

Article

Cumulative pregnancy rate after ICSI with cryopreserved testicular tissue in non-obstructive azoospermia



Dr Konstantinos Dafopoulos was born in 1968 in Greece. He studied medicine at Athens University in Greece and graduated in 1991. After obtaining his speciality degree in Obstetrics and Gynaecology in Greece he worked as a postgraduate fellow in reproductive medicine and endoscopic surgery at the Medical University of Lübeck under Professor Klaus Diedrich. He is currently a lecturer in Obstetrics and Gynaecology at the University of Thessalia in Greece under Professor Ioannis E Messinis. His special interests lie in the field of evidence-based reproductive medicine. He has published more than 15 papers in international peer-reviewed journals.

Dr Konstantinos Dafopoulos

Konstantinos Dafopoulos, Georg Griesinger, Askan Schultze-Mosgau, Yasser Orief, Beate Schöpfer, Nikos Nikolettos, Klaus Diedrich, Safaa Al-Hasani¹

Department of Obstetrics and Gynaecology, University of Schleswig-Holstein, Campus Lübeck, Ratzeburger Allee 160, 23538 Luebeck, Germany.

¹Correspondence: e-mail: sf_alhasani@hotmail.com

Abstract

The aim of the present study was to describe a simplified and inexpensive method of testicular tissue freezing, to assess the cumulative clinical pregnancy rate (CPR) by this technique, and to provide useful information for counselling couples with non-obstructive azoospermia. One hundred and sixty-five couples with non-obstructive azoospermic males pursuing assisted conception, from December 1995 to December 2002, were included. In all cases, the testicular tissue retrieved by open multiple-biopsy (both sides, by testicular sperm extraction) was frozen using a simple liquid nitrogen vapour freezing technique and was stored in liquid nitrogen thereafter. Only mature spermatozoa were used for intracytoplasmic sperm injection (ICSI) after thawing. Expected CPR were calculated using the Kaplan–Meier survival analysis. A total of 281 cycles were performed resulting in 53 clinical pregnancies. Crude and expected CPR (95% confidence intervals) after three cycles were 32.1 (25.7–40.1) and 55.7% (37.0–74.4) respectively. In conclusion, this simplified method for freezing testicular tissue resulted in a satisfactory outcome after ICSI in cases of non-obstructive azoospermia.

Keywords: cryopreservation, cumulative clinical pregnancy rate, ICSI, non-obstructive azoospermia, TESE

Introduction

Azoospermia is the cause of about 2% of infertility (Hull *et al.*, 1985) and results from absent sperm production, due to pituitary insufficiency, primary testicular failure or ejaculatory dysfunction (non-obstructive azoospermia), or from obstruction of the genital tract (obstructive azoospermia). Defective spermatogenesis and genital tract obstruction account for 60 and 40% of patients with azoospermia respectively (Matsumiya *et al.*, 1994), while hypogonadotrophic azoospermia and ejaculatory dysfunction are uncommon.

After the first reports of using spermatozoa from testicular tissue derived by testicular biopsy for intracytoplasmic sperm injection (ICSI) (Schoysman *et al.*, 1993; Devroey *et al.*,

1994), this method has become an established technique for assisted reproduction in cases of non-obstructive azoospermia.

The testicular tissue obtained is examined histologically to identify the cause of azoospermia and the recovered spermatozoa may be used immediately for ICSI or the tissue may be cryopreserved for future ICSI procedures. In the second case (Romero *et al.*, 1996; Salzbrunn *et al.*, 1996; Khalifeh *et al.*, 1997), part of this tissue is thawed on the day of oocyte retrieval after ovarian stimulation and the recovered spermatozoa or spermatids are injected in the oocytes. The remainder of the testicular biopsy specimens may be used in the future.

The results obtained by this technique are satisfactory, and so far many babies have been born in cases where this would

otherwise have been impossible (Ben-Yosef *et al.*, 1997; Gordon, 2000; Osmanagaoglu *et al.*, 2003).

