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Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa

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In 25 patients (14 suffering from obstructive azoospermia, six from non-obstructive azoospermia, three from asthenoazoospermia and two from absence of ejaculation) spermatozoa were extracted from testicular biopsies. Intracytoplasmic sperm injection (ICSI) with fresh testicular spermatozoa was performed in 18 cases; spermatozoa in excess were cryopreserved in pills. No pregnancies were achieved. In the remaining seven patients, testicular spermatozoa were retrieved and cryopreserved during a diagnostic testicular biopsy. After thawing, sperm motility was assessed in 17 cases (68%), and 18 ICSI with cryopreserved testicular spermatozoa were performed. The mean two-pronuclear (2PN) fertilization rate was 59%, the mean cleavage rate was 92%, and six clinical pregnancies were achieved, all of them still ongoing (pregnancy rate 33%). A comparison of the results of ICSI carried out with fresh or cryopreserved testicular spermatozoa showed that the mean 2PN fertilization rates per cycle (53 compared with 55%), mean cleavage rates per cycle (99 compared with 96%) and embryo quality were not significantly different. In conclusion, cryopreservation of testicular spermatozoa is feasible, even in patients with non-obstructive azoospermia, and the results of ICSI with frozen–thawed testicular spermatozoa are similar to those obtained using fresh testicular spermatozoa. Cryopreservation of testicular spermatozoa may avoid repetition of testicular biopsies to retrieve spermatozoa for successive ICSI cycles in patients in whom the only source of motile spermatozoa is the testicle.

Key words: intracytoplasmic sperm injection/male infertility/pregnancy/sperm cryopreservation/testicular biopsy

Introduction

High fertilization and pregnancy rates have been reported after intracytoplasmic sperm injection (ICSI) with spermatozoa obtained from testicle biopsy (Silber *et al.*, 1995a). Testicular sperm extraction (TESE) and ICSI were initially used in patients with otherwise intractable obstructive azoospermia, because of non-feasibility or previous failure of standard microsurgical procedures (Silber *et al.*, 1995a; Gil-Salom *et al.*, 1995a). More recently, TESE with ICSI has been shown to be useful in non-obstructive azoospermia, and some patients with severe spermatogenic failure and markedly elevated serum concentrations of follicle stimulating hormone (FSH) can now be fertile using this new approach (Devroey *et al.*, 1994, 1995a; Gil-Salom *et al.*, 1995b).

Cryopreservation of testicular spermatozoa involves difficulties, because of their usually low concentration and motility (Silber *et al.*, 1995b). Nevertheless, freezing and storing testicular spermatozoa for future use is of interest, because it may avoid repetition of testicular biopsy if pregnancy is not achieved after a first ICSI cycle, and further attempts could be carried out. Specifically in patients with non-obstructive azoospermia and small testes, cryopreservation is important, since multiple extensive testicular biopsies may represent a significant loss of testicular mass.

We have applied a method for testicular biopsy extracted spermatozoa cryopreservation in 'pills' (Nagase and Niwa, 1964), which enables the storage of spermatozoa in very small aliquots. Here, we report on: (i) the results obtained after thawing the first 25 samples of testicular biospy extracted spermatozoa, cryopreserved in pills; and (ii) the fertilizing ability of frozen–thawed testicular spermatozoa and the outcome in comparison with those of fresh testicular spermatozoa.

Materials and methods

Patients

Between February and December 1995, 25 patients underwent TESE and testicular spermatozoa were cryopreserved in pills. In 18 patients, a first ICSI cycle with fresh testicular spermatozoa was performed, and spermatozoa in excess after injecting all the metaphase II oocytes were frozen. Pregnancy was not achieved, and frozen testicular spermatozoa were thawed to perform a second ICSI cycle with them. In the remaining seven patients, spermatozoa were obtained during a diagnostic testicular biopsy, and were directly frozen for performing ICSI later on.

The mean female age was 32.7 years (range 27–41 years) and the mean male age was 39.1 years (range 32–52 years). Mean duration of infertility was 8 years (range 5–16).

