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Should diagnostic testicular sperm retrieval followed by cryopreservation for later ICSI be the procedure of choice for all patients with non-obstructive azoospermia?

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BACKGROUND: This was a retrospective study to determine if diagnostic testicular biopsy followed by cryopreservation should be the procedure of choice for all patients with testicular failure. METHODS: The first part of the study analysed 97 ICSI cycles scheduled with frozen–thawed testicular sperm for 69 non-obstructive azoospermia (NOA) patients. The second part focused on a subgroup of 32 patients who underwent 42 ICSI cycles with frozen and 44 cycles with fresh testicular sperm. Sperm characteristics, fertilization, embryo quality, pregnancy and implantation rates were evaluated. RESULTS: Part I: The average time needed to find sperm was 113 min per cycle and 17 min per individual sperm. Fertilization rate, embryo transfer rate, ongoing pregnancy and implantation rates were 58.4%, 83%, 20.8% and 11.3%, respectively. Part II: The search time per sperm was higher ($P = 0.016$) in frozen (18 min) than in fresh suspensions (13 min). A higher embryo transfer rate was observed in fresh cycles than in frozen cycles (93.2% vs 76.2%, $P = 0.028$). Fertilization, ongoing pregnancy and implantation rates were comparable for the two groups. CONCLUSIONS: Even in a programme with low-restrictive criteria for patient allocation and for sperm cryopreservation, diagnostic testicular biopsy followed by cryopreservation can be the procedure of choice for patients with testicular failure.

Key words: cryopreservation/ICSI/non-obstructive azoospermia/pregnancies/testicular sperm

Introduction

Since its introduction in 1993 (Craft *et al.*, 1993; Schoysman *et al.*, 1993), intracytoplasmic injection with testicular sperm has become a routine treatment procedure for patients with azoospermia, whether they suffer from obstructive azoospermia with normal spermatogenesis (OA) or non-obstructive azoospermia with testicular failure (NOA). High fertilization rates, pregnancy rates and implantation rates are obtained in obstructive patients (Devroey *et al.*, 1994, 1996; Nagy *et al.*, 1995; Silber *et al.*, 1995; Kahraman *et al.*, 1996; Fahmy *et al.*, 1997; Mansour *et al.*, 1997; De Croo *et al.*, 2000), and sperm can be retrieved in almost 100% of the cases (Tournaye *et al.*, 1997a). In the population of patients with NOA, however, the probability of finding sperm is only ~50% in a non-selected population (Tournaye *et al.*, 1997a, b), but fertilization and pregnancy rates can reach acceptable levels (Devroey *et al.*, 1996; Silber *et al.*, 1996; Schlegel *et al.*, 1997; Madgar *et al.*, 1998; Palermo *et al.*, 1999; Balaban *et al.*, 2001; Bukulmez *et al.*, 2001; Sousa *et al.*, 2002; Windt *et al.*, 2002). Vernaeve *et al.* (2003) demonstrated significantly lower fertilization and implantation rates in non-obstructive (306 cycles) compared to obstructive azoospermia (605 cycles). No reliable clinical, pre-operative parameter can predict the presence of sperm in the testis. Only testicular histology has been shown to have a clinical value in predicting sperm recovery during TESE (Mulhall *et al.*, 1997; Tournaye *et al.*, 1997a; Ezeh *et al.*, 1999; Schulze *et al.*, 1999; Su *et al.*, 1999; Seo and Ko, 2001). However, the recovery of fresh testicular biopsies at the day of oocyte retrieval is highly stressful for the couple as it implies a 50% risk of pointless ovarian stimulation of the female partner. Moreover, repeated testicular surgery in subsequent ICSI cycles may cause testicular devascularization and possibly permanent injury (Schlegel and Su, 1997; Ron-el *et al.*, 1998).

Preliminary diagnostic biopsy retrieval combined with cryopreservation of the tissue may overcome these problems. After its introduction for ICSI (Romero *et al.*, 1996), several groups reported comparable results in terms of fertilization, embryo quality, pregnancy and implantation rates with the use of fresh and frozen testicular sperm for patients with obstructive azoospermia (Prins *et al.*, 1999; Gil-Salom *et al.*, 2000; Wood *et al.*, 2002). The first reports on the use of frozen–thawed testicular sperm in non-obstructive patients were case reports (Romero *et al.*, 1996; Perraguin-Jayot *et al.*, 1997) or present data on a mixed population of mainly OA

and some NOA patients (Gil-Salom *et al.*, 1996; De Croo *et al.*, 1998; Palermo *et al.*, 1999; Huang *et al.*, 2000; Windt *et al.*, 2002). An additional problem in these early publications is the definition of testicular failure (NOA), which was not always clearly described and/or not always based on testicular histology. Nevertheless, acceptable results of ICSI with frozen–thawed testicular sperm of NOA patients have been described (Friedler *et al.*, 1997; Oates *et al.*, 1997; Ben-Yosef *et al.*, 1999; Habermann *et al.*, 2000; Kupker *et al.*, 2000; Sousa *et al.*, 2002).

In contrast to OA patients, however, where viable sperm can easily be retrieved from the frozen specimens, the impaired quality of the testicular tissue of NOA patients does not allow for cryopreservation and later use for ICSI in all cases. As has been demonstrated for ejaculated sperm, a significant decrease in sperm motility and viability by freezing and thawing also occurred for testicular sperm (Verheyen *et al.*, 1997). This implies that cases with extremely low numbers of sperm retrieved can hardly be considered candidates for cryopreservation. Preliminary diagnostic surgery and freezing can therefore still be considered a controversial approach for them. There is indeed a high chance that sperm may not be found at diagnostic retrieval due to its complete absence or to limited tissue retrieval and/or efforts for sperm searching. In order to overcome the risk that the frozen material is inadequate for injection upon thawing, some IVF centres define limits for testicular sperm quality suitable for freezing, and others only allocate patients for treatment on the basis of sufficient quality (motility) of a preliminary-thawed testicular sperm fraction. In this way, patient populations with NOA may differ greatly from one IVF clinic to another, which may define the results of ICSI.

In our IVF clinic, we offer couples with non-obstructive azoospermia any chance for having their own genetic offspring. While, historically, the use of freshly retrieved sperm has been our first-line approach, diagnostic surgery combined with cryopreservation is also offered. Couples are carefully counselled about the pros and cons of both strategies.

The aim of this retrospective study was to analyse the efficiency of the use of frozen–thawed testicular sperm from NOA patients in an IVF programme with low-restrictive criteria for treatment allocation and for cryopreservation of diagnostically retrieved sperm. The first part analysed the overall results of ICSI cycles planned with frozen–thawed sperm. The second part studied a subgroup of patients who underwent both cycles with frozen and cycles with fresh testicular sperm.

