

# The value of sperm pooling and cryopreservation in patients with transient azoospermia or severe oligoasthenoteratozoospermia

S.Lahav-Baratz, E.Rothschild, B.Grach, M.Koifman, H.Shiloh, D.Ishai and M.Dirnfeld<sup>1</sup>

IVF Unit, Department of Obstetrics and Gynecology, Carmel Medical Center and the Technion Faculty of Medicine, Haifa, Israel

<sup>1</sup>To whom correspondence should be addressed at: IVF unit, Department of Obstetrics and Gynecology, Carmel Medical Center, Michal 7 street, Haifa 34362, Israel. E-mail: dirnfeld\_martha@clalit.org.il

**BACKGROUND:** A transient state of azoospermia may occur due to toxic, environmental, infectious or iatrogenic conditions. Finding sperm in the ejaculate of such patients is often unpredictable and may be critical in IVF treatment. In the present study, the approach of pooling and cryopreservation of sperm is evaluated. Cryopreservation was performed in a unique group of patients in whom no sperm had been found in at least one previous sperm examination and in patients diagnosed as suffering from non-obstructive azoospermia in whom, occasionally, sperm were found. **METHODS:** A total of 157 semen pooling and cryopreservation procedures in 53 patients was performed between January 1998 and December 2000 in our centre. Forty five of these patients underwent an IVF–ICSI treatment during the study period. In 32 patients, fresh sperm were used to perform ICSI. In 13 patients no sperm were available, and the previously frozen sperm were used. **RESULTS:** Using our pooling system, 13 IVF–ICSI cycles were rescued. In seven patients with a previous testicular biopsy due to azoospermia, sperm cryopreservation was possible. Overall, 13 pregnancies (10 deliveries, two ongoing pregnancies and one missed abortion) were achieved. **CONCLUSION:** The introduction of semen banking for patients with transient azoospermia may increase the chance of pregnancy using their own sperm.

*Key words:* azoospermia/cryopreservation/semen pooling/sperm

## Introduction

Assisted reproductive technologies have enabled patients with severe male factor or azoospermia to father children using ICSI (Palermo *et al.*, 1992; Craft *et al.*, 1993; Van Steirteghem *et al.*, 1993; Tournaye *et al.*, 1994). Unfortunately, successful IVF–ICSI cycles in patients with severe male factor, i.e. severe oligoasthenoteratozoospermia (OAT) or non-obstructive azoospermia, can be complicated by unanticipated cancellation or additional testicular biopsy (TESE) when sperm are unavailable on the day of oocyte retrieval.

In certain patients defined as azoospermic, sperm may in time appear in the ejaculate. Spermatogenic arrest leading to azoospermia can occur due to heat, infection, hormonal or nutritional factors and may be reversible either spontaneously or after specific treatment (Martin-du Pan and Campana, 1993). States of transient azoospermia caused by genital infection have been reported and a sufficient number of motile sperm could be retrieved for injection after anti-inflammatory treatment (Montag *et al.*, 1999a,b).

Patients diagnosed as azoospermic may occasionally be found to have sperm in the ejaculate. When such a situation exists it may be defined as ‘virtual azoospermia’ (Tournaye *et al.*, 1995).

Fluctuations in sperm count and quality, although difficult to predict, are well documented. Annual variations in sperm count are significantly correlated with birth rate (Fisch *et al.*, 1997). Intra- and inter-individual changes of sperm parameters were observed in assisted reproduction patients as well as in the overall population (Ossenbuh, 1998).

Such fluctuations in sperm count in severe OAT patients may lead to difficulties in finding sperm in the ejaculate. A certain proportion of these patients will be found azoospermic on the day of oocyte retrieval.

In both transient azoospermia and/or severe OAT, pooling and cryopreservation of sperm may rescue IVF–ICSI cycles and prevent unnecessary testicular biopsy. It is difficult to predict which patients might benefit from sperm pooling and cryopreservation.

The present study was undertaken to evaluate the meticulous follow-up, pooling and cryopreservation of sperm for future use in such patients.