Various pieces of automated equipment are used for testicular tissue freezing before storage in liquid nitrogen. The present study describes a simplified and inexpensive method of testicular tissue freezing and assesses the CPR by this technique, providing useful information for counselling couples in cases of non-obstructive azoospermia.

Materials and methods

Patients

Four hundred and thirteen non-obstructive azoospermic males pursuing assisted conception, from December 1995 to December 2002, entered the testicular biopsy programme. In the present study, 165 (40%) patients with successful sperm recovery were included. In all cases, the testicular tissue retrieved by open multiple biopsy testicular sperm extraction (TESE) was cryopreserved and only mature spermatozoa (no early spermatids) were used for ICSI after thawing. Only cycles with embryos available for transfer were included. Five cycles where there was no fertilization (two cases) or cancellation of the ICSI for non-medical reasons (three cases) were excluded. Clinical pregnancy was defined as fetal heart activity in transvaginal ultrasound at 5–6 weeks after embryo transfer.

Retrieval of testicular tissue

Testicular tissue was obtained under local or general anaesthesia using an open multiple-biopsy technique (one cranial and one caudal scrotum incision in each testis). Incision of the tunica albuginea was chosen in an apparently avascular region to avoid testicular vessels, and testicular parenchyma was harvested from one cranial and one caudal site in each testis. Before closure of the tunica albuginea, 2 ml heparinized saline solution was rinsed underneath the tunica to minimize scarification. All patients underwent only one TESE procedure. From each part, a sample was fixed for histopathological examination. Testicular specimens in Ham's F10 medium were examined under $\times 40$ objective of phase-contrast microscopy to confirm the presence of spermatozoa and were subsequently frozen in up to 10 fractions using the following simplified freezing technique.

Freezing method of testicular tissue

The freezing method is a modification of the liquid nitrogen vapour technique first introduced by Sherman (1954) for spermatozoa. Spermatozoa were not extracted before freezing and the tissue in samples the size of a rice grain was frozen. The testicular tissue suspension samples were placed in 2 ml vials (Greiner, Frickenhausen, Germany) containing 0.5 ml HEPES-buffered medium consisting of modified Earle's balanced salt solution with 0.4% human serum albumin and 15% glycerol as a cryoprotectant (Sperm Freeze; Medicult, Hamburg, Germany). The samples with cryoprotectant after incubation for 15 min at room temperature were placed in canes and then suspended on the open top of a styrofoam box filled with liquid nitrogen and left there for 20–30 min (**Figure 1**). Afterwards, they were immersed in liquid nitrogen and stored until the time of ICSI.

Ovarian stimulation

Ovarian stimulation was performed using urinary or recombinant gonadotrophins [Menogon (Ferring Arzneimittel GmbH, Kiel, Germany) or Gonal-F (Serono International S.A., Geneva, Switzerland); Puregon (Organon, Oss, The Netherlands) respectively] and either gonadotrophin-releasing hormone (GnRH) agonists [Enantone Gyn (Takeda Pharma GmbH, Aachen, Germany) or Decapeptyl-Gyn Depot (Ferring Arzneimittel GmbH)] (205 cycles) or antagonists [(Cetrotide (Serono International S.A.) or Orgalutran (Organon)] (90 cycles) for pituitary suppression in various protocols, while in two cycles there was no pituitary suppression. Ovulation was induced by administration of either urinary or recombinant human chorionic gonadotrophin (HCG) [Choragon (Ferring Arzneimittel GmbH) or Ovitrelle (Serono International S.A.) respectively]. Oocyte retrieval was performed by transvaginal ultrasound guided puncture of follicles 36 h after the HCG injection with or without general anaesthesia. For luteal-phase support, progesterone [Utrogest (Dr Kade, Berlin, Germany) or Crinone (Serono International S.A.)] with or without additional HCG was utilized.

