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Predictive value of testicular histology in secretory azoospermic subgroups and clinical outcome after microinjection of fresh and frozen–thawed sperm and spermatids

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BACKGROUND: A retrospective study was carried out on 159 treatment cycles in 148 secretory azoospermic patients to determine whether histopathological secretory azoospermic subgroups were predictive for gamete retrieval, and to evaluate outcome of microinjection using fresh or frozen–thawed testicular sperm and spermatids. METHODS: Sperm and spermatids were recovered by open testicular biopsy and microinjected into oocytes. Fertilization and pregnancy rates were assessed. RESULTS: In hypoplasia, 97.7% of the 44 patients had late spermatids/sperm recovered. In maturation-arrest (MA; 47 patients), 31.9% had complete MA, and 68.1% incomplete MA due to a focus of early (36.2%) or late (31.9%) spermiogenesis. Gamete retrieval was achieved in 53.3, 41.2 and 93.3% of the cases respectively. In Sertoli cell-only syndrome (SCOS; 57 patients), 61.4% were complete SCOS, whereas incomplete SCOS cases showed one focus of MA (5.3%), or of early (29.8%) and late (3.5%) spermiogenesis. Only 29.8% of the patients had a successful gamete retrieval, 2.9% in complete and 77.3% in incomplete SCOS cases. In total, there were 87 ICSI, 39 elongated spermatid injection (ELSI) and 33 round spermatid injection (ROSI) treatment cycles, with mean values of fertilization rate of 71.4, 53.6 and 17%, and clinical pregnancy rates of 31.7, 26.3 and 0% respectively. CONCLUSIONS: Histopathological subgroups were positively correlated with successful gamete retrieval. No major outcome differences were observed between testicular sperm and elongated spermatids, either fresh or frozen–thawed. However, injection of intact round-spermatids showed very low rates of fertilization and no pregnancies.

Key words: non-obstructive azoospermia/spermatids/spermatogenesis/testicular sperm/testicular histopathology

Introduction

Non-obstructive azoospermia was suggested to be a treatable situation after it was shown that sperm could be retrieved from the testis in cases of maturation arrest (MA) and hypoplasia (Jow *et al.*, 1993). This was then extended to cases with Sertoli cell-only syndrome (SCOS), with injection of the extracted sperm being then successfully applied to the clinical treatment of patients (Craft *et al.*, 1993; Schoysman *et al.*, 1993; Devroey *et al.*, 1995). Studies have also suggested that the probability of finding sperm at treatment varied according to the diagnostic testicular biopsy, being ~95% for hypoplasia, 52 and 69% for complete and incomplete MA, and 22 and 90% for complete and incomplete SCOS respectively (Tournaye *et al.*, 1996, 1997; Silber *et al.*, 1997; De Croo *et al.*, 2000).

Most of the reported clinical series related to non-obstructive azoospermia describe only the clinical outcome associated with sperm microinjection, either fresh or frozen–thawed. Those studies evidenced relatively low fertilization rates (38–67%) but rather high pregnancy rates (40–60%) after ICSI in cases of SCOS, MA and hypoplasia (Silber *et al.*, 1996, 1997; Tournaye *et al.*, 1996; Mansour, 1998; Al-Hasani *et al.*, 1999a; De Croo *et al.*, 2000). On the contrary, analysis of the few studies which have used spermatids for treatment reveals that whereas late spermatid injections (n = 127) seem associated with low fertilization (48.2%) and acceptable clinical pregnancy (29.9%) rates, round spermatid injections (n = 216) appear not to be clinically useful (22.5% of fertilization and 3.2% of clinical pregnancy rates) (Fishel *et al.*, 1995, 1996; Hannay *et al.*, 1995; Tesarik *et al.*, 1995, 1996, 1999; Chen *et al.*, 1996; Mansour *et al.*, 1996, 1997; Tanaka *et al.*, 1996; Amer *et al.*, 1997; Antinori *et al.*, 1997a,b; Araki *et al.*, 1997; Sofikitis *et al.*, 1997, 1998b,c; Vanderzwalmen *et al.*, 1997;

Yamanaka *et al.*, 1997; Barak *et al.*, 1998; Bernabeu *et al.*, 1998; Kahraman *et al.*, 1998; Al-Hasani *et al.*, 1999b; Sousa *et al.*, 1999).

In the present study we present the Portuguese clinical and laboratory data from the consecutive treatment of 148 non-obstructive azoospermic patients, with normal karyotypes. It is shown that histopathology allows further subdivisions of patients with incomplete SCOS and MA, and that these subgroups evidence distinct prognostic values. The outcome with both fresh and frozen–thawed testicular-retrieved sperm and different subtypes of spermatids is also presented.

Materials and methods

All male patients were referred after urology evaluation and were selected based on normal karyotypes, patent excretory ducts (as confirmed by physical examination, hormone levels, spermiogram and ultrasonography), absence of cryptozoospermia (as demonstrated by two consecutive spermiograms under centrifuged specimens), and a diagnostic testicular biopsy showing hypoplasia, MA or SCOS. In almost all of the cases, patients had already had a diagnostic testicular biopsy a few years previously at another hospital. When patients accepted IVF with donor sperm, the treatment testicular biopsy either followed oocyte retrieval or was performed 1–3 days before. In the latter case, cells were cultured until use in IVF medium at 32°C, 5% CO_2 in humidified air. When donor sperm was not an option to the couple, the biopsy was performed in advance of ovarian stimulation, with germ cells being frozen 24 h later. In all cases, no treatment testicular biopsy was made <6 months after the diagnostic testicular biopsy. All therapeutical procedures followed the guidelines of the Ethical Committee, and informed consent was obtained from all patients after careful explanation of the treatment technique.