Indications for TESE and ICSI were obstructive azoospermia (n = 14), non-obstructive azoospermia (n = 6), total asthenozoospermia (n = 3) and absent ejaculation (n = 2). Aetiology of obstructive azoospermia was epididymal obstruction in seven cases, and congenital absence of vas deferens (CAVD) in another seven. Spermatozoa could not be retrieved from the epididymides due to severe scrotal inflammatory or post-surgical scarring (10 cases), proximal obstruction (two cases), or absence of the epididymides (two cases).

In patients with non-obstructive azoospermia, a previous history

of left varicocele or orchitis was noted in three and one cases respectively. Diagnostic testicular biopsy revealed incomplete Sertoli cell-only syndrome (n = 3), severe hypospermatogenesis (n = 2) and maturation arrest (n = 1). Serum FSH concentrations were within normal range (1–12 mIU/ml) in three patients, and were elevated in the other three (17, 18.9 and 20.5 mIU/ml respectively). Serum concentrations of luteinizing hormone (LH), testosterone and prolactin were normal.

In the three patients with total asthenozoospermia, severe oligozoospermia (<100 000 spermatozoa/ml) was also present. A previous history of chronic prostatitis was noted in two cases; in the other patient, the aetiology of oligoasthenozoospermia was idiopathic and the serum FSH concentration was 34.6 mIU/ml. Despite total asthenozoospermia in the ejaculate, motile spermatozoa could be obtained from testis biopsy specimens in all three cases. Two patients presented absent ejaculation (psychogenic and secondary to spinal cord trauma respectively), and in both, TESE was performed during a diagnostic testicular biopsy.

Women included in the study revealed normal findings in the routine infertility work-up. Normal karyotypes were assessed in all couples. In couples with CAVD, screening for mutations in the cystic fibrosis gene complex (DF508, G542X, N 1303K, 1717–1, W 1282X, G551 D, R553X and DI507) was performed in husband and wife before entering the programme, all the wives proving negative. Informed consent about the ICSI procedure was obtained from all patients. Our study was approved by the Ethical Committee at the Instituto Valenciano de Infertilidad, Spain.

Retrieval of spermatozoa from testicular tissue

Spermatozoa were retrieved by open testicular biopsies, which were performed under 2% mepivacaine spermatic cord block. Testicular biopsies were carried out in 18 cases on the same day scheduled for oocyte collection. In patients with obstructive azoospermia, the scrotal skin and tunica vaginalis were opened and the scrotal content was inspected. After assessing the non-feasibility of retrieving epididymal spermatozoa, a 0.5 cm incision was made in the tunica albuginea and one or two pieces of extruding testicular tissue were excised. In patients with non-obstructive azoospermia or total asthenoazoospermia, 2–4 biosies were obtained from one or both testicles.

TESE was performed as previously described (Devroey *et al.*, 1994; Silber *et al.*, 1995a). Testicular tissue was immediately placed in a Petri dish containing 2 ml of sperm medium (Medicult, Copenhagen, Denmark). To release the spermatozoa from the seminiferous tubules into the medium, the biopsy specimen was finely minced and dispersed mechanically with the help of two sterile slides. Thereafter, the presence of sperm cells in this effluent was checked under an inverted microscope (Diaphot; Nikon Corporation, Tokyo, Japan) at ×400 magnification. In all cases, sporadic free spermatozoa could be identified in a field of debris, red blood cells, immature cells and Sertoli cells. The fluid content of the dish was then aspirated with an automatic pipette, placed in a 10 ml Falcon tube (Beckton Dickinson, Lincoln Park, New Jersey, USA), and incubated at 37°C and 5% CO_2.

Just before the microinjection procedure, the fluid was centrifuged at 600 *g* for 5 min. The supernatant was removed, the pellet was gently resuspended, and 1 µl of this suspension was placed in 5 µl Flushing medium (Medicult) droplets on the microinjection dish. After microinjecting all metaphase II oocytes retrieved, excess spermatozoa were frozen.

