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Fertilization and pregnancy using intentionally cryopreserved testicular tissue as the sperm source for intracytoplasmic sperm injection in 10 men with non-obstructive azoospermia

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Testicular tissue extraction (TESE) to obtain spermatozoa for use with intracytoplasmic sperm injection (ICSI) has recently been employed in patients with non-obstructive azoospermia. Standard protocol is to retrieve a new sample of testis tissue on the day of oocyte recovery. Unfortunately, ~30% of men will possess no spermatozoa in their tissue, making ICSI an impossibility. We investigated whether testicular tissue that was intentionally obtained well before any planned ICSI cycle and cryopreserved could then serve as an efficacious sperm source in a subsequent ICSI cycle. This study reports on 10 men with non-obstructive azoospermia who did have spermatozoa found within their testis tissue at the time of TESE and who chose to use their frozen samples as the source of spermatozoa for a later cycle of ICSI. In 19 cycles the overall fertilization rate was 48%. Embryo transfer occurred in 89% of cycles. Two couples have achieved pregnancy (one ongoing, one delivered). All patients except one had multiple vials of frozen tissue remaining following their first cycle. This approach is offered as an alternative to repeated testicular tissue sampling, as the availability of spermatozoa is assured prior to the initiation of ovulation induction. This tissue can be harvested at the same time as diagnostic biopsy, thereby minimizing the number of surgical procedures.

Key words: azoospermia/cryopreservation/ICSI/spermatozoa/TESE

Introduction

Until recently, the only avenues of therapy open to the man with non-obstructive azoospermia were donor insemination, adoption, or child-free living. Dramatically, new roads have recently been paved that offer the promise of fatherhood to these unfortunate men with the realization that individual whole spermatozoa may actually be present in the surgically retrieved testicular tissue of a proportion of non-obstructive azoospermic men (Devroey *et al.*, 1994, 1995a; Gil-Salom *et al.*, 1995a,b; Kahraman *et al.*, 1996). Pioneering and innovative work by several investigators has shown that these spermatozoa could be isolated and injected directly into the oocyte cytoplasm, that fertilization and embryo development could occur and that pregnancy and live births could ensue (Schoysman *et al.*, 1993; Silber, 1995; Tournaye *et al.*, 1995, 1996). Numerous reports now document the efficacy of testicular sperm extraction (TESE) using freshly obtained testicular tissue to provide the source of spermatozoa for a simultaneous intracytoplasmic sperm injection (ICSI) attempt.

However, no spermatozoa can be isolated from the harvested testicular tissue from 25–30% of men who have non-obstructive azoospermia, even after exhaustive searching (Mulhall, 1996). Unfortunately, there are no reliable pre-operative parameters or clinical clues that predict whether spermatozoa will or will not be retrievable from the tissue of a particular individual. Therefore, a disastrous potential exists when using freshly obtained tissue in that no spermatozoa might be available for ICSI on the day of oocyte recovery. This outcome instantly negates the tremendous physical, emotional and financial effort and expense the couple endured to reach the point of oocyte harvest. An additional problem surfaces for those couples who require a second or third TESE/ICSI attempt. New tissue has to be harvested each time which is a costly, invasive event that, just possibly, may not always yield spermatozoa. Repeated testicular surgery may also lead to significant, permanent testicular injury (Schlegel, 1996). Finally, clinicians for both partners must coordinate their efforts, limiting the availability of attempts and the flexibility for scheduling treatment of the couples.