Ovarian stimulation

Female patients were treated with a long GnRH analogue suppression protocol combining buserelin acetate (Suprefact; Hoechst, Frankfurt, Germany) with pure FSH (pFSH) (Metrodin HP; Serono, Geneva, Switzerland) or recombinant FSH (rFSH) (Gonal F; Serono, Puregon; Organon, Oss, The Netherlands). Ovulation was induced with HCG (Pregnyl; Organon, Profasi; Serono). Oocytes were recovered from large ovarian follicles by ultrasonically-guided follicular aspiration, 35 h after HCG, using flush medium (Medicult, Copenhagen, Denmark).

Treatment testicular biopsy

The spermatic cord block was performed according to the three-finger technique (Li *et al.*, 1992; Gorgy *et al.*, 1998; Nudell *et al.*, 1998). Local anaesthesia was achieved with 5–6 ml of a 1:1 mixture of 1% lidocaine hydrochloride solution (Xylocaine 2% without epinephrine; Astra Pharmaceuticals International, Sweden) and 0.5% bupivacaine (Marcaine 0.5% without epinephrine; Astra). After a few minutes, a skin weal was raised in the scrotum adjacent to the middle region of the testis, a 1 cm transverse incision was made and the tunica vaginalis space entered. An incision of 0.5 cm then enabled excision of a small piece of the seminiferous tubules. A preliminary sample microscopic check at the end of each biopsy avoided unnecessary tissue sampling. In general, almost all cases with hypoplasia had sperm or spermatids in the first three samples collected at one testis, and these were enough for treatment and frozen storage. In MA, SCOS and, occasionally, in hypoplasia cases, 5–10 biopsies of the same testis at different locations were needed to find sperm or spermatids. When such cells were not found, the contralateral testis was also analysed whenever possible. After careful cleaning and haemostasis, the tunica albuginea, the vaginal, the scrotum layers and the skin were closed. The procedure took about 20–30 min and was performed entirely on an outpatient basis, enabling a rapid recovery with minimal complaints and total absence of surgical complications. Tramadol and nimesulide per os were given to relieve any discomfort in the first 24 h. Where needed, a new biopsy was scheduled only after a period of 6 months (Schlegel and Su, 1997).

Preparation of testicular samples

Each sample was expressed in Sperm Preparation Medium (SPM, Medicult) with surgical blades, and 10 µl were observed to confirm the presence or absence of sperm or elongated spermatids. The resultant fluid was washed with SPM, 2×5 min, by centrifuging at 500–600 *g*, and the pellet resuspended for 5 min in 2 ml of erythrocyte-lysing buffer (Verheyen *et al.*, 1995) using endotoxin-free, embryo and cell culture tested chemicals (Sigma, Barcelona, Spain). After washing, samples were digested (Crabbé *et al.*, 1997) for 1 h at 37°C, in a solution of SPM containing 25 µg/ml of crude DNase and 1000 IU/ml of collagenase-IV (Sigma). After a new wash, the pellet was resuspended in 50–100 µl of IVF medium (Medicult) and then incubated at 30–32°C, 5% CO_2 in air until use. For freezing, the sample was diluted with Sperm Freezing Medium (Medicult), exposed for 10–15 min to liquid nitrogen (LN_2) vapours, and finally immersed and stored in LN_2.

Selection of cells for microinjection

The distinction between round spermatids and Sertoli cell nuclei, cytoplasmic remnants and lymphocytes, as also the distinction between elongating spermatids, elongated spermatids and testicular sperm have been the subject of much debate, but clear criteria have been established (Tesarik, 1997, 1998; Aslam *et al.*, 1998; Lewis and McClure, 1998; Mansour *et al.*, 1998; Silber and Johnson, 1998; Silber *et al.*, 1998; Sofikitis *et al.*, 1998b; Sousa *et al.*, 1998, 1999; Tesarik *et al.*, 1998; Vanderzwalmen *et al.*, 1998). Briefly, isolated nuclei of Sertoli cells have an elevated border, a large nucleolus, no other visible internal structures, and shrink in 10% PVP-SPM (Medicult); round-shaped cytoplasmic remnants, blebbed-out from degenerating cells, have no internal visible organelles and shrink in PVP; lymphocytes have internal nuclear irregularities due to condensed patches of chromatin, they stick to the tip of a 6 µm inner micropipette and stretch with aspiration, and if left in culture they tend to attach and develop cytoplasmic extensions (pseudopodes) within 24 h. On the contrary, round spermatids have a smooth outline and inner aspect, the nuclear limit is clearly visible, the acrosomal vesicle is distinguishable as 1–2 large round vesicles (Golgi phase) or as a fine dark elongating region at one nuclear pole (cap phase), they deform and adapt their shape to the aspirating 6 µm inner micropipette, and do not shrink in PVP. Round spermatid injection (ROSI) was used when patients did not accept donor sperm. We have used intact round spermatids for injection because a slightly larger injection pipette is not associated with a higher rate of oocyte degeneration (Sousa *et al.*, 1999), at this stage the proximal centriole may still not be attached to the nuclear envelope (Holstein and Roosen-Runge, 1981), and because the cytoplasmic membrane and the nuclear envelope of the round spermatid is ruptured soon after contact with the ooplasm, enabling diffusion of the spermatid oocyte-activating substance and proper pronucleus formation (Sousa *et al.*, 1996, 1999). Because ionophores are not allowed for clinical use, oocytes were not activated through an induced intracellular calcium rise after ROSI (Tesarik and Sousa, 1995b).

On the contrary, elongating and elongated spermatids are very easy to distinguish. In the present series, whenever we found normal

elongating spermatids, we also found elongated spermatids, albeit after several hours of searching. When elongated spermatids could not be found but elongating spermatids were present, the latter had extensive nuclear and tail malformations and were not used for treatments. This means that we have only used elongated spermatids for injection (ELSI). However, we did not use elongated spermatids without a full length flagellum or with abnormal head morphology.