Thawing procedure

After oocyte retrieval, testicular tissue samples containing spermatozoa were thawed in a 37°C water bath for 3–5 min and prepared by using only the mechanical method for the ICSI. More specifically, after washing the testicular tissue in two drops of Ham's F-10 medium for a few seconds in order to remove the cryoprotectant, the tissue was minced with a scalpel (1 min of mincing is sufficient) in a centre-well dish. The minced tissue was incubated in 0.5–1 ml of Ham's F-10 medium, added in the centre-well dish, up to 5 h before used for ICSI procedure.

ICSI procedure

ICSI was performed as previously described (Al-Hasani *et al.*, 1995). The supernatant medium was centrifuged for 1–2 min in an Eppendorf tube. One microlitre from each pellet was added to one or two drops of medium under oil to be used for injection. In cases where no motile spermatozoa could be recovered, immotile spermatozoa were injected if they were of normal shape. If at least slight movements of the head or tail were observed, spermatozoa were considered motile which is suggestive of viability.

Statistical analysis

Expected CPR were calculated by life-table analysis using Kaplan–Meier survival analysis (Kaplan and Meier, 1958). Due to the small population size, life-table analysis was confined to the overall group and no analysis was performed for age subgroups, in order to avoid overestimation of the CPR. Furthermore, since the majority of males displayed mixed forms of spermatogenesis defects, life-table analysis was not performed for specific histological subgroups. Likewise, life-table analysis was not performed for different stimulation protocols, different ovulation protocols or different methods of luteal phase support, although these could have influenced pregnancy outcome, especially the luteal phase support. The CPR were expressed as cumulative percentage probabilities with 95% confidence

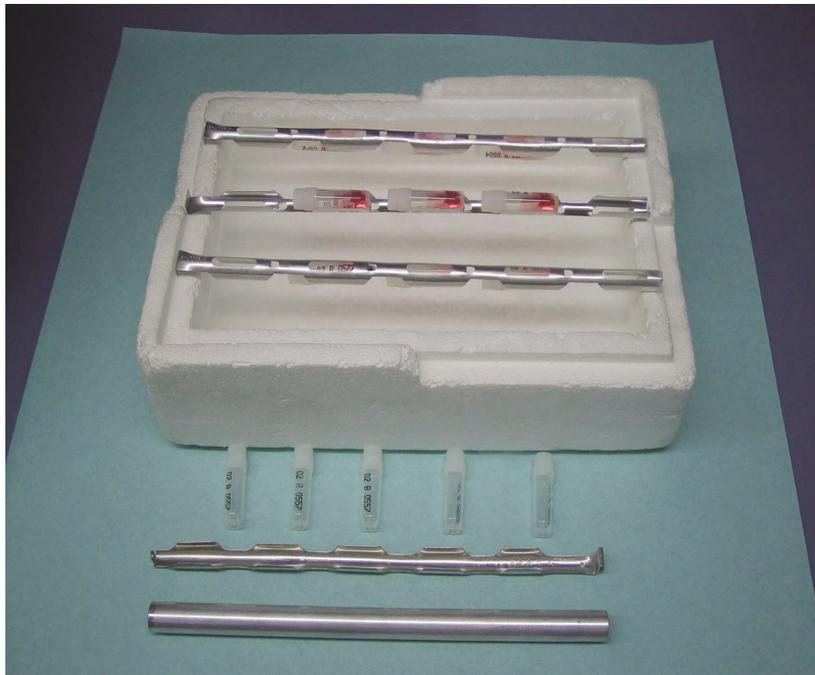


Figure 1. Canes with vials containing testicular tissue samples with cryoprotectant placed on the open top of a styrofoam box filled with liquid nitrogen.

intervals (95% CI). The chi-squared test was also used. Descriptive statistics are presented as mean \pm SEM. The statistical software package used was NCSS 2001 (Number Cruncher Statistical Systems, Kaysville, UT, USA).