In seven patients, spermatozoa were cryopreserved before the ICSI cycle, at the time of performing a biopsy for histological diagnosis. During this diagnostic biopsy, at least two pieces of testicular tissue were excised; the first for histological diagnosis and the second for sperm cryopreservation. The latter was processed as above, but after checking the presence of motile spermatozoa in the sperm suspension, all spermatozoa were frozen.

Cryopreservation of testicular spermatozoa

Before freezing, 1 ml of Test Yolk Buffer cryopreservation medium containing glycerol (Irvine Scientific, Santa Ana, CA, USA) was added to the sperm pellet obtained after centrifugation at room temperature. The mixture was homogenized and placed in a 37°C bath for 45 min. Thereafter, the mixture was again homogenized, and dispensed with the help of an automatic pipette in 10–15 100 µl droplets on a dry ice surface (Nagase and Niwa, 1964). Freezing of these microdrops occurred in ~1 min. Frozen droplets ('pills') were plunged into liquid nitrogen (–196°C) and stored.

For thawing, three pills were removed from the liquid nitrogen and placed in a 5 ml Falcon tube for 5 min at room temperature. The tube with the pills was then placed in a 37°C and 5% CO_2 chamber for 15 min. To remove the cryopreservation medium, the specimens were washed by centrifugation with 2 ml IVF medium (Medicult, Copenhagen, Denmark) at 600 *g* for 5 min. After elimination of the supernatant, the pellet was resuspended in 100 µl. This final suspension was incubated at 37°C and 5% CO_2 for 1 h. Thereafter, the presence of motile spermatozoa for ICSI was checked under an inverted microscope (Diaphot; Nikon Corporation).

In most cryopreserved samples, all spermatozoa were initially immotile after thawing, but after waiting for ~1 or 2 h, a very weak motility was evident in some spermatozoa. If no motile spermatozoa were observed, additional sets of three pills were thawed until sperm motility was found.

Ovarian stimulation

Ovarian stimulation was carried out by the association of leuprolide acetate (Procrin; Abbott SA, Madrid, Spain), human menopausal gonadotrophin (HMG, Pergonal; Serono Laboratories, Madrid, Spain), FSH (Neo-Fertinorm; Serono Laboratories) and human chorionic gonadotrophin (HCG, Profasi; Serono Laboratories). Vaginal ultrasound-guided follicle puncture took place 36–38 h after injection of HCG. The cumulus–corona cells were initially removed by exposure to Flushing medium (Medicult) and 80 IU of hyaluronidase (Hyaluronidase type IV-S; Sigma, St. Louis, MO, USA) for up to 1 min. After removing the corona cells, only metaphase II oocytes were injected.

ICSI procedure

ICSI procedures were carried out according to Van Steirteghem *et al.* (1993a,b) and Silber *et al.* (1995a,b) on an inverted microscope (Diaphot, Nikon Corporation) at ×400 magnification using the Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, New York, USA) equipped with two coarse positioning manipulators and with two three-dimensional hydraulic remote-control micro-manipulators (Narishige, Tokyo, Japan). After the microinjection procedure, the oocytes were incubated in 20 µl microdrops of IVF medium (Medicult) under lightweight mineral oil.

Fertilization was assessed 18 h after the injection by seeking the presence of pronuclei. Embryo cleavage was assessed 24 h thereafter. Embryo quality was analysed under the dissecting microscope before transfer, according to Conaghan *et al.* (1993). Briefly, grade 1 embryos contained intact and symmetrical blastomeres with no extracellular fragmentation. Embryos were scored as grade 2 if fragments were present, and as grade 3 if at least one cell had degenerated as well. If an embryo had only one blastomere intact, it was given a grade 4, and a completely fragmented embryo with no cells intact was scored as grade 5.

Table I. Results after thawing cryopreserved testicular spermatozoa

	Motile spermatozoa after thawing	
	Yes	No
Aetiology of infertility		
Obstructive azoospermia	9	5
Non-obstructive azoospermia	4	2
Total asthenozoospermia	2	1
Absent ejaculation	2	0
Serum FSH concentration (mIU/ml)		
<12	14	7
>12	3	1
Male partner's age (years)		
<35	9	4
35–40	5	2
>40	3	2
Total	17 (68%)	8 (32%)

FSH = follicle stimulating hormone.