The elongating spermatid is differentiated from the elongated spermatid mainly based on nuclear morphology (Holstein and Roosen-Runge, 1981). The former has a nucleus that is not fully elongated nor condensed and protrudes only slightly beyond the apical cell region. The elongated spermatid is differentiated from the testicular spermatozoon based on the upper limit of the basal cytoplasm. In a spermatozoon, the basal cytoplasm is confined to the midpiece region, which does not exhibit any continuity with the equatorial region of the spermatozoon head. This is made possible through the coalescence between the upper limit of the midpiece cytoplasmic membrane and the basal nuclear envelope. This point of fusion is named the posterior ring (Holstein and Roosen-Runge, 1981). So, the elongated spermatid is differentiated from a testicular spermatozoon when the upper limit of the basal cytoplasm is still above the nuclear base (Holstein and Roosen-Runge, 1981; Sousa *et al.*, 1999). It is also at the end of the elongated spermatid stage that the distal centriole is depolymerized. Because the distal centriole is committed to synthesize the axoneme, to which it remains tightly linked from the round spermatid stage (Holstein and Roosen-Runge, 1981), it has no intrinsic centrosome capability and thus spermatid microinjection does not introduce into the ooplasm an additional microtubule polymerizing centre (Sousa and Tesarik, 1994; El Shafie *et al.*, 2000). That is why, and as clinical series have already demonstrated, the distal centriole of microinjected spermatids does not preclude normal fertilization and embryo cleavage.

Microinjection and embryo culture

Cells were selected using an inverted Nikon microscope with Narishige micromanipulators (Nikon, Tokyo, Japan) and commercial micropipettes (4–5 μm for late spermatids/sperm, 6–8 μm for round spermatids; SweMed; Frolunda, Sweden) according to a published method (Sousa *et al.*, 1998, 1999). Oocytes were injected using the strong dislocation of the ooplasm (Tesarik *et al.*, 1994; Tesarik and Sousa, 1995a). Injected oocytes were cultured in IVF medium at 37°C with 5% CO_2 in air. After 2 days, they were transferred to M3 medium (Medicult). Normal fertilization was assessed 14–18 h after injection, and embryo quality was evaluated (Staessen *et al.*, 1995). Supernumerary embryos were frozen with embryo freezing medium or with the blastocyst freezing pack (Medicult), in an automatic freezing apparatus (Kryo 10 Series III; Planer, UK). All patients had luteal supplementation with three times daily intravaginal administration of 200 mg natural-micronized progesterone (Utrogestan; Jaba, Berlin, Germany). Pregnancy was confirmed by a rise in serum βHCG on 2 consecutive days, 2 weeks after embryo transfer. A clinical pregnancy was established by ultrasonography at 7 weeks gestation. All couples agreed to have a prenatal diagnosis, which was performed by amniocentesis at 16 weeks of pregnancy.

Statistics

When appropriate, correlation analysis were performed, and the significance of difference between the percentages of two groups were evaluated with the χ^2-test. Significance was accepted where $P < 0.05$.

Results

In total, there were 148 patients and 234 treatment cycles (159 with 87 ICSI, 39 ELSI and 33 ROSI cycles, and 75 cycles with donor sperm). Patients were subdivided acording to the histopathological analysis of the diagnostic testicular biopsy: 44 (29.7%) had hypoplasia, 47 (31.8%) MA, and 57 (38.5%) SCOS. Patients were selected after normal karyotypes were confirmed, and thus the present series excludes all testicular histologies associated with chromosomal abnormalities. The histopathological diagnosis was based on the study of at least 100 seminiferous tubule profiles. Tubular hyalinization, fibrosis and Kleinfelter-like cases were excluded from the present study, except when the karyotypes were normal and those specific cases were restricted to a focal attainment of the bioptic specimen.

Hypoplasia

In all patients, the diagnostic biopsy showed spermatogenesis up to the late spermatid or spermatozoa stages, which were recovered for treatment in 43 cases (97.7%). In one case, a total cell aplasia was found at treatment despite an extensive bilateral search of the testicular bioptic specimens. This may be explained by an intratesticular injury that has progressively destroyed the germinal epithelium during the 5 years between the diagnostic and the treatment testis biopsies or, alternatively, by an unfortunous diagnostic testis biopsy that was taken over a unique focus of spermatogenesis. In total, there were 53 cycles with ICSI, 11 cycles with ELSI, and three cycles using IVF or intra-uterine insemination (IUI) with donor sperm (one case with no remaining spermatids and one case with total cell aplasia).

The mean female age was 33 years (range 22–40), the mean male age was 36.1 years (range 27–55), and the mean duration of infertility was 6.7 years (range 1–18). Only 11.4% of the women showed associated pathology (three cases with hyperprolactinaemia, one case of endometriosis, and one case with tubal obstruction). About 50% of the male patients had increased FSH levels, 38.6% showed decreased testicular volume, and 13.6% had associated testicular pathology (three varicocele, two orchitis, one cryptorchidia). Most of the patients with decreased testicular volume exhibited increased FSH levels and had no associated local pathology.

In ICSI cycles using fresh sperm, there have been nine normal evolving pregnancies (five clinical, four ongoing), and four deliveries of healthy children. Of these 13 pregnancies, three are twin and two triplet. In ICSI with frozen–thawed sperm there were two biochemical pregnancies, one is presently clinical and three have delivered healthy babies. Of these four successful pregnancies, one is twin and one triplet. In fresh ELSI cycles, there is one ongoing pregnancy and three deliveries of healthy children. Of these four pregnancies, one is twin (Table I).