## Materials and methods

### *Patient population*

Sperm cryopreservation has been available in our centre since 1986. For this retrospective study we included patients in whom pooling

and cryopreservation were performed from January 1998 to December 2000. Eligibility criteria for inclusion in the study included: (i) patients with severe OAT in whom no sperm were found on at least one previous sperm examination (group 1); and (ii) patients diagnosed as suffering from non-obstructive azoospermia in whom, on at least one occasion, sperm were found in the ejaculate (group 2).

Exclusion criteria for the present study included patients in whom cryopreservation was performed prior to chemo-radiotherapy, and patients with difficulties in providing semen samples.

All patients were documented for past medical history: the duration of infertility, the hormonal profile and any history of hormonal therapy. History of testicular biopsy was also noted.

Patients were requested to provide an ejaculate to the IVF laboratory at least 3–4 times in a period of 1 month, at initiation of the IVF cycle, or before.

When the semen was contaminated with many leukocytes, appropriate antibiotic therapy was administered to patients before they entered the pooling programme.

### Sperm preparation and cryopreservation

Sperm analysis was performed using the Makler chamber (Sefi Medical Instruments, Haifa, Israel). All semen were then centrifuged at 600 *g* for 10 min. Five  $\mu$ l drops of the pellet were screened using an inverted microscope ( $\times 200$ ) magnification; the same investigator examined all samples.

In cases where sperm showed any indication of movement, a sample was frozen. An equal amount of cryopreservation medium was added to the sperm after centrifugation. TEST yolk buffer containing glycerol (Irvine Scientific, Irvine, CA, USA) was used as the freezing medium. Freezing protocol started at room temperature, then at a rate of  $-1.7^{\circ}\text{C}/\text{min}$  to  $-6^{\circ}\text{C}$  and then to  $-100^{\circ}\text{C}$  at a rate of  $-5^{\circ}\text{C}/\text{min}$ .

Thawing of samples was performed by warming the ampoules at  $37^{\circ}\text{C}$ . Equal volumes of human tubal fluid (HTF) medium containing 2.5% serum substitute supplement (Irvine Scientific) was then added. The samples were centrifuged at 600 *g* for 10 min, continuing with additional wash.

All patients were asked to abstain from intercourse for 72 h and to bring fresh semen on the day of oocyte retrieval. When sperm were not present in the fresh sample, we requested the patient provide an additional ejaculate. If sperm were again absent, thawing was performed.

### Protocol of ovarian stimulation, oocyte retrieval and ICSI procedure

Ovarian stimulation was performed in all women with long down-regulation protocol as previously described (Dirnfeld *et al.*, 1993).

ICSI procedure was carried out using a Nikon microscope with Nomarski optics with two three-dimensional manipulators (Narishige, Tokyo, Japan) as fully described (Ben-Yosef *et al.*, 1999). Fertilization was confirmed 16–20 h after injection by the presence of two distinct pronuclei under the inverted microscope. Cleavage was assessed 24 h later and the embryos were classified according to their morphological appearance. Up to three embryos were selected for transfer into the uterine cavity. In patients older than 38 years or in cases of repeated IVF failures, four embryos were replaced. Luteal supplementation consisted of i.m. progesterone in oil (Gestone<sup>®</sup>; Paines and Byrne, Greenford, Surrey, UK), 100 mg/day.

## Results

Between January 1998 to December 2000, 61 patients meeting the established criteria were referred to our laboratory. There

**Table I.** Characteristics of the patients that were referred for pooling and cryopreservation according to our criteria

	Severe OAT	Azoospermia	Total <i>P</i>
No. of cases	43	18	61
Age (male partner) (mean $\pm$ SD)	33.6 $\pm$ 6.6	33.2 $\pm$ 5.7	NS
Duration of infertility (mean $\pm$ SD)	5.0 $\pm$ 4.5	4.2 $\pm$ 3.2	NS
Patients with FSH > 12 IU/ml (male)	13 (30%)	5 (28%)	NS
Pooling and cryopreservation	42	11	53
No cryopreservation	1	7	8
Patients enrolled for IVF-ICSI with:			
fresh sperm	27	5	32
frozen-thawed sperm	8	5	13
Total	35	10	45

OAT = oligoasthenoteratozoospermia.

were 43 severe OAT patients (group 1), and 18 azoospermic patients (group 2).