Clinical pregnancy was determined by observing a gestational sac with fetal heart beat by means of an ultrasound scan at 7 weeks of pregnancy.

Statistical analysis

The mean two-pronuclear (2PN) fertilization rate per cycle and mean cleavage rate per cycle are expressed as the mean of the percentage values of the variables within each cycle. Mean number of blastomeres and mean fragmentation degree are also expressed as the mean of the values of the variables within each cycle. Results of ICSI with fresh and with cryopreserved testicular spermatozoa were compared by the Mann–Whitney U test. A P value of <0.05 was considered to be statistically significant. The analysis was carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA).

Results

Sperm survival after thawing

In 17 out of 25 frozen–thawed testicular sperm samples (68%), sperm motility could be assessed after thawing, and ICSI was performed with cryopreserved spermatozoa. In all of them, sufficient motile spermatozoa were recovered after thawing for microinjecting all the metaphase II oocytes retrieved after ovarian stimulation. In five cases it was necessary to thaw all the frozen pills to obtain enough spermatozoa for ICSI. In 11 cases, only three pills were thawed, the remaining being still stored for their eventual future use. In one couple, two successive ICSI cycles were carried out, as pregnancy had not been achieved either after the first attempt with fresh spermatozoa nor after a second cycle with cryopreserved spermatozoa.

In eight cases, no motile spermatozoa were observed after thawing; in these patients ICSI was carried out with fresh spermatozoa retrieved by a second testicular biopsy. Sperm survival after cryopreservation does not appear to be influenced by the aetiology of infertility, serum FSH concentration or the patient's age (Table I).

ICSI with cryopreserved testicular spermatozoa

Tables II and III show fertilization, embryo cleavage and embryo quality after 18 ICSI with cryopreserved testicular spermatozoa. Of the 185 intact metaphase II oocytes after ICSI, 106 (57%) were normally fertilized. There were no fertilization failures. Mean cleavage rate per cycle was 92% and a mean of 3.8 embryos were transferred into the uterine cavity in 18 transfers. A total of 24 good quality, supernumerary embryos were cryopreserved.

Table II. Oocyte characteristics and fertilization after intracytoplasmic sperm injection (ICSI) with cryopreserved testicular spermatozoa

No. cycles	187
No. cumulus–corona cells	267
No. metaphase II oocytes injected	206
Intact oocytes (% of injected)	185 (90)
No. 1PN oocytes	11
No. 2PN oocytes (% of intact)	106 (57)
No. 3PN oocytes	7
Mean 2PN fertilization rate per cycle[a]	59 ± 25
No. fertilization failures	0

PN = pronuclear.
[a]Values are mean ± SD of the fertilization rates within each cycle.

Table III. Embryo cleavage and embryo quality after intracytoplasmic sperm injection (ICSI) with cryopreserved testicular spermatozoa

No. cleaved embryos	95
Mean cleavage rate per cycle[a]	92 ± 15
Mean no. blastomeres per embryo[a]	3.3 ± 0.9
Mean embryo fragmentation degree[a]	1.6 ± 0.7
No. cycles with transfer (%)	18 (100)
Mean no. embryos per transfer[b]	3.8 ± 1.4
No. cryopreserved embryos	24
No. clinical pregnancies	6
Pregnancy rate per stated cycle	33%

[a]Values are mean ± SD of the percentage values of the variables within each cycle.
[b]Values are mean ± SD.

Six clinical pregnancies with cryopreserved testicular spermatozoa were achieved (one twin and five singleton), all of them still ongoing. This represents an ongoing pregnancy rate per started cycle and per transfer of 33%. The ongoing implantation rate was 10.3%.