Results

The ages (mean \pm SEM) of the male and female partners were 34.2 ± 0.6 and 32.2 ± 0.2 respectively. Histological diagnoses of the testicular biopsies showed some form of spermatogenesis defect, such as germ cell aplasia, maturation arrest, tubular sclerosis and mixed forms in the majority of cases. All patients had a normal karyotype.

A total of 297 cycles in 165 couples were performed, accounting therefore, for 1.8 ± 0.08 cycles performed for each couple (range 1–6). The mean interval between consecutive cycles was 6.4 ± 1.7 months. Eighty-seven couples underwent one, 43 underwent two, 21 underwent three, 11 underwent four, one underwent five and two underwent six cycles.

In 160 (53.8%) cycles motile spermatozoa were injected, while in the rest 137 cycles immotile spermatozoa were used for ICSI. The normal fertilization rate (number of two pronuclei oocytes/number of metaphase II oocytes injected) was $53 \pm 1\%$ and 2.6 ± 0.04 embryos were transferred (range 1–3) with a modified cumulative embryo score (Al-Hasani *et al.*, 1999) of 25.5 ± 0.7 . In particular, in 291 cycles the embryos were transferred on day 2 (758 embryos), while in the remaining six cycles, embryo transfer was performed on day 3 (17 embryos). From a total of 775 embryos 60 (7.7%), 66 (8.5%), 433 (55.9%), 47 (6.1%), 122 (15.7%), 46 (5.9%) and 1 (0.1%) reached the 2-, 3-, 4-, 5-, 6-, 8- and 10-cell stage respectively. From the day 3 embryos, 11 and 6 reached the 8- and 6-cell stage respectively. In

terms of embryo quality grading, according to the degree of fragmentation and regularity of blastomeres (Al-Hasani *et al.*, 1999), from a total of 775 embryos 302 (39%), 330 (42.6%) and 143 (18.5%) were of ideal, good and poor quality respectively. From the day 3 embryos, seven and 10 were of ideal and good quality respectively.

The clinical pregnancy rate per cycle was 20.9% (62/297) and the multiple pregnancy rate (twins) per cycle was 2% (6/297). The clinical pregnancy rate per couple or per TESE was 37.6% (62/165). Fifty-three (32.1%) of the 165 couples achieved at least one clinical pregnancy. In these 53 couples, each couple had an average of 1.17 ± 0.07 clinical pregnancies (range 1–4).

Crude and expected CPR were calculated considering the achievement of the first clinical pregnancy of an individual couple as the final event of the respective couple (281 cycles) and excluding the rest of the performed cycles (16 cycles). Therefore, a total of 165 couples underwent 281 cryo-TESE cycles resulting in 53 clinical pregnancies. Between these couples, 94 underwent one, 42 underwent two, 18 underwent three, eight underwent four, one underwent five and two underwent six cycles. The number of cycles performed per patient was 1.7 ± 0.06 (range 1–6) and the number of cycles required for achieving a clinical pregnancy was 1.5 ± 0.09 per patient (range 1–4). In 150 (53.4%) cycles motile spermatozoa were injected, while in the rest 131 cycles immotile spermatozoa were used for ICSI. The normal fertilization rate was $53 \pm 1\%$ and 2.6 ± 0.04 embryos were transferred (range 1–3) with a modified cumulative embryo score (Al-Hasani *et al.*, 1999) of 25.3 ± 0.7 .

Crude and expected CPR with 95% confidence intervals are presented in **Table 1**.

Table 1. Cumulative clinical pregnancy rates (CPR) after ICSI in non-obstructive azoospermia patients with sperm from cryopreserved testicular tissue.