The age, duration of infertility and FSH concentration in the two groups were similar (Table I). Testicular volume measurements were within normal limits in all except one patient for each group. Additionally, the mean number of semen samples collected per patient in the pooling process was similar in the two groups.

In 23 of the patients enrolled, testicular biopsy was previously recommended by the physician. In 13 of these patients, TESE procedure had been performed in the past. In seven patients of the 13, sperm were found and cryopreservation was performed. They were diagnosed as non-obstructive azoospermia. Histological findings on biopsy included hypospermatogenesis in five patients, Sertoli cells only in a left testis in one patient and maturation arrest in one patient.

In one patient defined as severe OAT and in seven patients defined as azoospermic, no sperm suitable for cryopreservation were found on repeated examination.

Cryopreservation of sperm was performed for 53 patients, 42 in group 1 and 11 in group 2.

Of 53 patients with cryopreserved sperm, 45 underwent IVF-ICSI treatment during the study period. In 32 (71%) patients, fresh sperm were used to perform ICSI, with no need to utilize the stored pooled sample. In 11 patients, no sperm were found in the fresh semen samples given on the day of oocyte retrieval and we used the frozen-thawed sperm. In two other patients very few motile sperm were found in the fresh semen, and we opted to thaw and use the frozen-thawed sample. To summarize, in 13 patients out of 45 (29%), fresh sperm were not available on the day of oocyte retrieval and frozen-thawed sperm were used.

Patient characteristics in terms of both male and female patients' age, duration of infertility, the number and maturity of the retrieved oocytes were similar in the two groups (Table II).

Fertilization rate, the number of transferred embryos and the number of embryos suitable for cryopreservation were similar in the fresh and frozen-thawed groups. Six fertilized oocytes from the fresh semen group and two in the frozen-

**Table II.** Outcome of ICSI procedure and embryo transfer in the fresh sperm versus frozen-thawed sperm in the pooling and cryopreservation groups

	Fresh	Frozen-thawed	P
No. of cases	32	13	
Age (female partner) (mean $\pm$ SD)	28.9 $\pm$ 4.9	28.8 $\pm$ 7.4	NS
No. of oocytes	416	192	
Oocytes per cycle (mean $\pm$ SD)	13.0 $\pm$ 6.5	14.6 $\pm$ 7.9	NS
No. of metaphase II oocytes	341	152	
Metaphase II oocytes per cycle (mean $\pm$ SD)	9.2 $\pm$ 5.0	11.3 $\pm$ 5.9	NS
2 PN (%)	179 (52.5)	71 (46.7)	NS
3 PN (%)	6 (1.7)	2 (1.3)	NS
No. of embryos transferred (mean $\pm$ SD)	2.7 $\pm$ 1.5	2.5 $\pm$ 1.1	NS
No. of embryos frozen (mean $\pm$ SD)	1.5 $\pm$ 2.3	1.9 $\pm$ 3.0	NS
Clinical pregnancy (%)	10/32 (31)	3/13 (23)	NS
Implantation rate (%)	16/80 (20)	4/29 (14)	NS

thawed group were found to have multiple pronuclei (3 PN). The difference was not significant.

The clinical pregnancy and the implantation rate were also similar in both groups (31 versus 23%, NS and 20 versus 14%, NS respectively) (Table II).

Ten pregnancies resulted from the fresh sperm group; six singleton pregnancies, two twin and two triplet, one reduced to twin, one reduced to singleton. The frozen-thawed group yielded three pregnancies; two singleton and one twin pregnancy.

Thirteen babies were delivered, and two pregnancies are ongoing. One singleton pregnancy from the frozen-thawed group resulted in a late abortion.

## Discussion

The population of patients considered for our pooling and cryopreservation programme were patients in whom the chance of finding sperm in their semen, particularly on the day of oocyte retrieval, was low and unpredictable.

Cryopreservation was performed in 98% of patients with severe OAT, and in 61% of patients previously defined as azoospermic. Moreover, during the study period, motile sperm were found in the ejaculate of 54% patients with a previous testicular biopsy and cryopreserved. Ron-El *et al.* performed sperm examination on the day of TESE and similarly found sperm in 17 of 49 (35%) patients with non-obstructive azoospermia (Ron-El *et al.*, 1997). In our study, at least seven TESE operations were avoided.