Maturation arrest

Of the 47 patients with MA, the diagnostic testicular biopsy showed 15 cases (31.9%) of complete MA, 17 cases (36.2%) had one focus of early spermiogenesis (round or elongating spermatids), and 15 cases (31.9%) exhibited one focus of late spermiogenesis (late spermatids or spermatozoa). After treatment, testicular biopsy confirmed complete MA in three patients, four had one focus with round spermatids, and

Table I. Outcome in hypoplasia

	ICSI *n* (%)		ELSI *n* (%)	
	F	FT	F	FT
Cycles	33	20	10	1
Injected oocytes	213	115	73	10
(mean, range)	6.5 (2–15)	5.8 (1–13)	7.3 (4–10)	–
Degenerated oocytes	19	11	4	4
Intact oocytes	194	104	69	6
Non-fertilized oocytes	20	24	26	3
1PN zygotes	14	8	7	0
2PN zygotes	157 (80.9)[a]	66 (63.5)	34 (49.3)	2 (33.3)
>2PN zygotes	3	6	2	1
Cleaved embryos (from 2PN)	153 (97.5)	60 (90.9)	32 (94.1)	2 (100)
Embryo grade (A+B)	134 (87.6)	48 (80)	24 (75)	1 (50)
No. embryos transferred (mean)	2.9	2.6	2.3	2
Day of embryo transfer (mean)	3.3	3.4	3.3	3
Cycles with embryo transfer	32	20	10	1
Clinical pregnancies	13	4	4	0

Fresh (F) and frozen–thawed (FT) gametes. [a]$P < 0.05$ to ICSI (FT) and ELSI (F, FT).

eight (53.3%) enabled recovery of sperm/late spermatids; MA patients with one focus of early spermiogenesis revealed very similar figures (3; 7; and 7 cases, 41.2%); on the contrary, 14 (93.3%) of the patients showing one focus of late spermiogenesis had recovery of sperm/late spermatids (one case with a focus of round spermatids). In total, there were 22 ICSI cycles, 21 ELSI cycles, 20 cycles with round spermatid injection (ROSI: 11 original cases and nine cases with absence of late spermatids/sperm in the second trial), 11 donor sperm IVF cycles (two after ICSI/ELSI, one after ROSI, and eight from couples with complete MA), and four donor sperm IUI cycles (one after ICSI/ELSI, three from patients with complete MA).

The mean female age was 31.9 years (range 24–41), the mean male age was 34.9 years (range 24–53), and the mean duration of infertility was 5.7 years (range 1–18). About 27.7% of the women showed associated pathology [six polycystic ovarian syndrome (PCOS), four hyperprolactinaemia, one endometriosis, one tubal obstruction, and one ovarian insufficiency]. About 38.3% of the male patients had increased FSH levels, 42.6% showed decreased testicular volume, and 25.5% had associated testicular pathology (three varicocele, nine cryptorchidia). In patients where no late spermatids/sperm could be recovered, there was a predominance of normal values of FSH (72.2 versus 58.6%), of normal testicular volume (77.8 versus 57.4%), and fewer cases of local pathology (5.6 versus 37.9%). Correspondingly, normal testicular volume appeared to be associated with normal FSH values (75%), decreased testicular volume was associated with higher FSH values (71.4%), and 45% of the cases with decreased testicular volume were associated with local pathology (chryptorchidia).

In ICSI cycles using fresh sperm, to date there is one ongoing pregnancy and three deliveries of healthy babies. Of these four pregnancies, one is twin. In ICSI with frozen–thawed sperm there is one ongoing pregnancy and two deliveries (healthy babies). In fresh ELSI cycles there were two biochemical and one clinical pregnancies, and two healthy deliveries. Of these three successful pregnancies, one is twin (Table II). Of the cycles with donor sperm, there were four IVF term pregnancies and one IUI term pregnancy with delivery of healthy children.

SCOS

Of the 57 patients with SCOS, the diagnostic testicular biopsy showed 35 cases (61.4%) of complete SCOS, three cases had one focus of primary spermatocytes (5.3%), 17 cases (29.8%) had one focus of early spermiogenesis (round or elongating spermatids), and two cases (3.5%) exhibited one focus of late spermiogenesis (late spermatids or spermatozoa). After treatment, testicular biopsy revealed complete SCOS in 31 patients, three had one focus with round spermatids, and one (2.9%) enabled recovery of sperm/late spermatids; all SCOS patients with one focus of primary spermatocytes had sperm/late spermatids recovered; SCOS patients with one focus of early spermiogenesis revealed five cases with round spermatids, and 12 cases with sperm/late spermatids (70.6%); of the two SCOS patients with one focus of late spermiogenesis, one had sperm/elongated spermatids recovered, whereas in the other case only Sertoli cells could be found. In total, there were 12 ICSI cycles, seven ELSI cycles, 13 ROSI cycles (eight original cases and five cases with absence of late spermatids/sperm in the second trial), 41 donor sperm IVF cycles (two after ICSI/ELSI, two after ROSI, and 37 from couples with complete SCOS), and 16 donor sperm IUI cycles (from patients with complete SCOS).

The mean female age was 30.8 years (range 19–42), the mean male age was 34.2 years (range 23–48), and the mean duration of infertility was 6 years (range 1–19). About 22.8% of the women showed associated pathology (eight PCOS, three hyperprolactinaemia, one endometriosis, one miomatosis). About 75.4% of the male patients had increased FSH levels, 71.9% showed decreased testicular volume, and 36.8% had associated testicular pathology (eight varicocele, six cryptorchidia, four adult parotiditis, one chemotherapy, two hypogonadism). Increased FSH levels were present in 58.8% of the patients with sperm/late spermatids, in 37.5% of the patients where only round

Table II. Outcome in maturation arrest (MA)

	ICSI *n* (%)		ELSI *n* (%)		ROSI *n* (%)	
	F	FT	F	FT	F	FT
Cycles	11	11	16	5	17	3
Injected oocytes	59	54	140	44	98	28
(mean, range)	5.4 (2–11)	4.9 (1–10)	8.8 (2–16)	8.8 (5–14)	5.8 (2–11)	9.3 (9–10)
Degenerated oocytes	7	2	8	3	12	2
Intact oocytes	52	52	132	41	86	26
Non-fertilized oocytes	12	13	39	11	36	13
1PN zygotes	5	4	10	10	32	7
2PN zygotes	34 (65.4)[a]	35 (67.3)[b]	79 (59.8)[c]	19 (46.3)[b,d]	15 (17.4)[a,c]	5 (19.2)[b,d]
>2PN zygotes	1	0	4	1	3	1
Cleaved embryos (from 2PN)	31 (91.2)	27 (77.1)	77 (97.5)	16 (84.2)	10 (66.7)	5 (100)
Embryo grade (A+B)	27 (87.1)	23 (85.2)	65 (84.4)	9 (56.3)	9 (90)	5 (100)
No. embryos transferred (mean)	2.3	1.9	3.2	2.2	1.5	1.7
Day of embryo transfer (mean)	3.5	3.9	3.5	3.8	3.3	3.7
Cycles with embryo transfer	11	9	16	5	6	3
Clinical pregnancies	4	3	3	0	0	0