	Treatment cycle number					
	1	2	3	4	5	6
No. patients	165	71	29	11	3	2
No. clinical pregnancies	32	16	4	1	0	0
No. non-pregnant	133	55	25	10	3	2
No. non-pregnant and discontinued	62	26	14	7	1	2
Dropout rate (%)	62/133 (46.6)	26/55 (47.3)	14/25 (56)	7/10 (70)	1/3 (33.3)	2/2 (100)
Crude CPR (%)	19.4	29.1	31.5	32.1	32.1	32.1
95% CI of crude CPR	14.2–26.5	22.9–36.9	25.2–39.5	25.7–40.1	25.7–40.1	25.7–40.1
Expected CPR (%)	19.4	41.9	55.7	64.8	64.8	64.8
95% CI of expected CPR	12.7–26.1	29–54.9	37–74.4	39–90.6	39–90.6	39–90.6
Clinical pregnancy rate per cycle (%)	32/165 (19.4)	16/71 (22.5)	4/29 (13.8)	1/11 (9.1)	0/3 (0)	0/2 (0)

Thirty-two of the 53 pregnancies (60.4%) were achieved during the first cycle, but there was a high dropout rate during the consecutive cycles. The clinical pregnancy rate per cycle (**Table 1**) did not differ significantly between the first four cycles (19.4 versus 22.5 versus 13.8 versus 9.1%) ($\chi^2 = 1.8$, d.f. = 3, $P = 0.618$), although the test was underpowered (power = 0.17).

Discussion

The successful outcomes after ICSI (Palermo *et al.*, 1992) established the potential of sperm recovery that is indicated for irreversible obstructive azoospermia, primary testicular failure, necrozoospermia and some cases of ejaculatory failure. One of these sperm recovery techniques used in assisted reproduction for azoospermia is TESE.

Spermatozoa recovered by TESE may be used fresh, or can be cryopreserved for future ICSI cycles. Fertilization, implantation and pregnancy rates after ICSI with fresh testicular tissues are similar to those with frozen samples in cases of non-obstructive azoospermia (Friedler *et al.*, 1997; Ben-Yosef *et al.*, 1999). Furthermore, the second option is more convenient, since it excludes the possibility of unfruitful commencement of ovarian stimulation and minimizes the repeats of the TESE procedure (Oates *et al.*, 1997) and its potential adverse local effect on the testis (Tash and Schlegel, 2001). An advantage of the open multiple-biopsy technique is the early detection of testicular neoplasia, which may be as high as 2.5% in males with non-obstructive azoospermia (Küpker *et al.*, 2000; Schultze-Mosgau *et al.*, 2003).

An essential step in testicular tissue cryopreservation is the freezing before plunging into liquid nitrogen. Various expensive apparatuses are used for computer-controlled freezing of testicular tissue. The simplified and inexpensive liquid nitrogen vapour technique originates from experience with sperm freezing and resulted in a satisfactory ICSI outcome, namely, crude and expected CPR of 32.1 and 64.8% respectively after four cycles.

However, it is not claimed that the simplified freezing technique is better than the automated one, since no real analysis on cryodamage and no comparison between the two techniques was performed in the present study. Cryodamage can only be evaluated by comparing initial and post-thawing parameters of viability (motility and hypo-osmotic swelling test on spermatozoa) and structural damage (morphology, DNA maturity, electronic microscopy). Several studies have focused on this. Overall there is a loss between 25 and 75% in viability after cryopreservation. Testicular spermatozoa are more susceptible to cryopreservation compared with ejaculated spermatozoa (Holden *et al.*, 1997; Nijs and Vanderelst, 2000; Nijs and Ombelet, 2001). Steele *et al.* (2000) did not detect any changes in DNA integrity after freezing and thawing of testicular spermatozoa, however. Isolation of testicular spermatozoa from the testicular debris before freezing results in higher survival rates and fertilizing potential after freeze–thawing than freezing of cell suspensions or intact tubuli. (Allan and Cotman, 1997; Crabbe *et al.*, 1999). Freezing of suspensions, intact tubuli or intact testicular biopsies can result in the release of toxic products after cell lysis. No evidence has been found for the protective effect of Sertoli cells present in the cell suspension during the cryopreservation process. Moreover, Sertoli cells have more DNA damage after freezing and thawing and this figure increases after culture *in vitro* (Tesarik *et al.*, 2000).

