Diagnostic testicular biopsy and cryopreservation of testicular tissue as an alternative to repeated surgical openings in the treatment of azoospermic men


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Between May 1996 and May 1998, 64 azoospermic patients underwent an investigative testicular biopsy combined with the cryopreservation of spermatozoa which were retrieved from a simultaneously examined fresh sample. Testicular tissue cryopreservation was carried out in 43 cases (67%) for late intracytoplasmic sperm injection (ICSI) attempts. In all, 23 couples underwent 26 assisted conception cycles; the fertilization rate was 64% with spermatozoa (139/218, 24 cycles), 40% with round spermatids (2/5, one cycle), and 69% with elongated spermatids (9/13, one cycle). The embryo cleavage rate was 84%. A mean number of 2.7 ± 0.7 embryos were replaced in 24 patients. In two cases, embryo quality was very poor and they were not transferred. Eight clinical pregnancies resulted (35% per patient and 33% per transferred cycle) with an implantation rate of 14.1%; two patients have already delivered and six are ongoing. In conclusion, the cryopreservation of testicular tissue during the first diagnostic biopsy is an alternative to repeated surgical openings and permits patients to initiate an ovarian stimulation cycle with the certitude of having spermatozoa available. Moreover, since only one straw is routinely used for each ICSI cycle, the frozen tissue remains as a sperm source for multiple attempts.

Key words: azoospermia/cryopreservation/intracytoplasmic sperm injection/testicular sperm extraction

Introduction

Intracytoplasmic sperm injection (ICSI) has been widely applied in association with microsurgical procedures to treat couples suffering from azoospermia. Clinical pregnancies and delivery of healthy babies have been achieved with surgically retrieved epididymal (Pryor et al., 1984; Temple Smith et al., 1985; Silber et al., 1994; 1995a; Gianaroli et al., 1996) and testicular spermatozoa (Schoysman et al., 1993a; 1993b; Devroey et al., 1995a; Silber et al., 1995b). However, one of the major drawbacks in the treatment of azoospermic patients resides in the eventual need of resorting to microsurgery at each ICSI attempt. Beyond the trouble for the patient, repeated operations can damage epididymal and testicular tissues. In order to avoid this inconvenience, percutaneous epididymal sperm aspiration and testicular sperm aspiration (TESA) were introduced with the aim of recovering sperm cells from epididymis and testis without repeated surgical openings (Craft and Tsirigotis, 1995; Craft et al., 1995). However, both procedures could be traumatic for epididymal and testicular tissues and fail to recover sperm cells, particularly in azoospermic patients with primary testicular dysfunction. Therefore, although TESA is a reliable method for azoospermic patients with complete spermatogenesis, open biopsy with TESE (testicular sperm extraction) remains the best option in cases of maturation arrest.

A valuable approach is represented by the cryopreservation of the microsurgically recovered spermatozoa, thus avoiding a second surgical operation for further ICSI attempts. Indeed, pregnancies and deliveries have been reported after injection of frozen–thawed epididymal (Devroey et al., 1995b) and testicular spermatozoa (Gil-Salom et al., 1996). Usually, oocyte and microsurgical sperm retrievals are scheduled for the same day. Therefore, the freezing of supernumerary sperm cells is carried out after performing oocyte insemination with the freshly isolated spermatozoa. Unfortunately, some cases (25–30%) have been reported where TESE failed to recover sperm in men with non-obstructive azoospermia (Mulhall et al., 1996). Therefore, a potential pitfall exists for those patients where no spermatozoa are available on the day of egg recovery. This condition is certainly stressful for the couple both physically, psychologically and financially.

The aim of the present study was to verify the feasibility of performing cryopreservation of testicular tissue during the first diagnostic biopsy and to subsequently use thawed sperm to inseminate the partner’s oocytes. The expected advantages are: (i) to minimize the risk of not having spermatozoa available at the time of ICSI; (ii) to avoid repeated surgical openings; and (iii) to schedule the treatment cycle at the couple’s convenience. In this paper, data regarding sperm recovery, fertilization and pregnancy rates with thawed testicular spermatozoa are presented.

Materials and methods

Patients

From May 1996 to May 1998, 64 men (mean age 35.3 ± 6 years, range 26–52) diagnosed with unexplained non-obstructive azoospermia by physical examination were included in this study. The patients had a testicular volume ≤15 ml and follicle stimulating hormone (FSH) blood level ≥12 IU/ml. After thorough examination,
the ejaculates revealed the complete absence of spermatozoa. Therefore, patients were invited to undergo a testicular biopsy combined with cryopreservation of testicular spermatozoa. Full explanation about this procedure was given and their informed consent obtained. During the surgical intervention, a sample of the testicular tissue was prepared for histopathological examination whereas a wet preparation was carried out and duly checked under an inverted microscope in order to assess the presence of sperm cells. When spermatozoa were found, the tissue was cryopreserved for late ICSI cycles.