Fresh (F) and frozen–thawed (FT) gametes. [a,b,c] $P < 0.05$ between same superscripts.

spermatids were recovered, and in 93.8% of the patients with complete SCOS. Similarly, in these three groups there was a predominance of decreased testicular volume (82.4, 75 and 65.6% respectively). Because the large majority of the patients in the study had decreased testicular volume and increased FSH, no association was found between these two parameters. There was also no association found between local pathology and any of the other parameters or subgroups of patients.

In ICSI cycles with fresh sperm there was one biochemical pregnancy, and in cycles with frozen–thawed sperm there were two deliveries of healthy babies (one twin). In fresh ELSI cycles there is one ongoing pregnancy and two deliveries of healthy children (Table III). Of the cycles with donor sperm, there are currently one ongoing and 15 normal term pregnancies in IVF cycles, and three normal term pregnancies after IUI.

Discussion

Sperm can be retrieved from the testis in cases of non-obstructive azoospermia, which includes hypoplasia, MA and SCOS. Several studies suggested that the presence of one focus of elongated spermatids/spermatozoa in the diagnostic biopsy (in incomplete MA or SCOS syndromes) correlates highly with successful sperm retrieval at treatment, and that neither the testis volume, male serum FSH, associated male pathology or professional status could be used as successful predictive factors (Devroey *et al.*, 1995; Gil-Salom *et al.*, 1995; Yemini *et al.*, 1995; Lewin *et al.*, 1996; Silber *et al.*, 1996; Tournaye *et al.*, 1997; Ezeh *et al.*, 1998a,c, 1999; Amer *et al.*, 2001). Although the presence of spermatocytes and spermatids in ejaculates, as detected by specific staining, seems to predict a successful testicular sperm retrieval, this was only noticed for cases with hypoplasia and focal spermiogenesis (Ezeh *et al.*, 1998c) or were not sufficiently correlated with the specific histopathological subtypes (Amer *et al.*, 2001). Other authors have also suggested that anti-Mullerian hormone seminal-plasma levels, as well as telomerase assays in testicular tissue, could predict the presence of spermatids in cases of SCOS (Fénichel *et al.*, 1999; Yamamoto *et al.*, 1999b; Schrader *et al.*, 2000). Data from our present series of 148 consecutive non-obstructive azoospermic patients confirm the importance of histopathology for a predictive prognosis, and further suggest that incomplete MA and SCOS cases may harbour other subdivisions with distinct prognosis, a finding that has been anticipated in very large series of diagnostic testicular biopsies from azoospermic men (Schulze *et al.*, 1999; Glander *et al.*, 2000).

In non-obstructive azoospermia, several studies have suggested that the testicular open-biopsy method appears superior for those cases that are expected to be difficult. Nevertheless excellent sperm retrieval success has been achieved with percutaneous testis aspiration, particularly in patients with a better prognosis as given by the presence of elongated spermatids or spermatozoa in the diagnostic testicular biopsy (Lewin *et al.*, 1996, 1999; Rosenlund *et al.*, 1998; Meng *et al.*, 2000). However, other studies suggest that even in cases of hypoplasia, percutaneous aspiration does not enable the recovery of sufficient sperm for injection and freezing (Ezeh *et al.*, 1998b; Mercan *et al.*, 2000).

Each biopsy should be immediately checked in order to avoid excessive multiple and bilateral sampling, but in most of the cases where the probability of finding sperm is worst, multiple biopsies seem to be needed (Hauser *et al.*, 1998; Amer *et al.*, 1999; Sousa *et al.*, 1999), although the recommended maximum number of biopsies ranges from one (Silber *et al.*, 1995, 1997; Verheyen *et al.*, 1995) to three (Hauser *et al.*, 1998; Amer *et al.*, 1999). Our present series confirm these findings, but establishes different approaches regarding the different subtypes of histopathologies, which show distinct inherent difficulties of finding a focus of spermiogenesis. In general, the number of cases with multiple and bilateral biopsies increased according to the severity of the diagnosis and to the type of spermatid retrieved. In hypoplasia, most of the patients had unilateral surgery (97% with sperm, 70% with late spermatids), with 1–3 fragments giving enough gametes for treatment and storage (72% with sperm, 57% with late spermatids). In MA, most of the patients also had unilateral

Table III. Outcome in Sertoli cell-only syndrome (SCOS)

	ICSI *n* (%)		ELSI *n* (%)	ROSI *n* (%)
	F	FT	F	F
Cycles	6	6	7	13
Injected oocytes	39	31	48	74
(mean, range)	6.5 (4-9)	5.2 (1-10)	6.9 (4-13)	5.7 (1-10)
Degenerated oocytes	6	1	7	4
Intact oocytes	33	30	41	70
Non-fertilized oocytes	9	7	10	34
1PN zygotes	3	3	9	25
2PN zygotes	21 (63.6)	19 (63.3)	21 (51.2)	11 (15.7)[a]
>2PN zygotes	0	1	1	0
Cleaved embryos (from 2PN)	20 (95.2)	14 (73.7)	21 (100)	9 (81.8)
Embryo grade (A+B)	16 (80)	14 (100)	20 (95.2)	9 (100)
No. embryos transferred (mean)	2.2	2.5	2.5	1.4
Day of embryo transfer (mean)	3.5	3.3	3.5	3.5
Cycles with embryo transfer	6	4	6	6
Clinical pregnancies	0	2	3	0