Materials and methods

Patient population

This retrospective study included 97 ICSI cycles scheduled with the use of frozen–thawed testicular sperm of NOA patients between January 1998 and December 2002. Sixty-nine male patients with non-obstructive azoospermia (mean male age 35.1, range 21–66) were included. Azoospermia was confirmed on at least two diagnostic semen samples after centrifugation at 1800 *g* and exploration of the entire pellet. All patients had a clinical work-up including a physical examination, hormonal assessment (FSH, LH and testosterone) and measurement of biochemical markers in seminal plasma. Transrectal ultrasound, karyotype analysis, assessment for Yq microdeletion, and analysis of the most frequent CFTR gene mutations were performed whenever indicated. Klinefelter patients were also included in the study. Preimplantation genetic diagnosis (PGD) by fluorescent *in situ* hybridization (FISH) was carried out on their embryos. Other cycles with frozen testicular sperm of non-Klinefelter NOA patients in which diagnostic aneuploidy screening had been carried out were excluded from the present study.

For all patients, the diagnosis of testicular failure was based on the clinical findings and on the histological examination of the testicular biopsy. Patients with germ-cell aplasia (Sertoli-cell only syndrome), maturation arrest or tubular sclerosis/atrophy, either with or without focal spermatogenesis on histology were included in the study. Patients with hypospermatogenesis were excluded.

Out of the 69 patients, 37 patients underwent only treatments with frozen testicular sperm while a population of 32 patients (mean male age 36.5, range 24–55) underwent ICSI cycles with frozen testicular sperm (42 cycles) as well as ICSI cycles with fresh testicular sperm (44 cycles). Some of them first had a fresh TESE cycle followed by a frozen TESE cycle, or vice versa. For those 32 patients, the results of ICSI with fresh testicular sperm and of ICSI with frozen testicular sperm were compared in a second part of the study.

At the start of the treatment, the mean age of the female patients was 32.0 (range 21–44) in the first part of the study. In part II of the study, the mean female age was 32.0 (range 21–45) in the fresh TESE cycles and 32.8 (range 22–45) in the frozen TESE cycles.

All couples were counselled about the procedure and signed an informed consent for their treatment.

Testicular sperm recovery

In patients with a clinical diagnosis of non-obstructive azoospermia, open excisional testicular biopsy retrieval was carried out under general anesthesia or occasionally under locoregional anesthesia, as previously described (Tournaye *et al.*, 1997b). After excision, testicular biopsy specimens were placed in a Petri dish with HEPES-buffered culture medium. Concomitant with the surgery, testicular biopsies were processed by mechanical shredding (Verheyen *et al.*, 1995) and the suspension was explored under an inverted microscope at 400× magnification. Whenever appropriate, additional biopsies from different regions of the testis were retrieved bilaterally. The surgery was discontinued when few, preferably motile, sperm had been observed or when a representative number of small biopsies had been excised randomly from both testes or when testicular volume did not allow for further excision. When no or only immotile spermatozoa were observed after mechanical shredding of all retrieved biopsies, lysis of the red blood cells (Verheyen *et al.*, 1995; Nagy *et al.*, 1997) and/or enzymatic digestion of the remaining tissue fractions with collagenase type IV (Crabbé *et al.*, 1997, 1998) was carried out. During surgery, a single small biopsy of each testis was sent for histological examination.

Testicular biopsies were retrieved either for diagnostic reasons ($n = 37$ patients) followed by freezing and later ICSI cycles with frozen–thawed testicular sperm, or for immediate fresh therapeutic use (ICSI) at the day of oocyte retrieval ($n = 32$ patients) and followed by ICSI cycles with frozen–thawed testicular sperm. The testicular cell suspension was frozen for later use, if at least one, preferably motile, sperm was observed after diagnostic retrieval or if, after injection of the mature oocytes at the day of biopsy retrieval, sufficient remaining spermatozoa were supposed to be available for a next ICSI treatment.

Preparation and cryopreservation of testicular suspensions

Testicular biopsies, either treated by mechanical methods or additionally by enzymatic methods, were cryopreserved if appropriate. The quality of the material was assessed for concentration and motility, if possible. The information obtained was mostly based on very few spermatozoa observed. The suspensions were prepared for freezing by dropwise dilution with Test-Yolk buffer (TYB) containing glycerol as cryoprotectant (Irvine Scientific, Santa Anna, CA), in a proportion 1:1 (v/v). The straws (Instruments de Médecine Véterinaire IMV, 61300 L'Aigle, France, or CBS high security straws, Cryo Bio System, L'Aigle, France) were loaded and equilibrated for 10 min in a 37°C water bath. The straws were rapidly frozen in liquid nitrogen vapour as follows: straws were horizontally placed at ~20 cm above the liquid nitrogen surface for 8 min, followed by 8 min at 10 cm above the surface. After this two-step procedure, the straws were finally immersed and stored in liquid nitrogen.

Thawing and preparation of testicular suspensions

At the day of oocyte retrieval, testicular suspensions were thawed only when mature metaphase II oocytes were available for injection. Thawing was performed at room temperature for 5–10 min. According to the quality of the fractions before freezing, only one or immediately more straws were thawed. The thawed suspension was washed by dropwise dilution with 5 ml Hepes-buffered medium. During addition, the tube was shaken in order to continuously obtain an homogeneous suspension. The mixture was centrifuged at 750 *g* for 5 min and the supernatant was discarded. After a second washing step, the remaining pellet was resuspended. Droplets of 10 μl were smeared at the bottom of a Petri dish and overlayed with 3.5 ml of paraffin oil. Individual spermatozoa were identified and selected on the basis of (twitching) motility and appropriate morphology, whenever possible. If no selection was possible due to an extremely low number of sperm present, also morphologically subnormal sperm and in some cases even immotile sperm were used for injection. Searching for sperm was mostly carried out by two or three technicians/embryologists and the duration of this procedure was registered. Whenever necessary, additional straw(s) were thawed in order to find motile sperm for injection. If no sperm at all were found after thawing and exploring all the frozen material, or occasionally if only immotile sperm were found after extensive search, a fresh testicular biopsy retrieval was carried out after consulting the patient. If the patient refused new surgery or if repeated surgery was not indicated, donor sperm was used as anticipated from a preliminary counselling, or the treatment was cancelled. Considering the maturity of the injected sperm, only morphologically mature-looking spermatozoa or elongated spermatids with a fully developed tail but a cytoplasmic droplet were injected. Round spermatids were never used for ICSI.