The use of frozen–thawed testicular tissue intentionally retrieved at a time remote from any ICSI cycle may solve these problems. Diagnosis of sperm availability is made prior to any ovarian stimulation and/or oocyte recovery. A separate diagnostic biopsy is not required, significantly altering the current approach that urologists employ in the evaluation of men with non-obstructive azoospermia. Both diagnostic biopsy for histological evaluation and therapeutic biopsy for documentation of the presence of whole spermatozoa within the tissue can be accomplished with one surgical endeavour. Couples in whom no spermatozoa are found during TESE are spared the physical and fiscal demands of ICSI. For those couples with the confirmed presence of spermatozoa within the harvested tissue, an ICSI cycle can be initiated any time that it is convenient. Since the tissue can be divided into multiple aliquots prior to cryopreservation, and each aliquot can potentially serve as a sperm source for a single ICSI attempt, numerous cycles may result from only one tissue retrieval. There would always exist the rare possibility that in those specimens with an extremely low number of spermatozoa to begin with, there would be no useable spermatozoa upon thawing and a fresh sample would need to be obtained. This

circumstance did not occur in the patients reported herein. Our approach is different to that suggested by Craft and Tsirigotis (1995), since the homogenized tissue itself is cryopreserved prior to isolation of individual spermatozoa. However, we agree with these authors who advocate a minimal amount of overall processing for these types of samples.

Based upon our favourable experience with frozen–thawed epididymal spermatozoa (Oates *et al.*, 1996), we sought to investigate if frozen–thawed testicular tissue obtained from men with non-obstructive azoospermia could serve as an efficacious sperm source for ICSI by documenting fertilization rates, embryo development and pregnancy achievement.

Materials and methods

Patient population

A group of 10 men (mean age: 35.8 years, range 28–43) had spermatozoa present within their fresh testicular parenchyma at the time of initial tissue harvesting (see below), and the resultant frozen–thawed specimens have been used as the sperm source for 19 ICSI procedures. All men had presented with normal volume azoospermia. History revealed cryptorchidism in two men (one had a unilateral orchidectomy) but no other relevant information was evident. Physical examination demonstrated testes of diminished size and consistency in nine patients while one had markedly atrophic gonads (patient no. 4). The vasa deferentia and epididymides were palpably normal in all patients. There were no varicoceles present. Mean serum follicle stimulating hormone (FSH) was 12.7 mIU/ml (range 3.9–25), indicating an appropriate compensatory response of the pituitary to seminiferous tubule dysfunction. Diagnostic testicular biopsy (accomplished either previously or at the time of TESE) demonstrated: Sertoli cell only in three patients, early maturation arrest (presence of spermatogonia and spermatocytes only; $n = 1$), late maturation arrest (presence of spermatogonia, spermatocytes and a few early spermatids; $n = 2$) and a mixed pattern consisting of a combination of the above ($n = 4$). Karyotypic analysis revealed a 47, XXY pattern in patient no. 4. If any question of obstruction existed, full scrotal exploration with the intent of performing reconstructive microsurgery was carried out. There was no evidence of obstructive pathology in any of these patients as assessed by their physical examination and FSH concentration or by further exploration of the vas and epididymis.

Ten female partners (mean age: 33.6 years, range 28–40) were fully evaluated prior to TESE with routine ovulatory, tubal, and uterine assessment. Five of the women were $\geqslant$35 years old. Patient no. 1's wife had mild endometriosis documented laparoscopically, patient no. 6's partner had a large submucous fibroid removed via hysteroscopy, and patient no. 7's spouse had undergone numerous prior cycles of artificial insemination by donor (AID) with and without ovarian stimulation with no resultant pregnancy. In the remaining seven women, no obvious abnormalities were found. All couples were given a full and complete explanation of the various options, including the use of fresh testicular tissue for ICSI. All chose an approach of tissue harvesting, determination of sperm presence or absence, cryopreservation of the tissue and use of a portion of the frozen–thawed tissue as the sperm source for a future ICSI cycle.

