**Ongoing pregnancies after intracytoplasmic injection using cryopreserved testicular spermatozoa**

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We report two clinical pregnancies occurring after intracytoplasmic sperm injection (ICSI) using cryopreserved spermatozoa obtained from testicular biopsy, made in two different infertility situations in our clinic. The first patient showed a secretory azoospermia associated with elevated serum follicular stimulating hormone (FSH) level and spermiogenesis maturation arrest. The second patient was affected by azoospermia resulting from bilateral epididymal obstruction. Spermatozoa present in the wet preparation of testicular biopsies made on the day of scrotal exploration were cryopreserved within the testicular tissue for both men. Intracytoplasmic injections were performed at a later date, using spermatozoa prepared from frozen–thawed tissues. In each case, three embryos were obtained and transferred in utero. The transfers resulted in a twin pregnancy for the first case, and in a singleton pregnancy for the second. Living foetuses were seen in the ultrasound scan at the 7th week and both pregnancies are proceeding to date beyond 30 weeks without complications.

**Key words:** cryopreserved testicular spermatozoa/intracytoplasmic sperm injection/male infertility/testicular biopsy

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**Introduction**

Ever since the first pregnancies obtained by intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992), this method of oocyte fertilization has become a useful tool in the treatment of male infertility. Indications for its use are increasing, especially with the possibility of using surgically obtained spermatozoa from the epididymis (Tournaye et al., 1994) and the testis (Silber et al., 1995). Cryopreservation of surgically obtained spermatozoa gave satisfactory results, thus limiting the number of necessary sperm epididymal collection procedures (Devroey et al., 1995). Recently, the first pregnancies after ICSI using frozen–thawed spermatozoa obtained from testicular biopsies have been reported in a number of countries (Fisher et al., 1996; Gil-Salom et al., 1996; Hovatta et al., 1996; Podsiadly et al., 1996; Khalifeh et al., 1997; Oates et al., 1997) in patients suffering from obstructive or non-obstructive azoospermia. Antinori et al. (1997) described successful fertilization and pregnancy using frozen–thawed round spermatids isolated from testicular tissue from a patient with maturation arrest of spermatogenesis.

In this report, we describe the first two cases to our knowledge in France, whereby testicular sperm samples were cryopreserved and later used successfully in ICSI attempts.

**Materials and methods**

**Patients**

Two infertile couples, in whom the husbands (aged 33 years) were affected by azoospermia secondary to severe spermatogenetic failure for the first and moderate spermatogenetic failure associated with bilateral epididymal obstruction for the second, came to our clinic for investigation of 6 and 3 years primary infertility respectively.

In the first case, right orchidectomy and left orchidopexy had been performed when the patient was 10 years old, after failure of human chorionic gonadotrophin (HCG) treatment for testicular ectopy. The patient showed azoospermia and reduced sperm volume with elevated serum follicle stimulating hormone (FSH) (16 mIU/ml). A diagnostic testis biopsy was performed to investigate the possibility of attempting intracytoplasmic sperm injection (ICSI), and it revealed severe spermatogenetic arrest, with round-spermatid blockade and hypospermatogenesis. In microscopic evaluation, only a few spermatozoa were observed in the wet preparation, some of which showed signs of vitality (limited tail movements). The decision was made to cryopreserve the testicular tissue with the spermatozoa.

In the second case, the patient had undergone surgery for bilateral hydrocoele 10 years previously, followed by epididymitis 1 year later. His serum FSH level was normal. Diagnostic surgical exploration revealed excretory azoospermia due to bilateral epididymis obstruction; testicular biopsy showed associated hypospermatogenesis. A few spermatozoa with subtle signs of vitality were observed, and the option of cryopreservation of testicular tissue with spermatozoa was retained.

In the two cases, histopathology of testicular tissue taken at the time of testicular surgery confirmed microscopic evaluation of hypospermatogenetic testicular activity.

Routine infertility tests in the wives (aged 33 and 25 years respectively) gave normal results, and the couples entered our in-vitro fertilization (IVF) programme to attempt ICSI with frozen–thawed testicular spermatozoa.

**Testicular spermatozoa observation and cryopreservation**

In each case, freshly extracted testicular tissue was immediately placed in a sterile Petri dish containing 0.5 ml of IVF medium (Medicult; OSI, Elancourt, France). The tissue was briefly dissected using two sterile slides. A drop of the fluid content of the dish was retained for observation of spermatozoa under the light inverted microscope. In both cases, one or two spermatozoa were observed in the wet preparation, some of which showed signs of vitality. In microscopic evaluation, only a few spermatozoa with subtle signs of vitality were observed, and the option of cryopreservation of testicular tissue with spermatozoa was retained.