Ovarian stimulation, oocyte retrieval and ICSI

Female patients underwent ovarian stimulation using urinary or recombinant FSH in combination with GnRH antagonists or agonists. Oocyte retrieval was carried out 36 h after injection of 10 000 IU human chorionic gonadotropin (hCG) by vaginal ultrasound-guided puncture of the ovarian follicles. Denudation of the cumulus cells was carried out by exposure to 10 IU of hyaluronidase (Van de Velde *et al.*, 1997). Nuclear maturation of the oocytes was inspected under a stereomicroscope. Only mature, metaphase II oocytes were used for ICSI. Searching for sperm and intracytoplasmic sperm injection were started shortly after thawing and preparation of the testicular suspensions. The procedure of intracytoplasmic sperm injection has been described previously (Joris *et al.*, 1998). Individual culture of the injected oocytes and embryos was performed in 25-μl droplets under paraffin oil during the entire culture period.

Fertilization, embryo evaluation and embryo transfer

Survival and fertilization of the oocytes were observed 16–18 h after injection under an inverted microscope at 400× magnification. Normal fertilization was confirmed by the presence of two distinct pronuclei (PN) with two distinct or fragmented polar bodies. Fertilization rates were expressed as the percentage of 2PN per injected metaphase II oocyte. Embryo development and quality were assessed daily until the moment of intrauterine transfer. Embryo quality was scored according to the number of blastomeres and the rate of fragmentation. Type A embryos had no anucleate fragments, type B embryos showed ≤20% fragments, type C embryos >20% and ≤50% fragments, and type D embryos >50% fragments. Only the results of embryo quality at day 2 are presented in this study.

Normal cleaving embryos up to type C for fragmentation were considered eligible for transfer. Intrauterine transfer was carried out at day 2 or 3, and occasionally at day 5 after injection.

A rise in serum hCG on two consecutive occasions from 11 days on after embryo transfer indicated pregnancy. A clinical pregnancy was defined by the presence of a gestational sac with fetal heart beat at ultrasonography after ~7 weeks of pregnancy. An ongoing clinical pregnancy was defined as a clinical pregnancy with fetal heartbeat beyond 20 weeks of pregnancy. The implantation rate is considered as the percentage of fetal sacs with heart beat on the number of embryos transferred.

Statistical analysis

Comparison of parameters between the pregnant and non-pregnant patients in the first part of the study was performed using the Mann–Whitney *U* test.

In the second part of the study on ICSI cycles with fresh and ICSI cycles with frozen testicular sperm in the same patients, comparison of sperm parameters, fertilization and embryo quality was performed using the Mann–Whitney U test. Data on embryo transfer, pregnancy and implantation rates were analyzed using the Chi-square test. Statistical tests were performed at the 5% level of significance.

Results

Part I: Results of ICSI with frozen–thawed testicular sperm in the overall NOA patient group (69 patients)

Sperm recovery after thawing

For 69 patients, 97 cycles were planned for ICSI with frozen–thawed testicular spermatozoa. The histological findings in the biopsy specimens were germ-cell aplasia (Sertoli cell-only syndrome) for 34 patients, maturation arrest for 18 patients and tubular sclerosis or atrophy for 8 patients. Nine patients with Klinefelter syndrome on karyotype analysis showed germ-cell aplasia or tubular sclerosis/atrophy. Frozen–thawed testicular sperm were found and used for ICSI in 77 out of the 97 scheduled cycles (79.4%). The frozen–thawed suspensions could not be used in 20 out of the 97 cycles (20.6%) despite extensive search for (motile) sperm. In 14 of these cycles, a fresh biopsy retrieval, planned as back-up procedure, was carried out at the day of oocyte retrieval. Donor sperm was used in five cases, as agreed at

the preliminary patient counselling in the period preceding the treatment. The treatment was completely cancelled in one case.

The proportion of cycles with successful sperm recovery after thawing was comparable for the different histological subgroups: in cycles of patients with germ-cell aplasia, frozen–thawed sperm could be used for injection in 41 of the 52 cycles (79%). In 16 of the 20 cycles of patients with maturation arrest (80%), and in 10 out of 12 cycles of patients with sclerosis/atrophy (83%), frozen–thawed testicular sperm could be injected. Sperm retrieval from the frozen–thawed suspensions of patients with Klinefelter syndrome was successful in 10 out of the 13 cycles (77%).

In the 77 cycles with frozen–thawed sperm used for ICSI, the average time needed per cycle to find the sperm was 113 min (range 3–384), and the average time per sperm was 17 min (range 2–85). In 63 cycles (82%), only motile sperm were injected, while only immotile sperm were used in six cycles (8%). In the eight remaining cyles (10%), insufficient motile sperm were found to inject all mature oocytes after extensive search, so that part of the oocytes were injected with immotile sperm. Overall, 87% of all mature oocytes were injected with a motile sperm. In all 41 cycles of patients with germ-cell aplasia, and in all 10 cycles of patients with sclerosis/atrophy, motile sperm were found to inject almost all the oocytes. The six cycles with only immotile sperm available for injection were carried out for patients with maturation arrest (4/16 cycles) and for patients with Klinefelter syndrome (2/10 cycles).

Fertilization and embryo development

The number of oocytes retrieved and the results of fertilization are presented in Table I. The mean values presented are the averages of the percentages of fertilization calculated per cycle. In the present study with frozen testicular sperm, the mean fertilization rate was 58.4% in 77 cycles. In six out of the 77 cycles (8%), none of the oocytes showed normal fertilization. The cycle characteristics that probably contributed to complete fertilization failure were: low number (1–4) of oocytes retrieved (three cycles), injection with only immotile sperm (three cycles), injection with a high proportion of immotile sperm (one cycle), injection of a proportion (5/18) of the M-II oocytes (one cycle) only. For six treatment cycles (8%) in which only immotile sperm were injected, total fertilization failure was observed in three cycles (with low oocyte numbers: 1, 1, 4). In three other cycles, however, fertilization was obtained but oocytes did not cleave in two of the cycles.

Table I. Number of oocytes and results of fertilization after ICSI with frozen testicular sperm of non-obstructive azoospermia (NOA) patients (mean ± SD)

	Frozen TESE–NOA
Cycles	77
COC/cycle	9.4 ± 5.0
Metaphase II/cycle	8.0 ± 4.3
% 2PN[a]	58.4 ± 26.3
% 1PN[a]	6.3 ± 15.6
% ≥3PN[a]	2.4 ± 5.2

COC, cumulus oocyte complex.
[a]The presented value is the average of the percentages of fertilization calculated per cycle.