Technique of testicular tissue extraction

After coordination of the time and date with the embryology laboratory of the Reproductive Science Center of Boston, MA, USA, therapeutic and diagnostic testicular biopsy as well as simultaneous 'wet preparation' were carried out. Using 0.25% bupivicaine as a local block of the spermatic cord supplemented with a small amount of i.v. sedation, the left hemiscrotum was opened for a short distance down to the surface of the testicular capsule. A 1 cm transverse incision was made in the tunica albuginea and the testis was compressed to extrude the seminiferous epithelium which was sharply excised. The single tissue sample obtained was divided into 10 individual 0.4 mm pieces and placed into 1 ml of test yolk buffer (TYB; Irvine Scientific, Santa Ana, CA, USA) in a 12 ml conical tube. A second 0.3 mm sample was extracted and blotted several times on a glass microscope slide in a drop of TYB prior to placement in Bouin's solution for formal histological analysis. The 'wet prep' so created was immediately viewed under $\times$400 magnification. If any whole, fully formed spermatozoa were seen scattered amongst the early spermatogenic cells, the incision was closed in layers as spermatozoa were expected to be found in adequate numbers upon formal processing (see below). However, if no spermatozoa could be identified, there existed no guarantee that spermatozoa would be present, even upon more rigorous search in the embryology laboratory. This possibility mandated removal of tissue from the contralateral testis in a fashion similar to the above. This approach maximized the chances that this operative procedure would be the only one required to answer the question of whether spermatozoa were indeed present and, if so, to provide enough tissue for several subsequent ICSI attempts. Following completion of tissue harvesting, the specimens were transported to the embryology laboratory for analysis, processing and cryopreservation. As stated above, the patient cohort for this study was composed only of those patients who had parenchymal spermatozoa and who went on to use it for ICSI. No complications occurred and all patients required a minimal amount of analgesics postoperatively. Formal scrotal exploration was carried out if the diagnosis of non-obstructive azoospermia was unclear based upon the clinical data. No patient in this series had an obstructive pathology.

Preparation and cryopreservation of testicular homogenates

Upon arrival at the laboratory, the biopsy specimens were allowed to settle and the supernatant was removed. A 5 µl aliquot was microscopically surveyed for the presence and number of spermatozoa. If spermatozoa were found, multiplication by 200 provided an estimate of the absolute number of spermatozoa present per ml. The supernatant was subsequently adjusted to a final concentration of 10% (v/v) glycerol (Sigma, St Louis, MO, USA)/TYB before being cooled to 4°C. The biopsy material was transferred to a sterile glass tube together with 1.0 ml of sterile glycerol (10% v/v) in TYB (TYB:glycerol) and the tissue homogenized using a loose fitting glass pestle and subsequently by repeated aspiration through a 16 gauge hypodermic needle. A 5 µl sample was searched using phase contrast microscopy for the presence of fully formed spermatozoa and the number/ml was calculated as described above. If spermatozoa were visualized, the homogenate was adjusted to a final volume of 5.0 ml of TYB:glycerol and either 500 µl or 1 ml aliquots were transferred to sterile labelled polypropylene tubes (Nunc Cryovials, USA Scientific Plastics, Ocala, FL, USA) and cooled to 4°C in a refrigerator. Using a programmable freezing unit all tubes were then cooled at –1°C/min to –7°C at which point ice crystal formation was induced before further cooling in a stepwise fashion to –30°C at 1°C/min and finally to –150°C at –5°C/min. All tubes were then transferred directly to liquid nitrogen and stored pending isolation of oocytes from the female partner.

Ovarian stimulation and isolation of oocytes

Pituitary gonadotrophin secretion in the female partner of each couple was suppressed by administration of leuprolide acetate 1 mg/day (Lupron, TAP Pharmaceutical, Deerfield, IL, USA) until serum oestradiol concentrations were $<$50 pg/ml and no follicles with

>10 mm diameter were visible on ultrasonographic images of each ovary. Suppression, once achieved, was maintained by continued administration of leuprolide acetate. Ovarian follicular development was induced by daily injections of exogenous gonadotrophin as either human menopausal urinary gonadotrophin (HMG; Pergonal, Serono, Randolph, MA, USA) or pituitary FSH (hFSH; Metrodin, Serono). Following day 6 of gonadotrophin administration, follicular development was monitored by serial ultrasound imaging of each ovary and serum oestradiol concentrations. A luteinizing dose of human chorionic gonadotrophin (HCG; Profasi, Serono: 10 000 IU) was injected when the serum oestradiol level exceeded 1000 pg/ml and three or more follicles of >17 mm diameter were evident. Ultrasound-guided transvaginal aspiration of follicular fluid was performed 36 h after HCG administration and oocytes isolated immediately from the aspirates. Cumulus cells were removed from each oocyte by incubation in hyaluronidase (Sigma; 80 units/ml) in Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL, Grand Island, NY, USA) supplemented with bovine serum albumin (BSA; Sigma; 0.1% w/v). The denuded oocytes were then rinsed and maintained in 30 ml drops of IVF-50 medium (Scandinavia IVF Science AG; Goteborg, Sweden) under mineral oil until injected with a single spermatozoon.