Fresh (F) and frozen–thawed (FT) gametes. [a]$P < 0.05$ to ICSI (F, FT) /ELSI.

surgery (83% with sperm, 71% with late spermatids), but these did not have enough gametes in 1–3 fragments (60% with sperm, 42% with late spermatids). In SCOS, the majority of the patients also had unilateral surgery (50% with sperm, 71% with late spermatids), but of these many fewer had enough gametes in 1–3 fragments removed (40% with sperm, 25% with late spermatids). In all other cases needing more biopsies, mature sperm or late spermatids were always found in the later specimens, whereas in bilateral biopsies they were retrieved in at least three samples from the contralateral testis. In cases with round spermatids or absence of gamete retrieval, only 31% had an unilateral sampling, and most had more than three specimens removed. This approach has thus enabled us to rescue mature gametes for treatment where the diagnostic testicular biopsy did not show any focus of late spermatids or spermatozoa, including 47% of the cases with MA (15/32) and 29% of the cases with SCOS (16/55). To achieve this difficult goal of careful search for a rare focus of spermiogenesis, we carried out small size (3 mm) testicular biopsies, enabling the exploration of different regions of the testis while avoiding excessive sampling, although this was also limited by the size and quality of the testis.

Another helpful tip that could guide successful sperm retrieval was given by the observation that seminiferous tubules are most probable to contain gametes if they appear larger and opaque (Schlegel, 1999; Amer *et al.*, 2000). Although we did not use microsurgery, our present series of 148 patients showed that very often dilated and opaque seminiferous tubules contained detached early germ cells but not spermatids or sperm. Nevertheless, because there is no individual certainty of success, it has also been suggested that the treatment testis biopsy should be performed before female ovarian stimulation, with the subsequent use of frozen–thawed gametes, which give normal fertilization and pregnancy rates (Gil-Salom *et al.*, 1996; Romero *et al.*, 1996; Friedler *et al.*, 1997; Al-Hasani *et al.*, 1999a; Ben-Yosef *et al.*, 1999; Sousa *et al.*, 1999, 2000). Another possibility is to perform the testis biopsy at least 1–2 days before oocyte retrieval, with the tissue being left in culture to enable a better gamete maturation and an increase in sperm motility (Zhu *et al.*, 1996; Liu *et al.*, 1997; Balaban *et al.*, 1999; Sousa *et al.*, 1999, 2000). The latter observation appears rather important, since immotile testicular sperm have been shown to elicit lower fertilization rates than motile testicular sperm (31 versus 61%), although motile sperm were found in the majority of reported cases, including SCOS ($n = 34$, 79%), MA ($n = 26$, 54%), or hypoplasia ($n = 10$, 70%) (Nagy *et al.*, 1998a). Regarding the present cases with microinjection, in hypoplasia most of the biopsies followed oocyte retrieval (40; 93%), and only three were performed 1 day (one case) or 2 days (two cases) before oocyte retrieval. The same applied to MA cases (41 cases at the same day: 93%; three cases 3 days before) and SCOS patients (18 cases in the same day; five cases 1 day and three cases 2 days before). Although this data does not allow statistical comparisons and we did not perform a quantitative study on the percentage of gametes showing motility during the period when motile sperm or late spermatids were found, motile gametes could also be recovered for treatment in all cases after 1–3 days of culture without any evidence of decreased quality. Furthermore, when gametes were initially immotile, after the culture period we were able to recover for injection at least in-situ motile cells, and when they initially exhibited only in-situ movements, in the majority of the cases we could find slow progressively motile gametes for injection. Similarly, we found no significant differences in the fertilization rate after injection of fresh ($n = 50$, 76%) or frozen–thawed ($n = 37$, 64.5%) sperm, and also after injection of fresh ($n = 33$, 55.4%) or frozen–thawed ($n = 6$, 44.7%) elongated spermatids, although frozen–thawed gametes showed a tendency for worse results. These findings were confirmed by pathology, except in the case of patients with hypoplasia, where the fertilization rate with frozen–thawed sperm was significantly lower.

Some clinical series showed that the relative frequency of finding sperm at treatment varied according to the diagnostic testicular biopsy, being ~25% for SCOS ($n = 6$–111), 48%

for MA (n = 1–76), and 74% for hypoplasia (n = 4–86) (Jow *et al.*, 1993; Devroey *et al.*, 1995; Lewin *et al.*, 1996, 1999; Friedler *et al.*, 1997; Amer *et al.*, 1999; Meng *et al.*, 2000). Some other studies presented a more detailed histopathological description, and showed that SCOS and MA cases could be subdivided into complete and incomplete syndromes. With this subdivision, the prognosis of finding sperm at treatment changed, with some studies showing drastic results, being 0% in complete SCOS (n = 3–11) or MA (n = 2–8), and 100% in incomplete SCOS (n = 2–7) or MA (n = 1–11) (Gil-Salom *et al.*, 1995; Yemini *et al.*, 1995; Kahraman *et al.*, 1996; Mulhall *et al.*, 1997; Ubaldi *et al.*, 1999; Westlander *et al.*, 1999). However, those findings could be due to the low number of patients studied, and thus contrasted with other larger clinical series, where the probability of finding sperm at treatment showed mean values of 95% in hypoplasia (n = 10–16), 52% in complete MA (n = 2–60), 69% in incomplete MA (n = 3–16), 22% in complete SCOS (n = 6–62) and 90% in incomplete SCOS (n = 2–50) (Tournaye *et al.*, 1996, 1997; Silber *et al.*, 1997; De Croo *et al.*, 2000). Our present results from 148 consecutive patients revealed very similar figures for hypoplasia (97.7%, n = 44), complete MA (53.3%, n = 15) and incomplete MA (65.6%, n = 32), but showed divergent results regarding SCOS cases (2.9% for complete SCOS, n = 35; 72.7% for incomplete SCOS, n = 22). However, in successful hypoplasia cycles, 82.8% (n = 53) had sperm for injection whereas 17.2% (n = 11) needed late spermatids; in MA cases the success rate was worst (61.7%, n = 29), with about half of the cycles using sperm (n = 22) and the other half using late spermatids (n = 21); and in SCOS cases the picture was again poor, with only 29.8% of the patients enabling retrieval of gametes for treatment (63.2% with sperm, n = 12; 36.8% with elongated spermatids, n = 7). These differences may be due to the differing origins of the populations. In fact, some cases of incomplete MA showed one focus of early spermiogenesis, and this subgroup had a similar rate of success for sperm retrieval as cases with complete MA, whereas patients with one focus of late spermiogenesis had a much higher chance of success (93.3%). Similarly, incomplete SCOS cases showed two further subgroups, one with a focus of premeiotic cells and another with one focus of early spermiogenesis, but these subgroups all had a very high chance of sucess for sperm retrieval.