Comparison between ICSI with fresh and cryopreserved testicular spermatozoa

In 12 couples a first ICSI cycle with fresh testicular spermatozoa was performed but, as pregnancy was not achieved, a second ICSI cycle with cryopreserved testicular spermatozoa was carried out. Comparison of results of ICSI with fresh versus cryopreserved testicular spermatozoa in these 12 couples shows that mean 2PN fertilization rates were not significantly different (Mann–Whitney U test, Table IV). Mean embryo cleavage rates per cycle, as well as embryo quality and mean number of embryos per transfer were also not significantly different in both groups (Mann–Whitney U test, Table V). Obviously, no pregnancies were obtained after the first ICSI cycle with fresh testicular spermatozoa.

Discussion

Cryopreservation of human spermatozoa has been widely used in assisted reproductive techniques. Usually, only normal semen samples are cryopreserved, because cryopreservation reduces sperm quality (Critser *et al.*, 1987; Hamerstedt *et al.*,

Table IV. Oocyte characteristics and fertilization after intracytoplasmic sperm injection (ICSI) with fresh and cryopreserved testicular spermatozoa in the same couples

	Fresh spermatozoa	Cryopreserved spermatozoa
No. cycles	12	12
No. cumulus–corona cells	146	167
No. metaphase II oocytes injected	109	131
Intact oocytes (% of injected)	94 (86)	114 (87)
No. 1PN oocytes	17	9
No. 2PN oocytes (% of intact)	49 (52)	58 (51)
No. 3PN oocytes	3	7
Mean 2PN fertilization rate per cycle[a,b]	53 ± 35	55 ± 21
No. fertilization failures	1	0

PN = pronuclear.
[a]Values are mean ± SD of the fertilization rates within each cycle.
[b]Not significant (Mann–Whitney *U* test).

Table V. Embryo cleavage and embryo quality after intracytoplasmic sperm injection (ICSI) with fresh and cryopreserved testicular spermatozoa in the same couples

	Fresh spermatozoa	Cryopreserved spermatozoa
No. cleaved embryos	48	55
Mean cleavage rate per cycle[a,b]	99 ± 4	96 ± 8
Mean no. blastomeres per embryo[a,b]	3.4 ± 1.2	3.4 ± 1.0
Mean embryo fragmentation degree[a,b]	1.5 ± 0.7	1.6 ± 0.9
No. cycles with transfer (%)	11 (92)	12 (100)
Mean no. embryos per transfer[b,c]	3.3 ± 1.5	3.4 ± 1.4
No. cryopreserved embryos	0	12
No. clinical pregnancies	0	6

[a]Values are mean ± SD of the percentage values of the variables within each cycle.
[b]Not significant (Mann–Whitney *U* test).
[c]Values are mean ± SD.

1990). However, high fertilization and pregnancy rates can now be achieved after ICSI, even with severely impaired semen parameters (Van Steirteghem *et al.*, 1993a,b; Nagy *et al.*, 1995a). Therefore, the possibility now exists to also cryopreserve defective semen samples. In such cases, semen quality will be worse after thawing, but if a few spermatozoa survive cryopreservation, they can be used for ICSI. This strategy is of great interest when dealing with valuable sperm samples, such as surgically retrieved spermatozoa from the epididymis or testicle.

Successful ICSI using frozen–thawed epididymal spermatozoa obtained by microsurgical epididymal sperm aspiration has been reported recently by Nagy *et al.* (1995b) and Devroey *et al.* (1995b). Epididymal spermatozoa showed a severely impaired motility after thawing. Nonetheless, high fertilization (45–56%), cleavage (73–82%) and clinical pregnancy (33–43%) rates were achieved.

However, cryopreservation of testicular spermatozoa involves more difficulties than that of epididymal spermatozoa, because of the lower number and motility. Craft and Tsirigotis (1995) recently reported that, in certain circumstances, it is possible to recover motile spermatozoa after thawing frozen testicular spermatozoa retrieved by percutaneous testicular sperm aspiration. We have used open biopsies because, in our experience, more spermatozoa are obtained by this technique than by percutaneous aspiration.

The cryopreservation technique we have used is not new (Nagase and Niwa, 1964), but it seems to be highly appropriate for freezing testicular spermatozoa. When using straws or ampoules, some spermatozoa remain attached to their walls; this is not the case when using pills. The small volume of the pills, each containing only a very small number of spermatozoa, permits the distribution of the few spermatozoa retrieved from testicular biopsy into multiple aliquots. This guarantees that only a minimal amount of spermatozoa will be thawed in excess, thus preserving the remaining frozen samples for future use.