Technique of ICSI—sperm thaw, isolation, manipulation and injection

Once isolation of oocytes from follicular aspirates was confirmed, an aliquot of frozen testicular homogenate, prepared as described above, was thawed at room temperature with occasional gentle inversion. Thawed homogenates were diluted with four volumes of DPBS:BSA and centrifuged at 250 *g* for 10 min at room temperature. The resultant pellet was resuspended in 500 µl of DPBS:BSA and 5 µl drops were overlayed with mineral oil and maintained at 37°C. Individual spermatozoa were identified and transferred to successive adjacent drops of DPBS:BSA and then polyvinylpyrrolidone (PVP 10% w/v; Scandinavia IVF Science AG) immediately prior to being placed in the cytoplasm of an oocyte under phase contrast microscopy. Spermatozoa isolated from thawed homogenates in this fashion appeared no different from freshly ejaculated spermatozoa with respect to overall morphology, affinity for capillary glass or susceptibility to mechanical damage. Approximately 90% of the spermatozoa that were selected demonstrated occasional tail twitching, indicating viability. Rarely, overt motility with poor forward progression was observed. Only a few oocytes were injected with morphologically normal spermatozoa completely devoid of any activity. There was no movement of any degree in the spermatozoa employed to inseminate the four retrieved oocytes in the only cycle of patient no. 4.

Between 4 and 8 h after isolation of oocytes, individual spermatozoa were injected into the cytoplasm of metaphase II oocytes, following mechanical 'immobilization' of the sperm tail with the injection pipette. The individual spermatozoa selected were aspirated into a glass pipette (~5 µm internal, 8 µm external diameter) preloaded with PVP and positioned in a 5 µl drop of DPBS:BSA adjacent to an oocyte immobilized by gentle vacuum suction at the tip of the glass holding pipette. The tip of the injection pipette was advanced until the oocyte plasma membrane was perforated. The single spermatozoon was ejected from the pipette together with less than 1 pl of PVP and the injection pipette was then withdrawn from the oocyte completely. The entire procedure was performed utilizing Hoffman modulation contrast optics. Following sperm injection, the oocytes were returned to individual 30 µl drops of IVF-50 medium and kept at 37°C in an atmosphere of 5% CO_2 in air. Fertilization, as evidenced by formation of a second pronucleus, was confirmed at 16–20 h post-injection. Oocytes containing two pronuclei (2PN) were considered fertilized and maintained for an additional 48 h in IVF-50 medium.

Intrauterine transfer of embryos

At 66–70 h post-injection just prior to transfer, the number of blastomeres, degree of fragmentation and symmetry of each embryo was noted. Embryos with the greatest number of blastomeres, the least fragmentation and greatest symmetry were ranked highest in quality. The statistical significance of differences in distribution of blastomeres, fragmentation pattern and symmetry between embryos resulting from the use of cryopreserved testicular spermatozoa, cryopreserved epididymal spermatozoa and freshly ejaculated spermatozoa were determined using the Kolmogorov–Smirnov method for comparing categorical distributions between independent samples.

For each patient, one to five of the highest quality embryos were selected for transfer, the actual number being determined by the total number of embryos available, the patient's age and prior reproductive history. The zonae of embryos selected for transfer were perforated using acidified Tyrode's solution (Sigma) and each embryo was transferred back to culture medium for 30–90 min prior to transcervical placement in the uterine lumen using a semi-rigid catheter (Cook Ob/Gyn, Spencer, IN, USA). Embryos that were not transferred were cryopreserved without undergoing assisted hatching. Beginning on the day of oocyte harvesting, progesterone 50 mg i.m. was administered to the women throughout the luteal phase.