In all, 23 patients underwent 26 assisted conception cycles. Induction of multiple follicular growth was performed through the administration of gonadotrophin releasing hormone analogues and exogenous gonadotrophins (Ferraretti et al., 1996). On the day of egg retrieval and after the isolation of oocytes, one straw of testicular sperm was thawed and spermatozoa were used for ICSI.

### Testicular tissue preparation and cryopreservation

Testicular samples were collected in Earle’s balanced salt solution (EBSS), supplemented with HEPES and 6% plasmanate, and allowed to settle for 15 min at room temperature. The specimens were rinsed twice and minced well into small pieces under a dissecting microscope, using two sterile slides. A drop of the suspension was examined under an inverted microscope (Olympus IMT2, Hoffman modulation contrast). If spermatozoa were present, the suspension was collected in 5-ml Falcon tubes and centrifuged at 600 g for 5 min. The supernatant was removed, the pellet diluted in 1 ml of in-vitro fertilization (IVF) medium (Scandinavian IVF Science AB, Gothenburg, Sweden) and homogenized using a Pasteur pipette. Cryopreservation was carried out following the protocol of Mahadevan et al. (1983). Briefly, the cryoprotectant Human Semen Preservation Medium (HSPM) was added to the homogenate in an equal volume (1:1; 0.1 ml/min). After balancing at 37°C for 5 min, the homogenate was loaded into 0.25-ml freezing straws which were first exposed to liquid nitrogen (LN) vapours for 15 min and then plunged into LN.

### Thawing and ICSI procedures

After isolation of oocytes, one straw was removed from LN and warmed immediately at 37°C. To remove the cryoprotectant, the thawed homogenate was washed twice in EBSS supplemented with 6% plasmanate and centrifuged at 600 g for 5 min. The pellet was then resuspended in 0.2 ml of IVF medium and a drop examined for the presence of sperm cells. When spermatozoa were present, the tissue was cryopreserved for late ICSI cycles.

### Fertilization assessment and embryo evaluation

Sixteen to 18 hours after injection, fertilization was assessed and monospermic zygotes were incubated in fresh medium. Twenty-four and 48 h later, cleaving embryos were observed and classified according to their morphological appearance. Embryo transfer took place 66–68 h after injection; only regularly developing embryos were transferred.
were transferred. Clinical pregnancies were confirmed by the presence of a gestational sac with fetal heart beat. The ratio between the number of gestational sacs with fetal heart beat and the total number of embryos transferred defined the implantation rate.

## Results

Following testicular biopsy, spermatozoa were found in 43 men and cryopreserved. In the remaining 21 patients, no sperm cells were detected during the fresh examination, as confirmed by the histological diagnosis.

Twenty-three couples with frozen testicular sperm underwent 26 ICSI cycles. The diagnostic biopsy revealed: 16 cases with partial maturation arrest, four with early and two with late maturation arrest, and one case with complete spermatogenesis. The total number of spermatozoa recovered ranged from less than 10 to 1.4\times 10^6. A total of 176 straws were frozen, with an average of 7.6 ± 3.3 per patient.

As depicted in Table I, 236 MII oocytes were injected with thawed haploid testicular cells: 218 from 24 cycles with spermatozoa, and 18 from two cycles (no. 4 and 19) with spermatids, as no spermatozoa were available after thawing. Normal fertilization resulted in 150 oocytes (64%), with similar percentages when using spermatozoa (64%) or spermatids (61% total; 40% with round spermatids and 69% with elongated spermatids). The zygotes obtained from four cycles (n = 38) were cryopreserved, since the patients were at high risk of developing ovarian hyperstimulation syndrome (OHSS); 23 of them were thawed in a subsequent cycle of replacement therapy.

Observation at 40 h after injection revealed 114 regularly cleaving embryos (84%). Twenty-four hours later, 76 embryos from 24 cycles had divided further to the 6–8-cell stage; of these, 64 were transferred, with an average of 2.7 embryos per transfer. In two cycles (no. 15 and 16) embryo replacement was not performed because of poor embryo development. As shown in Table I, clinical pregnancies were established in eight patients (seven singleton and one twin); of these, two went successfully to term (one with frozen thawed round spermatids) and six are still ongoing (one with elongated spermatids). The resulting clinical pregnancy rate was 35% per patient and 33% per transferred cycle, and the implantation rate was 14.1%. The cumulative results obtained are summarized in Table II.