The present method is therefore an alternative freezing technique resulting in a satisfactory outcome.

After thawing, it is preferable to mince testicular tissue instead of the enzymatic method for its dispersion, since this method is less time consuming with comparable resulting pregnancy rates (Baukloh, 2002). However, in spite of the risk the proteolytic enzymes to modify the sperm membrane proteins that trigger oocyte activation, influencing therefore the embryo quality after ICSI, the optimal method of obtaining spermatozoa is still under debate (Verheyen *et al.*, 1995; Crabbe *et al.*, 1997; Baukloh, 2002).

Counselling of infertile couples should include information about the treatment outcome and success, which is apparently of paramount importance.

Although cumulative clinical pregnancy (Shulman *et al.*, 2002) and delivery rates (Osmanagaoglu *et al.*, 2003) after ICSI with freshly recovered testicular spermatozoa in patients with obstructive and non-obstructive azoospermia have been recently reported, such data are lacking in cases of non-obstructive azoospermia with utilization of cryopreserved testicular spermatozoa.

In the present study, crude and expected CPR were 31.5 and 55.7% after three cycles respectively. Although no reliable conclusion can be drawn for four cycles and beyond due to the small number of cases, the respective figures remaining unchanged thereafter were 32.1 and 64.8%. Life-table analysis is generally believed to give an overestimate in cumulative studies, due to the assumption that the patients who continue and those who quit have the same chances of success (clinical pregnancy) (Stolwijk *et al.*, 2000).

Furthermore, the results showed that the clinical pregnancy rate per cycle did not change significantly for the first four cycles, although in absolute figures there was a decrease. Similar clinical pregnancy rates per cycle were found during the first five cycles in a recent study (Shulman *et al.*, 2002), but in this study fresh testicular spermatozoa were utilized for ICSI and no separate analysis for obstructive and non-obstructive azoospermia was performed. The expected CPR in the present study after six cycles (64.8%) was similar to the respective rate (74.17%) found in the study of Shulman *et al.* (2002). In spite of methodological differences between the study of Shulman *et al.* (2002) and ours, it seems that there are no differences in expected CPR after six cycles whether fresh or frozen testicular tissue is used for treatment of non-obstructive azoospermia. This observation could be added to the benefits of utilization of cryopreserved testicular tissue for non-obstructive azoospermia treatment.

On the other hand, Osmanagaoglou *et al.* (2003) found that after a few cycles, the delivery rate per cycle decreases compared with the first two cycles in patients with obstructive and non-obstructive azoospermia, where fresh testicular spermatozoa were utilized for ICSI. Since in this study delivery rates per cycle have been investigated, these results cannot be properly compared with the present results.

Although 60.4% of the clinical pregnancies were achieved after the first cycle, the consistently high dropout rate and the possible unchanged clinical pregnancy rate per cycle, which resulted in 64.8% expected CPR after four cycles, suggest that there is value in performing at least four cryo-TESE cycles. Economic constraints, psychological factors, denial of further treatment due to job, moving and organizational problems, various medical problems, other treatment options, fears regarding the complications of the procedure, treatment-independent pregnancy and other factors have been identified as reasons for dropout (Land *et al.*, 1997; Osmanagaoglou *et al.*, 1999). In Germany, the first four IVF attempts were paid for by health insurance during the present study period; therefore, the CPR after four cycles is not affected by this leading cause of dropout rate. Besides the aforementioned

factors, another important parameter reducing the dropout rate and subsequently increasing the crude CPR is the availability of testicular spermatozoa for consecutive cycles. All of the patients had undergone one TESE procedure. It is interesting that in this study, the dropout rates for each cycle were lower than the respective rates reported by Osmanagaoglou *et al.* (2003) where fresh testicular spermatozoa were utilized in non-obstructive azoospermia, suggesting that repeating the TESE procedure might contribute to the dropout phenomenon.