Results

This group of 10 men represents those for whom spermatozoa had been seen in their harvested tissue and the cryopreserved specimens had been used for ICSI. This subgroup was derived from the overall population of non-obstructive azoospermic patients, in whom spermatozoa have been found at TESE in 60% of cases.

Tissue harvesting and processing (Table I)

Six patients (60%) required only unilateral tissue retrieval while the remainder had bilateral TESE. The average number of cryopreserved vials was 8.4 (range 5–12). Two patients had 500 000 cryopreserved spermatozoa per vial, while the remaining eight had a mean number of 250 spermatozoa per vial (range 5–900).

Intracytoplasmic sperm injection (Table II)

A total of 19 cycles of ICSI was carried out: five couples went through one cycle while two couples underwent two, two couples accomplished three and one couple completed four cycles. The results are summarized in Table II. Out of the 19 cycles, 17 went to embryo transfer (89%). Two couples (nos 3 and 8) have achieved twin pregnancies for an ongoing per cycle pregnancy rate of 11%, a per transfer rate of 12%, and a per couple rate of 20%. One couple (no. 9) had an early biochemical pregnancy [cycle 9(b)] which quickly reverted to normal values indicating an early spontaneous loss. This event was not included in the calculation of either pregnancy rate or embryo implantation rate. The embryo implantation rate is 8% (four fetuses/50 embryos transferred). It should be noted that the eight oocytes retrieved from the female partner of patient no. 7 were characterized as 'dark, irregular, grainy and vacuolated.' This was consistent with an age-related effect (age 40 years) and her history of failed AID was, in retrospect, probably predictive of the poor oocyte quality. The female partner of patient no. 2 was also a poor responder, with only

Table I. Results of testicular tissue cryopreservation

Patient	Testicular histology	TESE (uni vs bilateral)	No. of vials cryopreserved	Cryovial volume (μl)	Total no. of spermatozoa/vial
1	Sertoli cell only	Unilateral	9	500	500
2	Early maturation arrest	Unilateral	5	1000	40
3	Late maturation arrest	Unilateral	10	500	500 000
4	Sertoli cell only	Bilateral	4	500	5
5	Mixed pattern	Unilateral	5	1000	100
6	Mixed pattern	Bilateral	8	500	50
7	Mixed pattern	Bilateral	12	500	900
8	Sertoli cell only	Bilateral	11	500	150
9	Late maturation arrest	Unilateral	10	500	500 000
10	Mixed pattern	Unilateral	10	500	240

Table II. Results of intracytoplasmic sperm injection (ICSI) using cryopreserved testicular spermatozoa

Patient no. (cycle)	Female age (years)	No. cryo vials used	No. vials remaining	No. oocytes injected	Fertilization rate (%)	No. resultant embryos	No. embryos transferred	No. embryos cryopreserved	Pregnancy (yes/no)	Result of pregnancy
1 (a)	35	1	8	10	60	6	4	1	No	
1 (b)		1	7	15	73	11	4	0	No	
1 (c)		1	6	11	27	3	3	0	No	
2 (a)	39	1	4	5	40	2	1	0	No	
2 (b)		1	3	1	0	0	0	0	No	
3 (a)	37	1	9	10	40	4	3	0	No	
3 (b)		1	8	9	89	8	5	0	Yes – twins	Delivered
4	28	4	0	4	25	1	1	0		
5 (a)	30	1	4	4	75	3	3	0	No	
5 (b)		2	2	9	33	3	3	0	No	
5 (c)		1	1	8	25	2	2	0	No	
5 (d)		1	0	7	43	3	2	0	No	
6	31	2	6	5	40	2	2	0	No	
7	40	1	11	8	0	0	0	0	No	
8	28	1	10	12	33	4	3	0	Yes – twins	Ongoing
9 (a)	30	1	9	3	100	3	3	0	No	
9 (b)		1	8	7	71	5	4	0	Biochemical	Early loss
9 (c)		1	7	8	75	6	4	0	No	
10	38	1	9	13	46	6	3	2	No	
Total				149	48	72	50	2		
Average	34	1.3		7.8[a]		3.8[a]	2.9[a]	0.30	11%/cycle	
Range		(1–4)		(1–15)		(0–11)	(1–5)		20%/couple	

[a]Per cycle.

five and one oocytes available for microinjection in her two cycles, respectively. She was aged 39 years at the time of her two stimulation attempts.