In relation to the clinical outcome, most of the series only reported ICSI cycles, without specifying results per pathology or making reference to spermatid injection cycles. In those series, the fertilization rate after ICSI was shown to vary between 39–69% and the cleavage rate between 66–97% (n = 13–179), the high quality embryo rate was 56–77% (n = 19) and the pregnancy rate per cycle with embryo transfer showed a mean of 33% (Devroey *et al.*, 1995, 1996; Silber *et al.*, 1995; Kahraman *et al.*, 1996; Fahmy *et al.*, 1997; Friedler *et al.*, 1997; Mansour *et al.*, 1997; Ghazzawi *et al.*, 1998; Houritz *et al.*, 1998; Madgar *et al.*, 1998; Lewin *et al.*, 1999; De Croo *et al.*, 2000; Mercan *et al.*, 2000). In our present series of 87 ICSI cycles, the fertilization (71.4%), cleavage (91.7%), high quality embryo (85.9%) and clinical pregnancy (31.7%) rates were similar.

On the contrary, only a few series presented detailed clinical outcomes after sperm injection per type of pathology. In SCOS cases, the fertilization rate after ICSI was 38–44% (n = 40), the cleavage rate was 79% (n = 22), the high quality embryo rate was 57–61% (n = 40), and the mean pregnancy rate per cycle with embryo transfer was 40% (n = 59). In cases of MA, the fertilization rate was similar (42–46%, n = 20), the cleavage rate was 61% (n = 13), the high quality embryo rate was higher (80–86%, n = 20), and the mean pregnancy rate per cycle with embryo transfer was 57% (n = 40). Finally, in hypoplasia, the fertilization rate was higher (67%, n = 11), the cleavage rate was 83% (n = 11), the high quality embryo rate was 71% (n = 11), and the pregnancy rate per cycle with embryo transfer was 54–60% (n = 46) (Tournaye *et al.*, 1996; Silber *et al.*, 1996, 1997; Al-Hasani *et al.*, 1999a; De Croo *et al.*, 2000). These rates support previous findings (Nagy *et al.*, 1998b), which showed no significant differences between ejaculated and testicular sperm regarding timing of oocyte activation, pronucleus formation and embryo cleavage. In our 87 ICSI cycles, the rates of fertilization, cleavage and high embryo quality were relatively higher in all three syndromes (63.5, 85, 88.2% respectively in SCOS, n = 12; 66.3, 84.1, 86.2% respectively in MA, n = 22; and 74.8, 95.5, 85.4% respectively in hypoplasia, n = 53), although the clinical pregnancy rate was lower (20% in SCOS, 35% in MA, 32.1% in hypoplasia), showing a similar rate to the largest series of cycles using either ejaculated or testicular sperm injection (Mansour, 1998; Bonduelle *et al.*, 1999).

Including the largest clinical series presented by our group (n = 59), the analysis of all reported cases using elongated spermatid injections (n = 166) demonstrates that, in comparison with ICSI, late spermatids appear to be associated with a significantly lower fertilization rate (48.4%), but relatively similar cleavage (90%) and pregnancy (28.9%) rates (Table IV) (Fishel *et al.*, 1995, 1996; Tesarik *et al.*, 1995, 1996, 1999; Chen *et al.*, 1996; Mansour *et al.*, 1996; Amer *et al.*, 1997; Antinori *et al.*, 1997a; Araki *et al.*, 1997; Vanderzwalmen *et al.*, 1997; Barak *et al.*, 1998; Bernabeu *et al.*, 1998; Kahraman *et al.*, 1998; Sofikitis *et al.*, 1998c; Al-Hasani *et al.*, 1999b; Sousa *et al.*, 1999). However, the present analysis by type of pathology did not show significant differences between ICSI and ELSI cycles, with either fresh or frozen–thawed gametes, except in the fertilization rate in hypoplasia cases. Although two cases of congenital malformations and chromosome aneuploidy were recently described (Zech *et al.*, 2000), the overall results do not signal an increase of risk in comparison with ejaculated or testicular sperm (Tarlatzis and Grimbizis, 1999).

On the contrary, results from intact round spermatid injections (n = 249), including the largest clinical series from our group (n = 91), show significantly lower fertilization (21.8%) and pregnancy (2.8%) rates (Table V) (Tesarik *et al.*, 1995, 1996, 1999; Amer *et al.*, 1997; Antinori *et al.*, 1997a,b; Vanderzwalmen *et al.*, 1997; Yamanaka *et al.*, 1997; Barak *et al.*, 1998; Bernabeu *et al.*, 1998; Kahraman *et al.*, 1998; Al-Hasani *et al.*, 1999b; Sousa *et al.*, 1999). These findings suggest that the presence of an oocyte-activating substance in round spermatids (Sousa *et al.*, 1996) is not enough to