Another major issue that should be considered in viewing the present results is the situation in Germany, where the regulations of the embryo protection law (Embryonenschutzgesetz, ESchG) prohibit embryo selection for transfer. It is possible that the CPR achieved here might be even higher after transfer of the qualitatively excellent embryos.

In conclusion, counselling of couples with non-obstructive azoospermia may include the information that they have 55.7 and 64.8% likelihood of clinical pregnancy after three and four ICSI cycles with cryopreserved testicular spermatozoa. It seems that the liquid nitrogen vapour freezing method of testicular tissue obtained from such patients is related to a satisfactory outcome after ICSI, in spite of the restrictive regulations of the embryo protection law in Germany.

References

- Al-Hasani S, K pker W, Baschat AA *et al.* 1995 Mini-swim up: a new technique of sperm preparation for intracytoplasmic sperm injection. *Journal of Assisted Reproduction and Genetics* **12**, 428–433.
- Al-Hasani S, Demirel LC, Schoepper B *et al.* 1999 Pregnancies achieved after frozen-thawed pronuclear oocytes obtained by intracytoplasmic sperm injection with spermatozoa extracted from frozen-thawed testicular tissues from non-obstructive azoospermic men. *Human Reproduction* **14**, 2031–2035.
- Allan JA, Cotman AS 1997 A new method for freezing testicular biopsy sperm: three pregnancies with sperm extracted from cryopreserved sections of seminiferous tubule. *Fertility and Sterility* **68**, 741–744.
- Baukloh V 2002 Retrospective multicentre study on mechanical and enzymatic preparation of fresh and cryopreserved testicular biopsies. *Human Reproduction* **17**, 1788–1794.
- Ben-Yosef D, Yogev L, Hauser R *et al.* 1999 Testicular sperm retrieval and cryopreservation prior to initiating ovarian stimulation as the first line approach in patients with non-obstructive azoospermia. *Human Reproduction* **14**, 1794–1801.
- Crabbe E, Verheyen G, Tournaye H *et al.* 1997 The use of enzymatic procedures to recover testicular germ cells. *Human Reproduction* **12**, 1682–1687.
- Crabbe E, Verheyen G, Tournaye H *et al.* 1999 Freezing of testicular tissue as a minced suspension preserves sperm quality better than whole-biopsy freezing when glycerol is used as cryoprotectant. *International Journal of Andrology* **22**, 43–48.
- Devroey P, Liu J, Nagy Z *et al.* 1994 Normal fertilization of human oocytes after testicular sperm extraction and intracytoplasmic sperm injection (TESE and ICSI). *Fertility and Sterility* **62**, 639–641.
- Friedler S, Raziel A, Soffer Y 1997 Intracytoplasmic injection of fresh and cryopreserved testicular sperm in patients with non-obstructive azoospermia: a comparative study. *Fertility and Sterility* **68**, 892–895.
- Gordon UD 2002 Assisted conception in the azoospermic male. *Human Fertility (Camb)* **5**, S9–S14.
- Holden CA, Fuscaldo GF, Jackson P *et al.* 1997 Frozen-thawed