Table II. Results of embryo development and fragmentation after ICSI with frozen testicular sperm of non-obstructive azoospermia (NOA) patients (mean ± SD)

	Frozen TESE–NOA
Embryo development (day 2)	
% 2-cell embryos	13.3 ± 19.7
% 3–4-cell embryos	59.6 ± 31.6
% 5–8 cell embryos	18.8 ± 24.4
% uncleaved	6.7 ± 16.6
Embryo fragmentation (day 2)	
% type A	14.5 ± 23.5
% type B	66.0 ± 29.4
% type C	7.5 ± 14.8
% A + B + C	89.3 ± 20.9

The results of embryo development and fragmentation rate on day 2 are presented in Table II. In 64 of the 77 treatment cycles (83%), embryos were available for intrauterine transfer. The reasons for having no embryos available for transfer in 13 cycles were: no fertilization (six cycles), no cleavage of fertilized oocytes (six cycles), and only aneuploid embryos available in a Klinefelter case (one cycle).

Pregnancy and implantation rates

Data on pregnancy and implantation rates are summarized in Table III. The positive hCG rate per cycle with frozen sperm was 28.6% and 34.4% per cycle with embryo transfer. The clinical pregnancy rate was 20.8% per cycle (16/77) and 25.0% per transfer (16/64). Six pregnancies were not ongoing: one biochemical pregnancy, four early miscarriages and one extra-uterine pregnancy. The 16 ongoing pregnancies resulted in 14 live-born deliveries, 13 singletons and one twin. One pregnancy ended in a still-born delivery, and one was terminated after prenatal diagnosis because of a trisomy 18.

When subdivided according to the histological pattern in the testicular tissue, most pregnancies resulting in live-born children were obtained in the category of patients with germ-cell aplasia (8/34 patients or 23.5%) and in the category of sclerosis/atrophy (3/8 or 37.5%). Only one child was born (1/18 or 5.6%) in the subgroup of patients with maturation

Table III. Results of pregnancy and implantation rates after ICSI with frozen testicular sperm of non-obstructive azoospermia (NOA) patients

	Frozen TESE–NOA
Pos hCG per cycle (%)	22/77 (28.6)
per ET (%)	22/64 (34.4)
Clinical PR per cycle (%)	16/77 (20.8)
per ET (%)	16/64 (25.0)
Implantation (FHB/replaced embryo)	17/150 (11.3)
Deliveries	14 (13 singletons + 1 twin)

Pos, positive; ET, embryo transfer; PR, pregnancy rate; FHB, fetal heart beat.

Table IV. Sperm concentration and percentage motility for different subgroups of cycles

	Concentration (millions/ml)	Motility (%)	Cycles with only immotile sperm injected (%)
Cycles with frozen suspensions not used	0.0042[a,b] ± 0.0061	15 ± 15	8/18 (44%)
Cycles with frozen sperm injected	0.0565[a] ± 0.0860	17 ± 19	15/66 (23%)
Cycles resulting in a clinical pregnancy	0.0332[b] ± 0.0863	20 ± 18	5/15 (33%)

[a] $P = 0.034$, Mann–Whitney test.
[b] $P < 0.001$, Mann–Whitney test.

arrest and one child in the subgroup of patients with Klinefelter syndrome (1/9 or 11.1%).

Association between sperm characteristics and outcome parameters

In order to assess any parameter at the level of the IVF laboratory that could be associated with pregnancy, characteristics of the pregnant cycles and non-pregnant cycles were compared. The total duration of searching for sperm—a parameter that reflects the ability of sperm selection for ICSI and indirectly also the quality of the injected sperm—was comparable between the pregnant and non-pregnant group (112 min vs 103 min). Similarly, the mean search time per spermatozoon (15 min vs 14 min) and the proportion of oocytes injected with motile sperm (93% vs 96%) showed no difference. Also fertilization rate (64.3% vs 64.9%), proportion of good-quality embryos (98% vs 91%) and number of embryos transferred (2.5 vs 2.4) did not differ between the pregnant and non-pregnant groups.

For most of the scheduled cycles with frozen–thawed testicular sperm, the pre-freeze quality characteristics of the testicular suspensions could be registered. In order to check for possible relationships, a comparison in sperm characteristics was made between cycles in which the frozen–thawed suspension could not be used for ICSI, cycles in which the frozen–thawed sperm could be used for injection, and out of these the subgroup of cycles that resulted in a clinical pregnancy. It should be taken into account that the characteristics of sperm quality are often based on the observation of a very limited number of sperm (often <10) and should therefore be considered as rough estimates rather than quantitative measurements. Results of concentration and motility are presented in Table IV. The cycles where no frozen–thawed sperm was used for injection were characterized by a significantly lower sperm concentration (0.0042 millions/ml) before freezing than in the cycles with frozen sperm injected (0.0332 millions/ml) and in the cycles that resulted in a clinical pregnancy (0.0565 millions/ml). The percentage motility, however, was similar in the three groups.

Part II: Results of ICSI in a subgroup of NOA patients (32 patients) who underwent cycles with fresh and with frozen–thawed testicular sperm

Thirty-two patients underwent ICSI cycles with fresh testicular sperm (44 cycles) as well as ICSI cycles with frozen testicular sperm (42 cycles). Among them, there were 18 with germ-cell aplasia, 9 with maturation arrest and 3 with sclerosis/atrophy. Two Klinefelter patients also underwent ICSI cycles with fresh and with frozen testicular sperm.

The rank of trial was 1.5 for the cycles with fresh and 2.2 for the cycles with frozen sperm ($P < 0.001$ in the Mann–Whitney U test). The characteristics of the cycles considering the sperm searching procedure are summarized in Table V. The data show that sperm suitable for injection can be found more rapidly in the fresh than in the frozen specimens. The proportions of cycles and the proportion of oocytes injected with motile sperm, however, were comparable.

Table V also presents the number of oocytes retrieved and the results of fertilization. For none of these parameters were differences observed between the cycles performed with fresh and the cycles with frozen testicular sperm for the same patients. Only one failed fertilization (1/44 or 2.3%) occurred in the fresh TESE arm, while three (3/42 or 7.1%) occurred in the frozen TESE arm. Embryo quality characteristics were comparable between the cycles with fresh or frozen testicular sperm (data not shown).