Before freezing, 1 ml of medium containing glycerol as cryoprotect-
Sperm was added to the content of the Petri dishes at room temperature. Sperm Freeze in the first case (J.C.D. SA, Gauville, France) and Freezing Medium, a test yolk buffer cryopreservation medium, in the second case (Irvine Scientific-Clinisciences, Montrouge, France). The mixture was homogenized and in each case aspirated into three 0.5 ml straws. The straws were arranged in the cryochamber of a Nicoolbag MS21 apparatus (Companie Francaise des Produits oxygénes, France), and exposed to liquid nitrogen vapour for 90 min, before being plunged into the liquid nitrogen. The technique used to freeze the tissue did not require a computerized program, and was similar to that of manual freezing utilizing only liquid nitrogen vapour.

**IVF procedure**

Ovarian stimulation was carried out with a combination of Nafarelin (Synarel; Syntex, Puteaux, France), highly purified FSH (Metrodin HP; Serono, Boulogne, France) and HCG (Gonadotrophine Chorionique Endo; Organon, Saint-Denis, France). Vaginal ultrasound-guided follicle puncture took place 38 h after injection of HCG. Intravaginally administered progesterone (Utrogestan; Besins-Iscovesco, Paris, France) was used for luteal phase support.

**Oocyte and spermatozoa preparations for ICSI**

After oocyte retrieval, the cumulus–corona cells were initially removed by exposure to IVF medium (Ferticult Fertipro; J.C.D. SA, Gauville, France in the first case, and Medicull; Osi, Elancourt, France in the second case) and 50 IU of hyaluronidase (Hyaluronidase Choay; Sanofi Winthrop, Gentilly, France) for up to 1 min. After removal of the corona cells, metaphase II oocytes were found to be present. At this point, one testicular sample straw was rapidly thawed at 37°C; the contents were then placed into a sterile Petri dish and the pieces of testicular tissue were finely dissected with the help of two sterile slides. At this time, sperm density in the fluid was assessed to determine the need for additional straws.

All of the fluid in the Petri dish was aspirated into a syringe and transferred to a 5 ml sterile Falcon tube (Beckton and Dickinson, Rutherford, NJ). To remove the cryopreservation medium, specimens were washed twice by centrifugation with IVF medium (Ferticult Fertipro; J.C.D. SA, Gauville, France for the first case, and Medicull; Osi, Montrouge, France for the second case). After thawing and fine dissection of testicular tissue, most spermatozoa were initially immobile, but 1 h after cryoprotectant removal, very weak motility was evident in about 1%. In the two cases described, enough motile spermatozoa were recovered after preparation for injection of all the metaphase II oocytes collected. To achieve this, all the three stored straws were thawed in the first case, but only one straw was used for the second case, the two remaining straws being conserved for possible future ICSI attempts. Microinjection results are presented in Table I.

**ICSI**

The ICSI procedure carried out was similar to that previously described by Van Steirteghem et al. (1993), using an inverted microscope (Olympus IMT2, France) at ×400 magnification with the Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvile, NY 11548, USA) equipped with two coarse positioning manipulators, one three-dimensional mechanical and one three-dimensional hydraulic remote control micro-manipulators (Narishige, Tokyo, Japan). After the microinjection procedure, the oocytes were incubated in IVF medium (Ferticult Fertipro; J.C.D. SA, Gauville, France for the first case, and Medicull; Osi, Montrouge, France for the second case).

Fertilization was assessed 15–18 h after ICSI by the presence of pronuclei. Embryo cleavage was evaluated 24 h later and on the 3rd day, the number of blastomeres and embryo quality was determined: grade 1 embryos contained intact and symmetrical blastomeres without extracellular fragmentation; if moderate fragmentation or little blastomere asymmetry was present, embryos were scored grade 2; no grade 3 embryos, completely fragmented, were observed in this study. On day 3 after ICSI, embryos were transferred into the endometrial cavity using a Frydman catheter (CCD; Paris, France).

In our clinic, we routinely carry out embryo transfer on day 3 after oocyte retrieval, in both IVF and ICSI protocols, because at this point we can better observe embryo viability and thus more critically select embryos for transfer. This also imitates the natural physiological process in which embryos enter the endometrial cavity for implantation on day 5 after ovulation.