- epididymal spermatozoa for intracytoplasmic sperm injection. *Fertility and Sterility* **67**, 81–87.
- Hull M, Glazener C, Kelly N *et al.* 1985 Population study of causes, treatment, and outcome of infertility. *British Medical Journal* **291**, 1693–1697.
- Kaplan EL, Meier P 1958 Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association* **5**, 457–481.
- Khalifeh F, Sarraf M, Dabit S 1997 Full-term delivery following intracytoplasmic sperm injection with sperm extracted from frozen–thawed testicular tissue. *Human Reproduction* **12**, 87–88.
- Küpker W, Schlegel PN, Al-Hasani S *et al.* 2000 Use of frozen–thawed testicular sperm for intracytoplasmic sperm injection. *Fertility and Sterility* **73**, 453–458.
- Land J, Courtar D, Evers J 1997 Patient dropout in an assisted reproductive technology program: implications for pregnancy rates. *Fertility and Sterility* **68**, 278–281.
- Matsumiya K, Namiki M, Takahara S *et al.* 1994 Clinical study of azoospermia. *International Journal of Andrology* **17**, 140–142.
- Nijs M, Ombelet W 2001 Cryopreservation of human sperm. *Human Fertility (Camb)* **4**, 158–163.
- Nijs M, Van Der Elst J 2000 Biological aspects of testicular sperm extraction. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **92**, 1–6.
- Oates RD, Mulhall J, Burgess C *et al.* 1997 Fertilization and pregnancy using intentionally cryopreserved testicular tissue as the sperm source for intracytoplasmic sperm injection in 10 men with non-obstructive azoospermia. *Human Reproduction* **12**, 734–739.
- Osmanagaoglu K, Tournaye H, Camus M *et al.* 1999 Cumulative delivery rates after intracytoplasmic sperm injection: 5 year follow-up of 498 patients. *Human Reproduction* **14**, 2651–2655.
- Osmanagaoglu K, Vernaev V, Kolibianakis E *et al.* 2003 Cumulative delivery rates after ICSI treatment cycles with freshly retrieved testicular sperm: a 7-year follow-up study. *Human Reproduction* **18**, 1836–1840.
- Palermo G, Joris H, Devroey P *et al.* 1992 Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* **340**, 17–18.
- Romero J, Remohi J, Minguez Y *et al.* 1996 Fertilization after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. *Fertility and Sterility* **65**, 877–879.
- Salzbrunn A, Benson D, Holstein A *et al.* 1996 A new concept for the extraction of testicular spermatozoa as a tool for assisted fertilization (ICSI). *Human Reproduction* **11**, 752–755.
- Schoysman R, Vanderzwalmen P, Nijs M *et al.* 1993 Pregnancy after fertilisation with human testicular spermatozoa. *Lancet* **342**, 1237.
- Schultze-Mosgau A, Ludwig M, Schulze B *et al.* 2003 Incidence of neoplasia in male patients undergoing testicular sperm extraction. 19th Annual Meeting of ESHRE, Madrid.
- Sherman J 1954 Freezing and freeze–drying of human spermatozoa. *Fertility and Sterility* **5**, 357.
- Shulman A, Menashe K, Laor A *et al.* 2002 Cumulative pregnancy rate following IVF and intracytoplasmic sperm injection with ejaculated and testicular spermatozoa. *Reproductive BioMedicine Online* **4**, 151–156.
- Steele E, McClure N, Lewis S 2000 Comparison of the effects of two methods of cryopreservation on testicular sperm DNA. *Fertility and Sterility* **74**, 450–453.
- Stolwijk A, Wetzels A, Braat D 2000 Cumulative probability of achieving an ongoing pregnancy after in-vitro fertilization and intracytoplasmic sperm injection according to a woman's age, subfertility diagnosis and primary or secondary subfertility. *Human Reproduction* **15**, 203–209.
- Tash J, Schlegel P 2001 Histologic effects of testicular sperm extraction on the testicle in men with nonobstructive azoospermia. *Urology* **57**, 334–337.
- Tesarik J, Mendoza C, Anniballo R *et al.* 2000 In-vitro differentiation of germ cells from frozen testicular biopsy specimens. *Human Reproduction* **15**, 1713–1716.
- Verheyen G, De Croo I, Tournaye H *et al.* 1995 Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. *Human Reproduction* **10**, 2956–2959.

Received 31 August 2004; refereed 10 September 2004; accepted 6 January 2005.