In eight of our cases, no motile spermatozoa were recovered after thawing. We do not know precisely why these cases failed; sperm survival after cryopreservation does not seem to be influenced by the aetiology of infertility, serum FSH concentration or the patient's age. Three cases that did not render motile spermatozoa after thawing were patients with severe spermatogenic failure and very poor sperm quality. However, this is probably not the only reason for cryopreservation failure, because it was possible to recover motile spermatozoa after thawing in four additional patients with non-obstructive azoospermia and with few spermatozoa present in their testicles (two of the patients had elevated serum FSH concentrations). These cases confirm the feasibility of cryopreserving testicular spermatozoa, even in subjects with non-obstructive azoospermia.

In most thawed samples, all spermatozoa were initially immotile, but after waiting for 1 or 2 h, they showed some weak motility that verified their vitality. In some cases, recovery of motility after thawing took several hours. This pattern of delayed motility has also been observed after TESE. Hence, the tails of the spermatozoa should still be broken before microinjection, despite their weak motility (Silber *et al.*, 1995b).

The 55% mean 2PN fertilization rate after ICSI with cryopreserved testicular spermatozoa was comparable to that observed after ICSI with fresh testicular spermatozoa in the same couples (53%). Embryo cleavage and embryo quality were also similar in both groups. These results show that, despite the poor quality of testicular spermatozoa, it is possible to cryopreserve them, and that cryopreserved testicular spermatozoa retain their integrity for successful ICSI, working as well as fresh testicular spermatozoa, confirming our preliminary experience in two cases (Romero *et al.*, 1996). Six of our patients became pregnant, and they are, to the best of our knowledge, the first reported clinical pregnancies after ICSI with cryopreserved testicular spermatozoa.

Three patients in this series suffered from severe oligo-asthenozoospermia, without a single motile spermatozoon in the ejaculate. Microinjection of immotile spermatozoa has been shown to have a poor prognosis in ICSI (Nagy *et al.*, 1995a). It has been hypothesized that, in some cases, immotility may be secondary to senescent degeneration, and perhaps to delayed epididymal transport (Silber, 1995), and consequently in such patients, ICSI with testicular spermatozoa may offer a better prognosis. Our experience tends to support this concept,

since in three cases in this series with total asthenozoospermia in the ejaculate, motile spermatozoa were retrieved from testicular biopsies. In two of them, a previous history of chronic genital tract infection was present, and one may speculate that spermatozoa may die during genital tract transit.

In seven patients, spermatozoa obtained during a diagnostic testicular biopsy for histologic evaluation were directly cryopreserved to perform ICSI later on. This approach alleviates the need of a second testicular biopsy the day of oocyte collection to retrieve spermatozoa for ICSI. Oates *et al.* (1996) recently reported a similar strategy that dissociates microsurgical sperm retrieval and ICSI: intentionally cryopreserved epididymal spermatozoa were used at a later date for ICSI with great efficacy. In our cases, a single testicular biopsy was all the scrotal surgery the patient had to undergo, so improving his acceptability to ICSI. In addition, cryopreserved testicular spermatozoa may be used in several ICSI cycles, as shown by one of our cases in which two successive ICSI cycles with cryopreserved testicular spermatozoa were performed.

In conclusion, cryopreservation of testicular spermatozoa is feasible, even in patients with non-obstructive azoospermia, and ICSI with frozen–thawed testicular spermatozoa gives rise to fertilization and cleavage rates comparable to that obtained after ICSI with fresh testicular spermatozoa. Cryopreservation of testicular spermatozoa may avoid repetition of testicular biopsies to retrieve spermatozoa for successive ICSI cycles in patients in whom the only source of motile spermatozoa is the testicle.

Note added at Proof:

On April 23, 1996, the first two babies were born by Caesarian section. They were a female (2320 g) and a male (2280 g). They are completely normal and healthy.

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