Table VI shows the results of embryo transfer, pregnancy rates and implantation rates. When fresh testicular sperm was

Table V. Comparison of sperm characteristics in the ICSI cycles with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 non-obstructive azoospermia (NOA) patients

	Fresh TESE	Frozen TESE	Mann–Whitney
Cycles	44	42	
Search time/cycle (min)	81	110	$P = 0.053$
Search time/sperm (min)	13	18	$P = 0.016$
% oocytes injected with motile sperm	82.3	83.7	NS
Cycles injected with only motile sperm (%)	33/44 (75)	31/42 (74)	NS[a]
Cycles injected with only immotile sperm (%)	3/44 (7)	4/42 (10)	NS[a]
COC/cycle	10.5 ± 6.2	9.3 ± 5.2	NS
Metaphase II/cycle	9.1 ± 5.8	7.6 ± 4.2	NS
% 2PN	58.0 ± 24.2	59.3 ± 25.5	NS
% 1PN	7.0 ± 11.0	7.8 ± 19.2	NS
% ≥ 3PN	3.6 ± 8.3	1.9 ± 4.9	NS

[a] Chi-square test.

Table VI. Results of embryo transfer, pregnancy and implantation rates after ICSI with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 non-obstructive azoospermia (NOA) patients

	Fresh TESE	Frozen TESE	Chi-square
Cycles	44	42	
Transfers (%)	41 (93.2)	32 (76.2)	$P = 0.028$
Embryos/ET	2.6	2.5	NS
Pos hCG/cycle (%)	9/44 (20.4)	8/42 (19.0)	NS
Pos hCG/ET (%)	9/41 (21.9)	8/32 (25.0)	NS
Clinical PR/cycle (%)	7/44 (15.9)	6/42 (14.3)	NS
Clinical PR/ET (%)	7/41 (17.1)	6/32 (18.7)	NS
Implantation rate (%)	8/105 (7.6)	6/81 (7.4)	NS

ET, embryo transfer; Pos, positive; PR, pregnancy rate.

used, significantly more cycles ($P = 0.028$ in the Chi-square test) resulted in embryo transfer than with frozen testicular sperm (93.2% vs 76.2%). The reasons for having no embryos available for transfer in cycles with fresh and frozen sperm were: no fertilization (one vs three cycles), no or poor cleavage (two vs six cycles) and only aneuploid embryos (no vs one cycle). The rates of positive hCG and the ongoing clinical pregnancy rates were comparable with the use of fresh and frozen sperm. Similarly, no differences were observed in the implantation rates.

Discussion

The advantages of cryopreserving testicular sperm for ICSI are well known. After reporting the first ICSI trials with frozen testicular sperm (Hovatta *et al.*, 1996; Romero *et al.*, 1996), cryopreservation and successful microinjection of testicular sperm has become routine in patients with obstructive azoospermia. Preliminary cryopreservation as a routine procedure for non-obstructive cases, however, remains a controversial issue and the limits of this strategy have never been defined. Attitudes may differ highly between IVF centres, ranging from very strict criteria for freezing and therefore a strong selection of patients allocated to ICSI with frozen sperm, to exclusion for treatment of only those cases where no sperm is observed after an extensive search. It is evident that the outcome of ICSI strongly depends on these criteria for patient acceptance. Within our attitude of offering the couple the maximal chance of obtaining their genetically own offspring, we applied poorly restrictive criteria towards the quality of the biopsies considered eligible for freezing. Testicular suspensions from diagnostic retrieval were frozen if one sperm, motile or immotile, was observed, either after mechanical treatment (Verheyen *et al.*, 1995), or after enzymatic digestion (Crabbé *et al.*, 1997). Preliminary thawing of a frozen fraction for diagnostic reasons was never performed, in order not to waste valuable genetic material. Immotile sperm were injected only if no motile cells were available after extensive search, or if a fresh biopsy was impossible or undesirable. A fresh biopsy retrieval was, however, mostly scheduled as back-up procedure. Most groups do not describe their criteria for allocation of NOA patients, either for ICSI with fresh or with frozen testicular sperm. This makes it difficult to interpret the results of comparative studies between OA and NOA patients, or between the use of fresh and frozen sperm of NOA patients, which may explain discrepancies in the literature. Considering our current attitude for patient admission, the results of ICSI with frozen testicular sperm in the present study were acceptable and comparable with those reported in the literature. Once sperm could be retrieved, high fertilization rates per injected oocyte were achieved. The 2PN rate (58.4%) was comparable or slightly higher than the rate obtained with frozen sperm by Oates *et al.* (1997; 48% in 19 cycles), Friedler *et al.* (1997; 44% in 9 cycles), Gianaroli *et al.* (1999; 64% in 26 cycles), Kupker *et al.* (2000; 45% in 135 cycles), and in the multicentre study by Baukloh (2002; 48%). Fertilization rate even shows the tendency to be higher than in the study by Vernaeve *et al.* (2003) on 306 cycles with fresh testicular sperm (48.5%), although these data were generated in the same centre and using the same procedures. Complete fertilization failure in 8% of the cycles was mostly associated with a low oocyte number and/or the use of immotile sperm for injection. Embryo quality in terms of cleavage stage and fragmentation at day 2 were within the expectations and similar to the observations with ejaculated sperm (Nagy *et al.*, 1995). Pregnancy rates achieved in the present series of cycles were also comparable to the values reported in the literature. Although the implantation rate of 11.3% (in 77 cycles) is limited, it bears comparison with the values achieved in other groups (8–11% by Friedler *et al.*, 1997; Oates *et al.*, 1997; Ben-Yosef *et al.*, 1999).

One drawback associated with using frozen sperm for NOA patients within our current attitude is the substantial risk (~20%) of not finding sperm suitable for injection, despite extensive efforts. It is evident that this observation is linked to our unrestrictive criteria for testicular sperm freezing. Unfortunately, information on the probability of using the frozen testicular suspensions for ICSI is hardly presented in the literature. On the one hand, sufficient numbers of motile sperm may have been ensured either before freezing, or after diagnostic thawing of a fraction before starting treatment in some studies. On the other hand, the cycles in which no cryopreserved sperm was available for injection may have been excluded from the literature report. The information, however, is of paramount importance in establishing the limits to using frozen testicular sperm for NOA patients. In our series of 20 cycles without frozen sperm, a back-up fresh retrieval was successfully carried out in 14 cycles.

