic men. *Hum Reprod*. 1999;14(4):1034-8.
11. Prins GS, Dolgina R, Studney P, Kaplan B, Ross L, Niederberger C. Quality of cryopreserved testicular sperm in patients with obstructive and nonobstructive azoospermia. *J Urol*. 1999;161(5):1504-8.

12. Habermann H, Seo R, Cieslak J, Niederberger C, Prins GS, Ross L. In vitro fertilization outcomes after intracytoplasmic sperm injection with fresh or frozen-thawed testicular spermatozoa. *Fertil Steril*. 2000;73(5):955-60.
13. Dafopoulos K, Griesinger G, Schultze-Mosgau A, Orief Y, Schöpper B, Nikolettos N, et al. Factors affecting outcome after ICSI with spermatozoa retrieved from cryopreserved testicular tissue in non-obstructive azoospermia. *Reprod Biomed Online*. 2005;10(4):455-60.
14. Dafopoulos K, Griesinger G, Schultze-Mosgau A, Orief Y, Schöpper B, Nikolettos N, et al. Cumulative pregnancy rate after ICSI with cryopreserved testicular tissue in non-obstructive azoospermia. *Reprod Biomed Online*. 2005;10(4):461-6.
15. Amer M, Haggag SE, Moustafa T, Abd El-Naser T, Zohdy W. Testicular sperm extraction: impact of testicular histology on outcome, number of biopsies to be performed and optimal time for repetition. *Hum Reprod*. 1999;14(12):3030-4.
16. Liu J, Tsai YL, Katz E, Compton G, Garcia JE, Baramki TA. Outcome of in-vitro culture of fresh and frozen-thawed human testicular spermatozoa. *Hum Reprod*. 1997;12(8):1667-72.
17. Al Hasani S, Küpker W, Baschat AA, Sturm R, Bauer O, Diedrich C, et al. Mini-swim-up: a new technique of sperm preparation for intracytoplasmic sperm injection. *J Assist Reprod Genet*. 1995;12(7):428-33.
18. Veeck L. Preembryo grading and degree of cytoplasmic fragmentation. In: Veeck L, editor. *An atlas of human gametes and conceptuses*. London: Parthenon publishing; 1999. p. 46-50.
19. Gil-Salom M, Romero J, Minguez Y, Rubio C, De los Santos MJ, Remohi J, et al. Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. *Hum Reprod*. 1996;11(6):1309-13.
20. Küpker W, Schlegel PN, Al-Hasani S, Fornara P, Johannisson R, Sandmann J, et al. Use of frozen-thawed testicular sperm for intracytoplasmic sperm injection. *Fertil Steril*. 2000;73(3):453-8.
21. Lin MH, Morshedi M, Srisombut C, Nassar A, Oehninger S. Plasma membrane integrity of cryopreserved human sperm: an investigation of the results of the hypoosmotic swelling test, the water test, and eosin-Y staining. *Fertil Steril*. 1998;70(6):1148-55.
22. Jezek D, Knuth UA, Schulze W. Successful testicular sperm extraction (TESE) in spite of high serum follicle stimulating hormone and azoospermia: correlation between testicular morphology, TESE results, semen analysis and serum hormone values in 103 infertile men. *Hum Reprod*. 1998;13(5):1230-4.
23. Tournaye H, Verheyen G, Nagy P, Ubaldi F, Goossens A, Silber S, et al. Are there any predictive factors for successful testicular sperm recovery in azoospermic patients? *Hum Reprod*. 1997;12(1):80-6.
24. Schlegel PN, Su LM. Physiological consequences of testicular sperm extraction. *Hum Reprod*. 1997;12(8):1688-92.
25. Sheikh A, Fouad S, Hussein M. Fresh and frozen testicular tissue in conjunction with intra-cytoplasmic sperm injection in the treatment of obstructive and nonobstructive azoospermia. *Hum Reprod*. 1998;13:117-8.
26. Friedler S, Raziel A, Soffer Y, Strassburger D, Komarovsky D, Ronel R. Intracytoplasmic injection of fresh and cryopreserved testicular spermatozoa in patients with nonobstructive azoospermia--a comparative study. *Fertil Steril*. 1997;68(5):892-7.
27. Oates RD, Mulhall J, Burgess C, Cunningham D, Carson R. Fertilization and pregnancy using intentionally cryopreserved testicular tissue as the sperm source for intracytoplasmic sperm injection in 10 men with non-obstructive azoospermia. *Hum Reprod*. 1997;12(4):734-9.
28. Watkins W, Nieto F, Bourne H, Wutthiphan B, Speirs A, Baker HW. Testicular and epididymal sperm in a microinjection program: methods of retrieval and results. *Fertil Steril*. 1997;67(3):527-35.
29. Devroey P, Liu J, Nagy Z, Goossens A, Tournaye H, Camus M, et al. Pregnancies after testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermia. *Hum Reprod*. 1995;10(6):1457-60.
30. Van Steirteghem A, Nagy P, Joris H, Janssenswilten C, Staessen C, Verheyen G, et al. Results of intracytoplasmic sperm injection with ejaculated, fresh and frozen-thawed epididymal and testicular spermatozoa. *Hum Reprod*. 1998;13 (Suppl 1):134-42.