Embryo development

Embryo development at 40–45 h post-insemination for zygotes resulting from ICSI using cryopreserved testicular spermatozoa was compared with that for zygotes resulting from ICSI using fresh ejaculated and cryopreserved epididymal spermatozoa (Table III). Distribution of blastomeres, the degree of fragmentation and overall symmetry of embryos in each group at 40–45 h post-injection were compared. Development of embryos resulting from ICSI using cryopreserved testicular spermatozoa was not significantly different from that in the other two ICSI groups (freshly ejaculated and cryopreserved epididymal specimens).

Discussion

ICSI represents a great advance in the therapy of male factor infertility. ICSI is such a powerful technique that even frozen–thawed epididymal spermatozoa from men with unreconstructable ductal abnormalities can be used quite efficiently with conception rates equivalent to those cycles in which freshly obtained epididymal spermatozoa are employed (Chan *et al.*, 1995; Devroey *et al.*, 1995b; Oates *et al.*, 1996). Our present study attempts to further this evolution by eliminating some of the most important potential deficiencies that presently exist when using freshly obtained testis tissue from men with non-obstructive azoospermia. As our data confirm, viable fully formed spermatozoa can be retrieved from intentionally frozen–thawed testicular tissue (Romero *et al.*, 1996). In addition, these spermatozoa are capable of contributing to fertilization, embryo development and ongoing pregnancy. Embryos generated from the use of these spermatozoa are similar in quality (blastomere number, fragmentation pattern and symmetry) to embryos resulting from ICSI utilizing freshly ejaculated spermatozoa and cryopreserved epididymal spermatozoa in our programme, indicating that embryo development is not adversely affected by either the cryopreservation process or by the location of origin of the spermatozoa. The low embryo implantation rate of 8% may reflect subtle embryo deficiency

Table III. In-vitro development of embryos following ICSI using fresh ejaculated sperm (n = 1371), cryopreserved (cryo) epididymal spermatozoa (n = 82) and cryopreserved testicular spermatozoa (n = 72). Embryos were scored at 45–50 h post-ICSI; values are percentages

A. Blastomere number

	1	2	3	4	5	6	7	8	9	10
Fresh ejaculated	13.1	41.1	15.1	19.0	4.7	2.8	1.5	2.5	0.1	0.0
Cryo epididymal	13.4	45.1	11.0	25.6	2.4	1.2	1.2	0.0	0.0	0.0
Cryo testicular	27.8	34.2	13.9	16.5	2.5	3.8	0.0	1.3	0.0	0.0

No statistically significant difference existed between groups in blastomere number.

B. Blastomere fragmentation pattern

	A	B	C	D
Fresh ejaculated	14.4	49.0	31.6	4.9
Cryo epididymal	6.7	49.3	37.3	6.7
Cryo testicular	10.3	46.6	36.2	6.9

A: <10% fragmentation B: 10–30% C: 30–50% D: >50% fragmentation. No statistically significant difference existed between groups in degree of fragmentation.

C. Blastomere symmetry

	Good	Fair	Poor
Fresh ejaculated	26.5	56.5	17.0
Cryo epididymal	13.3	64.0	22.7
Cryo testicular	22.8	59.6	17.5

Good: blastomeres of equal size/regular shape. Fair: some variation in blastomere size/shape. Poor: blastomeres of unequal size/embryo irregular in shape.
No statistically significant difference existed between groups in blastomere symmetry.

that occurs when testicular spermatozoa are used from patients with spermatogenic failure, as previously suggested (Hovatta *et al.*, 1995; Tournaye *et al.*, 1996). A larger series of patients will help answer this question. We therefore offer, as an alternative approach, frozen–thawed testicular tissue as the source of spermatozoa for ICSI in those patients with non-obstructive azoospermia in whom testicular tissue is the only place spermatozoa may be found. By temporally dissociating TESE and ICSI, a number of potential pitfalls with the fresh tissue/ICSI combination technique are avoided.