A second point of discussion is the use of immotile frozen–thawed sperm for injection. In the ICSI programme, motility is used as an indicator for viability. It has been shown previously that immotile fresh testicular sperm can successfully be used for ICSI, notwithstanding the fact that the fertilization rate was lower (46% in 14 cycles) than with motile spermatozoa (65% in 159 cycles, Nagy *et al.*, 1998). The difference was more pronounced when only non-obstructive cases were considered (31.2% vs 60.8%). In general, the use of immotile sperm for ICSI results in reduced fertilization rates, and complete immotility is one of the major causes of fertilization failure in the ICSI programme, as demonstrated by Liu *et al.* (1995) in 29 out of the 76 cycles without fertilization. Despite the extremely low numbers and poor motility of testicular NOA sperm in our programme, motile sperm could be found for injection in an unexpectedly high proportion of cycles (92%). The proportion of cycles with only motile sperm injected (82%) is comparable to the observation by Ben-Yosef *et al.* (1999; 72%). Besides its effect on fertilization, the use of immotile sperm also affects pregnancy and implantation rates. Only in one cycle (of a Klinefelter patient), an ongoing clinical pregnancy occurred but resulted in a stillborn delivery. A reduced pregnancy rate with immotile frozen–thawed sperm was also described by Fischer *et al.* (1998). Although *in vitro* culture of frozen–thawed testicular sperm may improve motility in obstructive cases, it was ineffective and unpredictable when

only immotile sperm of non-obstructive cases were cultured (Liu *et al.*, 1997). These observations stress the importance of motility for ICSI with cryopreserved testicular sperm of NOA patients. Surprisingly, Gianaroli *et al.* (1999) needed to inject almost exclusively immotile sperm after thawing and obtained a fertilization rate of 64%. It may be questioned whether real immotility was distinguished from the hardly visible twitching motility in their study. Based on our results and those achieved by most other authors, it seems preferable to consider a fresh biopsy retrieval if the embryologist is faced with the presence of only real immotile sperm after thawing. For each individual case, this option should be discussed beforehand between the clinician and the patient.

A third drawback of using cryopreserved testicular sperm in a low restrictive programme is the high proportion of cycles without embryo transfer. The transfer rate of 83% in the present study was similar to the transfer rate (86%) in the study with fresh testicular sperm by Vernaeve *et al.* (2003). Within the same patient population (Part II), however, the transfer rate was significantly lower ($P = 0.028$) in the frozen (76%) than in the fresh cycles (93.2%). This observation was due to the higher prevalence of fertilization failure but also to absent or impaired embryo cleavage in the frozen cycles.

This is the first study to report on the duration of sperm searching in patients with testicular failure. It is well known and confirmed by this report that the search procedure is cumbersome and time-consuming for embryologists and technicians, depending on the severity of the cases that are allocated for treatment. In suspensions that showed no sperm after mechanical disruption, enzymatic digestion was mostly carried out and proved effective in 10 out of 18 patients. The long searching time indicates the severity of testicular failure and the low-restrictive attitude for sperm freezing in our patient population.

Based on the pre-freezing quality of the testicular suspensions, it seems difficult to define cut-offs for what to freeze and what not to freeze (Table IV). The lowest sperm numbers before freezing were indeed observed in samples that could not be used for ICSI upon thawing, but individual data indicate that no clear prediction is possible. Extremely low sperm numbers were also observed in samples that were used for ICSI and even in samples that resulted in ongoing pregnancies. Also the parameter motility had no predictive value. Even in one third of the samples that had led to ongoing pregnancies, no motility was observed before cryopreservation, while predominantly motile sperm could be injected from the thawed specimens after extensive search. This means that testicular suspensions can hardly be excluded for freezing on the basis of extremely low numbers and/or absence of motility.

For the different histological patterns in the testis, the probability of sperm availability after thawing was similar. Most of the pregnancies were, however, obtained for patients with germ-cell aplasia or tubular sclerosis/atrophy. Only two children were born from patients with maturation arrest (1/16 cycles) or patients with Klinefelter syndrome (1/10 cycles). For Klinefelter patients, this reduced chance is not related to the abnormal karyotype, as PGD was performed on the transferred embryos. It might, however, be correlated with the higher probability of only finding immotile frozen–thawed sperm for injection as observed in these populations. Recently, Staessen *et al.* (2003) reported an implantation rate of 12.8% obtained after 26 transfers of PGD-tested embryos for 20 couples of which the man had a 47,XXY karyotype.

In the present study, the overall outcome of cycles carried out with frozen–thawed testicular sperm is not inferior to that with fresh testicular sperm in the same patient group, despite the greater difficulty of finding sperm suitable for injection, and despite the higher risk of having no embryos of sufficient quality for transfer. The results seemed not to be biased by a difference in female age between the two arms of the study, being 32.0 years for the fresh TESE and 32.8 years for the frozen TESE cycles. Although there was a difference in rank of trial, showing that more patients first had a fresh TESE cycle followed by a frozen TESE cycle, the opposite also occurred. These observations indicate that, besides its damaging effects (Verheyen *et al.*, 1997; Nogueira *et al.*, 1999), freezing testicular sperm eliminates the most deficient sperm. This selection may, on the one hand, also explain why fertilization (48.5%), clinical pregnancy (15.4%) and implantation rates (8.6%) in the study by Vernaeve *et al.* (2003) with fresh testicular sperm were somewhat lower than those in the present study with frozen testicular sperm. On the other hand, it may be postulated that the population by Vernaeve *et al.* (2003) includes proportionally more difficult cases (without cryopreservation) than the population in the present study.

Although diagnostic retrieval and cryopreservation of testicular sperm is the first-line approach in several IVF centres, the limits of this approach should not be overlooked. Notwithstanding our minimal restriction criteria for sperm freezing, the results of ICSI with frozen–thawed testicular sperm were acceptable, at least once sperm were found for injection. Cryopreservation has several advantages as well as a number of drawbacks. Careful counselling with information on the pros and cons of cryopreservation is therefore of paramount importance.

It may be concluded from the present study that, even in a programme with low-restrictive criteria for patient allocation and cryopreservation of testicular sperm, diagnostic testicular sperm retrieval followed by cryopreservation can be the procedure of choice. In order to counteract the reasonable risk of not finding sperm or only immotile sperm, scheduling fresh surgery as back-up or counselling the couple for donor sperm as back-up is recommended. The use of totally immotile sperm after thawing should be discouraged on the basis of the present data.

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References

Balaban B, Urman B, Isiklar A, Alatas C, Mercan R, Aksoy S and Nuhoglu A (2001) Blastocyst transfer following intracytoplasmic injection of ejaculated, epididymal or testicular spermatozoa. Hum Reprod 16,125–129.

Baukloh V, German Society for Human Reproductive Biology (2002) Retrospective multicentre study on mechanical and enzymatic preparation of fresh and cryopreserved testicular biopsies. Hum Reprod 17,1788–1794.