Table IV. Review of outcome in elongated spermatid injection (ELSI) cycles

Author	Cycles	MII injected	2PN zygotes	Cleaved (from 2PN)	Clinical pregnancies
Fishel *et al.*, 1995, 1996	1	10	1	1	1
Tesarik *et al.*, 1995, 1996	4	23	14	14	0
Chen *et al.*, 1996	1[a]	13	4	2	0
Mansour *et al.*, 1996	15[b]	105	40	–	2
Amer *et al.*, 1997	3	34	19	18	2
Araki *et al.*, 1997	9	130	55	55	3
	14	–	–	–	5
Antinori *et al.*, 1997a	17	123	71	55	3
Vanderzwalmen *et al.*, 1997	8	36	23	23	3
Barak *et al.*, 1998	13	137	49	–	0
Bernabeu *et al.*, 1998	1	7	3	3	1
Kahraman *et al.*, 1998	3	31	24	17	2
Sofikitis *et al.*, 1998	13	79	52	41	2
Al Hasani *et al.*, 1999b	2	18	10	–	2
Sousa *et al.*, 1999	20	166	74	69	9
Tesarik *et al.*, 1999	1	12	6	6	1
	2	–	–	–	2
Present results	39	315	155	147	10
Total (%)	166	1239	600/1239 (48.4)	451/501 (90)	48/166 (28.9)

[a]Case of obstructive azoospermia without sperm in epididymus.
[b]Four ELSI cycles (two liveborn) + 11 mixed ELSI/ROSI cycles.

Table V. Review of outcome in round spermatid injection (ROSI) cycles

Author	Cycles	MII injected	2PN zygotes	Cleaved (from 2PN)	Viable clinical pregnancies
Tesarik *et al.*, 1995, 1996	7	39	14	14	2[a]
Amer *et al.*, 1997	56	610	110	79	0
Antinori *et al.*, 1997a,b	21	150	82	62	3[b]
Vanderzwalmen *et al.*, 1997	32	260	57	49	1[b]
Yamanaka *et al.*, 1997	9	53	34	30	0
Barak *et al.*, 1998	8	37	10	–	1[a]
Bernabeu *et al.*, 1998	8	69	7	7	0
Kahraman *et al.*, 1998	20	199	51	31	0
Al Hasani *et al.*, 1999	4	49	9	–	0
Sousa *et al.*, 1999	50	394	43	43	0
Tesarik *et al.*, 1999	1	6	2	2	0
Present results	33	200	31	24	0
Total (%)	249	2066	450/2066 (21.8)	341/431 (79.1)	7/249 (2.8) 4/246 (1.6)

[a]Patients with previous late spermatids/sperm in ejaculates.
[b]Patients without previous late spermatids/sperm in ejaculates/diagnostic testicular biopsy.

ensure proper fertilization, and confirm the low developmental potential of the embryos derived by this method (Aslam and Fishel, 1999; Balaban *et al.*, 2000). Injection of intact round spermatids was originally proposed due to the uncertainty about the position of the proximal centriole and of the site of concentration of the oocyte-activating substance. In the human, the proximal centriole, which is responsible for centrosome inheritance and for the zygote and embryo centrosome cycle (Sousa and Tesarik, 1994; Tesarik *et al.*, 1998; El Shafie *et al.*, 2000), is firmly attached to the basal nuclear envelope only at a late point in the round spermatid stage (Holstein and Roosen-Runge, 1981), whereas the oocyte-activating substance is known to be concentrated much later at the equatorial region of the spermatozoon head (Parrington *et al.*, 1996; Heyers *et al.*, 2000). However, data from animal experiments have shown that an alternative procedure, round spermatid nucleus injection (ROSNI), is superior to injection of intact round spermatids (Ogura *et al.*, 1993; Sofikitis *et al.*, 1994, 1996a,b, 1998b,c; Kimura and Yanagimachi, 1995; Yamamoto *et al.*, 1999a). Although successful ROSNI data as applied to humans remains scarce, it may prove to be a better alternative to ROSI, although most pregnancies ended in abortions (Hannay *et al.*, 1995; Tanaka *et al.*, 1996; Sofikitis *et al.*, 1996a, 1997, 1998b,c). Notwithstanding, experiments in the human showed that ROSI is not adversely affected by the use of a slightly larger microinjection pipette (as shown by the very low oocyte degeneration rate: Tables II, III), nor by the presence of a larger cytoplasmic layer surrounding the round spermatid nucleus (rupture of the cytoplasmic membrane and of the nuclear envelope is rapidly achieved after microinjection)

(Sousa *et al.*, 1996). Similarly, the human round spermatid has been shown to harbour an oocyte-activating substance that is already capable of inducing free calcium intracellular oscillations (Sousa *et al.*, 1996), a fact also proven by recent successful secondary spermatocyte injections followed by healthy human births (Sofikitis *et al.*, 1998a). On the contrary, in-vitro culturing of round spermatids (Cremades *et al.*, 1999, 2001; Sousa *et al.*, 2002) suggests that the low developmental potential of such cells may instead be due to a genetic block (Aslam *et al.*, 1998; Tesarik *et al.*, 1998; Sousa *et al.*, 2000). Other authors also suggested a new culture medium for rendering round spermatids more viable (Sofikitis *et al.*, 1994, 1996a,b, 1997, 1998a,b,c; Yamamoto *et al.*, 1999a). However, the medium used here has all the supplements required for a short (24 h) culture period (lactate, glucose, iron, vitamins and synthetic serum substitute). Furthermore, long-term in-vitro culturing experiments with much more specialized and enriched media were also unable to induce a proper maturation and developmental potential of round spermatids (Cremades *et al.*, 1999, 2001; Sousa *et al.*, 2002). These findings thus strongly suggest that isolated round spermatids retrieved from cases of complete MA or from cases of SCOS with a focus of germ cells arrested at meiosis may represent occasional events of meiosis completion, with round spermatids still harbouring important genetic defects responsible for their poor developmental competence, and that for these cases better treatment alternatives should be sought (Tesarik *et al.*, 2001).

Note added in proof

All ongoing pregnancies from HP, MA and SCOs have resulted in the birth of healthy babies.

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