By extracting testicular tissue on a day completely different from oocyte harvesting, a full, exhaustive and non-pressured examination of the tissue may be accomplished. For those in whom spermatozoa are found, the news is reassuring. The couple can then begin preparing for the rigours of an ICSI cycle, content with the knowledge that their efforts will indeed lead to an opportunity for fertilization and pregnancy. Since multiple vials of cryopreserved material are routinely obtained, the patient may not require any further TESE procedures and the couple is free to proceed into an ICSI cycle at any time appropriate and convenient for them. For those men in whom intensive search detects no whole spermatozoa, the news is devastating but at least no ICSI cycle with its attendant phlebotomy, ultrasonography, injections, and oocyte retrieval was accomplished in vain. It is possible that when only a very few spermatozoa are seen, there may be none available upon thawing and plans for an immediate fresh tissue harvesting may need to be in place should this situation arise. This did not occur to the patients in this study.

Use of frozen–thawed tissue simplifies the management of the non-obstructive azoospermic male. If the diagnosis of non-obstructive azoospermia is secure based on all available clinical evidence, the couple should be informed of the option of TESE and this should be carried out simultaneous with diagnostic testis biopsy. No formal diagnostic biopsy need precede TESE. Since the only way the spermatozoa are eventually used is in combination with ICSI, the couple's decision to employ ICSI if spermatozoa are found in the tissue must predate the acquisition of the tissue. All couples should undergo counselling regarding ICSI in terms of its risks, benefits, financial aspects, etc. At the same time, a frank discussion of the genetic aetiologies underlying spermatogenic failure as well as the implications for transmission to any offspring should occur (Silber *et al.*, 1995). Once both are accomplished, the couple can make an informed choice of whether ICSI is a procedure that they will employ should spermatozoa indeed be present. Only then should testicular tissue harvesting be scheduled. When tissue is extracted, immediate analysis in the embryology laboratory, if possible, or at least in the operating room, will dictate whether more tissue need be obtained. If spermatozoa are present, calculation of the number of whole spermatozoa/unit volume is essential to maximize the number of vials cryopreserved, each hopefully providing enough spermatozoa for a subsequent ICSI cycle.

Our study design anticipated the thoughts of prior authors who suggested that intentional cryopreservation of testicular tissue from men with non-obstructive azoospermia may be efficacious and would alleviate the necessity for subsequent testicular tissue extractions performed on the day of oocyte recovery (Gil-Salom *et al.*, 1996; Salzbrunn *et al.*, 1996). This is a similar strategy to that which we use for epididymal spermatozoa (Oates *et al.*, 1996). The data in the publication of Gil-Salom *et al.* (1996) is a compilation of the use of frozen–thawed testicular tissue from men with both normal spermatogenesis (the overwhelming majority, 76%) and non-obstructive azoospermia (only 24%). This present report focuses only on the non-obstructive azoospermic male and provides detailed information on fertilization, embryo development and pregnancy in this select group when frozen–thawed tissue is used.

In conclusion, since there is no way to predict with certainty the availability of testicular spermatozoa in an individual patient with non-obstructive azoospermia, a tissue sample should be inspected prior to initiation of an ICSI cycle, ideally at the same time as a diagnostic biopsy. If spermatozoa are present, the tissue can be cryopreserved and become the 'sperm source' for multiple, future ICSI cycles. Only one tissue extraction – both diagnostic and therapeutic in nature – is usually required. As our data suggest, the use of intentionally frozen–thawed testicular spermatozoa obtained from men with non-obstructive azoospermia is efficacious and its use may overcome many of the inherent problems that arise with the use of freshly retrieved tissue.

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