Ben-Yosef D, Yogev L, Hauser R, Yavetz H, Azem F, Yovel I, Lessing JB and Amit A (1999) Testicular sperm retrieval and cryopreservation prior to initiating ovarian stimulation as the first line approach in patients with non-obstructive azoospermia. Hum Reprod 14,1794–1801.

Bukulmez O, Yucel A, Yarali H, Bildirici I and Gurgan T (2001) The origin of spermatozoa does not affect intracytoplasmic sperm injection outcome. Eur J Obstet Gynecol Reprod Biol 94,250–255.

Crabbé E, Verheyen G, Tournaye H and Van Steirteghem A (1997) The use of enzymatic procedures to recover testicular germ cells. Hum Reprod 12,1682–1687.

Crabbe E, Verheyen G, Silber S, Tournaye H, Van de Velde H, Goossens A and Van Steirteghem A (1998) Enzymatic digestion of testicular tissue may rescue the intracytoplasmic sperm injection cycle in some patients with non-obstructive azoospermia. Hum Reprod 13,2791–2796.

Craft I, Bennett V and Nicholson N (1993) Fertilising ability of testicular spermatozoa. Lancet 342,864.

De Croo I, Van der Elst J, Everaert K, De Sutter P and Dhont M (1998) Fertilization, pregnancy and embryo implantation rates after ICSI with fresh or frozen–thawed testicular spermatozoa. Hum Reprod 13, 1893–1897.

De Croo I, Van der Elst J, Everaert K, De Sutter P and Dhont M (2000) Fertilization, pregnancy and embryo implantation rates after ICSI in cases of obstructive and non-obstructive azoospermia. Hum Reprod 15, 1383–1388.

Devroey P, Liu J, Nagy Z, Tournaye H, Silber SJ and Van Steirteghem AC (1994) Normal fertilization of human oocytes after testicular sperm extraction and intracytoplasmic sperm injection. Fertil Steril 62,639–641.

Devroey P, Nagy P, Tournaye H, Liu J, Silber S and Van Steirteghem A (1996) Outcome of intracytoplasmic sperm injection with testicular spermatozoa in obstructive and non-obstructive azoospermia. Hum Reprod 11, 1015–1018.

Ezeh UI, Taub NA, Moore HD and Cooke ID (1999) Establishment of predictive variables associated with testicular sperm retrieval in men with non-obstructive azoospermia. Hum Reprod 14,1005–1012.

Fahmy I, Mansour R, Aboulghar M, Serour G, Kamal A, Tawab NA, Ramzy AM and Amin Y (1997) Intracytoplasmic sperm injection using surgically retrieved epididymal and testicular spermatozoa in cases of obstructive and non-obstructive azoospermia. Int J Androl 20,37–44.

Fischer R, Baukloh V, Naether OGJ and Schulze W (1998) Use of cryopreserved testicular sperm. In: Filicori M and Flamigni C (eds), Treatment of Infertility. Communications Media for Education, pp 403–409.

Friedler S, Raziel A, Soffer Y, Strassburger D, Komarovsky D and Ron-el R (1997) Intracytoplasmic injection of fresh and cryopreserved testicular spermatozoa in patients with nonobstructive azoospermia–a comparative study. Fertil Steril 68,892–897.

Gianaroli L, Magli MC, Selman HA, Colpi G, Belgrano E, Trombetta C, Vitali G and Ferraretti AP (1999) Diagnostic testicular biopsy and cryopreservation of testicular tissue as an alternative to repeated surgical openings in the treatment of azoospermic men. Hum Reprod 14,1034–1038.

Gil-Salom M, Romero J, Minguez Y, Rubio C, De los Santos MJ, Remohi J and Pellicer A (1996) Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Hum Reprod 11,1309–1313.

Gil-Salom M, Romero J, Rubio C, Ruiz A, Remohi J and Pellicer A (2000) Intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Mol Cell Endocrinol 169,15–19.

Habermann H, Seo R, Cieslak J, Niederberger C, Prins GS and Ross L (2000) In vitro fertilization outcomes after intracytoplasmic sperm injection with fresh or frozen–thawed testicular spermatozoa. Fertil Steril 73,955–960.

Hovatta O, Foudila T, Siegberg R, Johansson K, von Smitten K and Reima I (1996) Pregnancy resulting from intracytoplasmic injection of spermatozoa from a frozen–thawed testicular biopsy specimen. Hum Reprod 11, 2472–2473.

Huang FJ, Chang SY, Tsai MY, Kung FT, Lin YC, Wu JF and Lu YJ (2000) Clinical implications of intracytoplasmic sperm injection using cryopreserved testicular spermatozoa from men with azoospermia. J Reprod Med 45,310–316.

Joris H, Nagy Z, Van de Velde H, De Vos A and Van Steirteghem A (1998) Intracytoplasmic sperm injection: laboratory set-up and injection procedure. Hum Reprod 13 (Suppl. 1),76–86.

Kahraman S, Ozgur S, Alatas C, Aksoy S et al. (1996) High implantation and pregnancy rates with testicular sperm extraction and intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia. Hum Reprod 11,673–676.

Kupker W, Schlegel PN, Al-Hasani S, Fornara P, Johannisson R, Sandmann J, Schill T, Bals-Pratsch M, Ludwig M and Diedrich K (2000) Use of frozen–thawed testicular sperm for intracytoplasmic sperm injection. Fertil Steril 73,453–458.

Liu J, Nagy Z, Joris H, Tournaye H, Smitz J, Camus M, Devroey P and Van Steirteghem A (1995) Analysis of 76 total fertilization failure cycles out of 2732 intracytoplasmic sperm injection cycles. Hum Reprod 10, 2630–2636.

Liu J, Tsai YL, Katz E, Compton G, Garcia JE and Baramki TA (1997) Outcome of in-vitro culture of fresh and frozen–thawed human testicular spermatozoa. Hum Reprod 12,1667–1672.

Madgar I, Hourvitz A, Levron J, Seidman DS, Shulman A, Raviv GG, Levran D, Bider D, Mashiach S and Dor J (1998) Outcome of in vitro fertilization and intracytoplasmic injection of epididymal and testicular sperm extracted from patients with obstructive and nonobstructive azoospermia. Fertil Steril 69,1080–1084.

Mansour RT, Kamal A, Fahmy I, Tawab N, Serour GI and Aboulghar MA (1997) Intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia. Hum Reprod 12,1974–1979.

Mulhall JP, Burgess CM, Cunningham D, Carson R, Harris D and Oates RD (1997) Presence of mature sperm in testicular parenchyma of men with nonobstructive azoospermia: prevalence and predictive factors. Urology 49,91–95.