The state of 'virtual' azoospermia, defined as the occasional presence of few sperm in the ejaculate, is distinguished from 'absolute' azoospermia, defined as the lack of sperm in the ejaculate (Tournaye *et al.*, 1995). It is probable that such a state existed in these patients in whom sperm were not available on the day of oocyte retrieval and pooled frozen-thawed sperm were used. Our results demonstrate that meticulous search and sperm cryopreservation can rescue IVF-ICSI cycles and prevent TESE procedures, donor sperm usage, or cycle cancellation in a select group of patients.

Cryopreservation techniques are simple and safe (Chernos

and Martin, 1989; Morris *et al.*, 1999). Advances in cryopreservation technology enable us to freeze individual sperm for eventual ICSI (Cohen *et al.*, 1997; Montag *et al.*, 1999a,b).

The cryopreservation procedure did not harm the sperm's ability to fertilize or the pregnancy rates in our study (Table II). Our results are similar to pregnancy and implantation rates described by others with fresh versus frozen sperm (Ron-El *et al.*, 1997; Ben-Yosef *et al.*, 1999).

Predicting the presence of sperm in the ejaculate of patients with transient azoospermia is not easy. Patients' age, duration of infertility, FSH concentration or even testicular volume were not predictive of sperm presence in our study.

Histological examination following testicular biopsy was also unreliable as a predictor. In our study, in patients who had undergone TESE due to azoospermia in the past, with histological findings including hypospermatogenesis, Sertoli cells only and maturation arrest, sperm were indeed found on sperm analysis. Nevertheless, it is important to note that sperm for ICSI were found in all of their previous biopsies.

Patients with azoospermia in whom sperm were previously found in sperm analysis and those with severe OAT with fluctuations in sperm count are candidates for the pooling programme.

Good anamnesis will reveal other patients with possible transient azoospermia secondary to spermatogenic arrest. This may be caused by several factors, including: nutritional (vitamin A deficiencies, zinc deficiencies caused by sickle-cell anaemia and renal insufficiency); certain antibiotics; heat (sauna, febrile diseases); infection and various endocrinopathies (Martin-du Pan and Campana, 1993).

Appropriate diagnosis and treatment is important in these patients to enable them to benefit from pooling and cryopreservation. Therapeutic options to be considered include the alpha sympathomimetic class of drugs, which have been shown positively to affect sperm count and motility (Kohn and Schill, 1994) and adjuvant hormonal therapy (Dirnfeld *et al.*, 2000).

On the other hand, patients with a well documented history of azoospermia in repeated examinations, patients with obstructive azoospermia, and patients with azoospermia due to known genetic abnormalities, are not candidates for the pooling programme.

One of the dilemmas encountered with the practice of pooling cryopreservation is at what frequency and intervals to perform the sperm analysis for the purpose of eventual cryopreservation. The duration of sperm production and maturation is 74 days (Speroff *et al.*, 1994). Sperm recovery may take more than one 74 day cycle, which may be too long a period for patients who desire infertility treatments. It is also a long period for postponement of IVF treatment in women above 38 years of age or in poor responders. However, the fluctuations in sperm number can also be referred to epididymal dysfunction, which lasts 8-10 days. So a second sperm analysis can actually be repeated after a week or two.

For our patients, we recommended sperm banking be performed every week or two at least 3-4 times prior to oocyte retrieval. Even when pooling and cryopreservation was not possible, we found the technique beneficial. We were able to prepare ourselves for the possible necessity of TESE or other

alternatives. Our patients were also more prepared for dealing with the possibility of cycle cancellation, donor sperm usage or additional TESE.

Semen banking has already been widely used in patients undergoing chemo-radiotherapy (Agarwa, 2000). The method is simple and inexpensive.

The introduction of semen banking for other indications, such as patients with transient azoospermia, as part of the standard procedure in the IVF laboratory will prevent TESE and increase those patients' chances of achieving pregnancy using their own sperm.

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