## Discussion

The intentional cryopreservation of epididymal spermatozoa and testicular tissue before ICSI treatment is efficacious and may overcome many of the inherent problems that could arise using fresh testicular tissue (Oates et al., 1996, 1997; Podsiadly et al., 1996; Romero et al., 1996). Additionally, the efficacy of cryopreserving testicular spermatozoa on the day of diagnostic testicular biopsy has been reported, with no significant differences in terms of fertilization and embryo developmental rates compared with those of fresh testicular spermatozoa (Gil-Salom et al., 1996).

In the current study, the testicular tissue obtained during the first diagnostic testicular biopsy was cryopreserved when the fresh examination revealed the presence of sperm cells. This strategy had the aim of combining the histological diagnosis with the storage of male gametes in order to perform late ICSI cycles. Open testicular biopsy rather than percutaneous aspiration has been used in order to obtain a specimen of good quality sufficient for both histological evaluation and the cryopreservation procedure. In fact, the testicular aspiration technique does not easily allow cryopreservation due to the limited number of sperm cells which are generally recovered (Tournaye et al., 1998). Although a more recent study reported that percutaneous testicular sperm aspiration through 19G butterfly needles permitted the evaluation of the presence or absence of mature spermatids in azoospermic men as efficiently as open biopsy, it did not, however, allow a complete evaluation of the different phases of spermatogenesis (Rosenlund et al., 1998).
As our study confirms, either motile or immotile spermatozoa recovered from the cryopreserved testicular tissue are capable of fertilization, organize embryo development and yield pregnancies (64%, 84% and 33% respectively). The results obtained do not differ from those achieved after ICSI with fresh testicular spermatozoa, as reported in a previous study where the percentages of fertilization, cleavage and pregnancy were 59%, 95% and 29 % respectively (Gianaroli et al., 1998). This is in agreement with the data reported by Nagy et al. (1998) and suggests that the poor motility of testicular spermatozoa recovered after thawing does not negatively affect the fertilization potential and embryo viability after ICSI.

In two patients, the number of sperm cells recovered by TESE was reported to be less than 10 in the whole sample. After thawing, no spermatozoa were found; therefore, spermatids from the same preparation (round spermatids in patient no. 4 and elongated spermatids in patient no. 19) were used for ICSI. The identification of viable round spermatids was assessed according to the criteria reviewed by Van der Zwalmen et al. (1998). The two patients became pregnant: one has already delivered a healthy baby girl and the second is ongoing. These results support the previous data reported in animals (Ogura et al., 1996) and in the human (Antinori et al., 1997), demonstrating that thawed spermatids maintain integrity and potential capacity to achieve fertilization and full embryo development up to delivery. However, since the use of immature sperm cells to assist fertilization could invoke some problems related to the state of DNA maturity, genomic imprinting and the possible transmission of genetic factors responsible for spermatogenic defects, these patients should be thoroughly counselled about possible risks and meticulously followed before and after conception.

On the other hand, embryos from four patients (no. 9, 13, 19, and 21) were not transferred during the fresh cycle, since they were at high risk of developing OHSS. Therefore, all their zygotes were cryopreserved, in order to prevent the onset of OHSS as a consequence of a pregnancy-related human chorionic gonadotrophin rise (Amso et al., 1990). The zygotes were later thawed, cultured and transferred in a subsequent hormonal replacement therapy cycle, yielding two pregnancies. This strategy represents a valuable safety measure in the prevention of the OHSS, even in cases of frozen-thawed testicular spermatozoa.

In conclusion, the current results confirm previous data (Oates et al., 1997) and suggest that the cryopreservation of testicular tissue during the first diagnostic biopsy is feasible and represents a useful tool to assist fertilization in patients whose only source of spermatozoa is the testicle. One advantage is that it permits the couple to undergo an ICSI cycle with the certainty of having spermatozoa present at the time of oocyte insemination. Indeed, the detection of sperm cells in a previous investigative biopsy does not exclude the possibility of not finding spermatozoa in a second opening on the day of oocyte retrieval. In fact, the occurrence of focal spermatogenesis is especially frequent in cases of azoospermia from maturation arrest, ductal obstruction or general cell aplasia. Therefore, the cryopreservation of sperm cells from a unique biopsy operation will prevent this troublesome event which is particularly tragic for infertile couples that do not accept heterologous insemination. Second, multiple ICSI cycles can be performed with the material recovered during the TESE procedure that has been frozen in single straws, of which only one is routinely thawed at each insemination cycle. Thus, the merging of the most advanced techniques in the reproductive medicine field opens a real possibility of treatment for azoospermic patients whose chances of conceiving are now becoming consistently high without the discomfort and inconvenience of repeated surgical operations.

References


Received on August 5, 1998; accepted on December 9, 1998