Nagy Z, Liu J, Cecile J, Silber S, Devroey P and Van Steirteghem A (1995) Using ejaculated, fresh, and frozen–thawed epididymal and testicular spermatozoa gives rise to comparable results after intracytoplasmic sperm injection. Fertil Steril 63,808–815.

Nagy ZP, Verheyen G, Tournaye H, Devroey P and Van Steirteghem AC (1997) An improved treatment procedure for testicular biopsy specimens offers more efficient sperm recovery: case series. Fertil Steril 68,376–379.

Nagy ZP, Joris H, Verheyen G, Tournaye H, Devroey P and Van Steirteghem AC (1998) Correlation between motility of testicular spermatozoa, testicular histology and the outcome of intracytoplasmic sperm injection. Hum Reprod 13,890–895.

Nogueira D, Bourgain C, Verheyen G and Van Steirteghem AC (1999) Light and electron microscopic analysis of human testicular spermatozoa and spermatids from frozen and thawed testicular biopsies. Hum Reprod 14, 2041–2049.

Oates RD, Mulhall J, Burgess C, Cunningham D and Carson R (1997) 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 12,734–739.

Palermo GD, Schlegel PN, Hariprashad JJ, Ergun B, Mielnik A, Zaninovic N, Veeck LL and Rosenwaks Z (1999) Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men. Hum Reprod 14,741–748.

Perraguin-Jayot S, Audebert A, Emperaire JC and Parneix I (1997) Ongoing pregnancies after intracytoplasmic injection using cryopreserved testicular spermatozoa. Hum Reprod 12,2706–2709.

Prins GS, Dolgina R, Studney P, Kaplan B, Ross L and Niederberger C (1999) Quality of cryopreserved testicular sperm in patients with obstructive and nonobstructive azoospermia. J Urol 161,1504–1508.

Romero J, Remohi J, Minguez Y, Rubio C, Pellicer A and Gil-Salom M (1996) Fertilization after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Fertil Steril 65,877–879.

Ron-El R, Strauss S, Friedler S, Strassburger D, Komarovsky D and Raziel A (1998) Serial sonography and colour flow Doppler imaging following testicular and epididymal sperm extraction. Hum Reprod 13,3390–3393.

Schlegel PN, Palermo GD, Goldstein M, Menendez S, Zaninovic N, Veeck LL and Rosenwaks Z (1997) Testicular sperm extraction with intracytoplasmic sperm injection for nonobstructive azoospermia. Urology 49, 435–440.

Schlegel PN and Su LM (1997) Physiological consequences of testicular sperm extraction. Hum Reprod 12,1688–1692.

Schulze W, Thoms F and Knuth UA (1999) Testicular sperm extraction: comprehensive analysis with simultaneously performed histology in 1418 biopsies from 766 subfertile men. Hum Reprod 14 (Suppl. 1),82–96.

Seo JT and Ko WJ (2001) Predictive factors of successful testicular sperm recovery in non-obstructive azoospermia patients. Int J Androl 24, 306–310.

Schoysman R, Vanderzwalmen P, Nijs M, Segal-Bertin C, Geerts L and Van de Casseye M (1993) Successful fertilization by testicular spermatozoa in an in-vitro fertilization programme. Hum Reprod 8,1339–1340.

Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H and Devroey P (1995) High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. Hum Reprod 10,148–152.

Silber SJ, van Steirteghem A, Nagy Z, Liu J, Tournaye H and Devroey P (1996) Normal pregnancies resulting from testicular sperm extraction and intracytoplasmic sperm injection for azoospermia due to maturation arrest. Fertil Steril 66,110–117.

Sousa M, Cremades N, Silva J, Oliveira C, Ferraz L, Teixeira da Silva J, Viana P and Barros A (2002) Predictive value of testicular histology in secretory azoospermic subgroups and clinical outcome after microinjection of fresh and frozen-thawed sperm and spermatids. Hum Reprod 17, 1800–1810.

Staessen C, Tournaye H, Van Assche E, Michiels A, Van Landuyt L, Devroey P, Liebaers I and Van Steirteghem A (2003) PGD in 47,XXY Klinefelter's syndrome patients. Hum Reprod Update 9,319–330.

Su LM, Palermo GD, Goldstein M, Veeck LL, Rosenwaks Z and Schlegel PN (1999) Testicular sperm extraction with intracytoplasmic sperm injection for nonobstructive azoospermia: testicular histology can predict success of sperm retrieval. J Urol 161,112–116.

Tournaye H, Verheyen G, Nagy P, Ubaldi F, Goossens A, Silber S, Van Steirteghem AC and Devroey P (1997a) Are there any predictive factors for successful testicular sperm recovery in azoospermic patients? Hum Reprod 12,80–86.

Tournaye H, Camus M, Vandervorst M, Nagy Z, Joris H, Van Steirteghem A and Devroey P (1997b) Surgical sperm retrieval for intracytoplasmic sperm injection. Int J Androl 20 (Suppl. 3),69–73.

Van de Velde H, Nagy ZP, Joris H, De Vos A and Van Steirteghem AC (1997) Effects of different hyaluronidase concentrations and mechanical procedures for cumulus cell removal on the outcome of intracytoplasmic sperm injection. Hum Reprod 12,2246–2250.

Verheyen G, De Croo I, Tournaye H, Pletincx I, Devroey P and Van Steirteghem AC (1995) Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. Hum Reprod 10,2956–2959.

Verheyen G, Nagy Z, Joris H, De Croo I, Tournaye H and Van Steirteghem A (1997) Quality of frozen–thawed testicular sperm and its preclinical use for intracytoplasmic sperm injection into in vitro-matured germinal-vesicle stage oocytes. Fertil Steril 67,74–80.

Vernaeve V, Tournaye H, Osmanagaoglu K, Verheyen G, Van Steirteghem A and Devroey P (2003) Intracytoplasmic sperm injection with testicular spermatozoa is less successful in men with nonobstructive azoospermia than in men with obstructive azoospermia. Fertil Steril 79,529–533.

Windt ML, Coetzee K, Kruger TF, Menkveld R and van der Merwe JP (2002) Intracytoplasmic sperm injection with testicular spermatozoa in men with azoospermia. J Assist Reprod Genet 19,53–59.

Wood S, Thomas K, Schnauffer K, Troup S, Kingsland C and Lewis-Jones I (2002) Reproductive potential of fresh and cryopreserved epididymal and testicular spermatozoa in consecutive intracytoplasmic sperm injection cycles in the same patients. Fertil Steril 77,1162–1166.

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