**Results**

After thawing and fine dissection of testicular tissue, most spermatozoa were initially immobile, but 1 h after cryoprotectant removal, very weak motility was evident in about 1%. In the two cases described, enough motile spermatozoa were recovered after preparation for injection of all the metaphase II oocytes collected. To achieve this, all the three stored straws were thawed in the first case, but only one straw was used for the second case, the two remaining straws being conserved for possible future ICSI attempts. Microinjection results are presented in Table I.

Positive pregnancy tests were obtained 2 weeks after embryo transfers, as indicated by serum β-HCG concentrations. Transvaginal ultrasound scan at 7 weeks showed normal fetal heart activities in two gestational sacs in the first case, and a singleton living fetus in the second case. Today, those pregnancies are currently at 34 and 30 weeks of gestation respectively, and proceeding without complications.

**Note added at proof**

Three healthy infants were born. A female and a male, weighing 1720 g and 2280 g respectively in case 1; a male weighing 2850 g in case 2.

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### Table I. Results after ICSI with frozen--thawed testicular spermatozoa

<table>
<thead>
<tr>
<th>No. oocytes injected</th>
<th>No. intact oocytes</th>
<th>2 PN oocytes in each embryo</th>
<th>No. cleaved embryos</th>
<th>Embryo quality</th>
<th>No. embryo transferred</th>
<th>No. blastomeres of each embryo transferred</th>
<th>Clinical pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Couple 1</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>3 grade 1</td>
<td>3 grade 1</td>
<td>5, 6, 6 Twin</td>
</tr>
<tr>
<td>Couple 2</td>
<td>11</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>3 grade 1</td>
<td>3 grade 1</td>
<td>7, 8, 8 Singleton</td>
</tr>
</tbody>
</table>

PN = pronuclei
Discussion

In this preliminary report, we demonstrate that testicular spermatozoa within testicular tissue can be successfully cryopreserved for ICSI, giving rise to normal fertilization and clinical pregnancies. Case 1 confirms the feasibility of cryopreserving testicular spermatozoa, even in a subject with non-obstructive azoospermia, as previously described (Gil-Salom et al., 1996; Oates et al., 1997). Our results show that, despite poor quality and relative immaturity, testicular spermatozoa retain their integrity after cryopreservation for successful ICSI. This agrees with a previously published successful fertilization and pregnancy following frozen–thawed round spermatid injection (Antinori et al., 1997), and confirms the data recently published by Tournaye et al. (1997), emphasizing the difficulty of prognosis using common clinical or biological parameters.

We used glycerol as cryoprotectant with or without yolk in the cryopreservation medium, and two different IVF media, without affecting the results. Previous published pregnancies resulting from frozen–thawed testicular sperm injection have used glycerol as cryoprotectant with (Gil-Salom et al., 1996; Khalifeh et al., 1997; Oates et al., 1997) or without yolk association (Fisher et al., 1996; Hovatta et al., 1996). Here, the protocol used for testicular tissue freezing and thawing was the same as that for freezing and thawing ejaculated spermatozoa. Our preliminary work showed better spermatozoal survival and motility after thawing when whole testicular tissue was frozen than when spermatozoa were extracted before freezing. Hence, we chose this method rather than isolated testicular spermatozoa cryopreservation. The same practice was used in two recently published studies (Fisher et al., 1996; Hovatta et al., 1996), and a similar approach was described by Salzbrunn et al. (1996).

Cryopreservation of testicular spermatozoa should be considered as a genuine therapeutic choice to avoid repeated surgery. It can be used in different situations:

(i) During routine exploration of normogonadotrophic azoospermia, when deferentangiography and testicular biopsy are performed. Despite the presence of obstructive azoospermia, serum FSH level is not a valid parameter for predicting successful testicular sperm recovery in these patients, so that histopathology is required to give an accurate prognosis, as recently described by Tournaye et al. (1997). A piece of testicular tissue reserved for biopsy is quickly observed by light microscopy, and if it contains spermatozoa, it can be cryopreserved. Should curative surgery be impossible or should it fail, then cryopreservation of testicular spermatozoa on the day of surgery yields a source of spermatozoa for future ICSI without repeated surgery. That suggests that all such routine explorations should be carried out in an assisted reproductive techniques (ART) clinic near to an ART laboratory, to enable the feasibility of ICSI using testicular tissue.

(ii) When testicular biopsy is made on the day of ovarian puncture, excess isolated spermatozoa can be cryopreserved, especially in patients with non-obstructive azoospermia, or hypergonadotrophic hypogonadal azoospermia with severe hypospermatogenic production and a high serum FSH level. In these cases, histopathology often reveals germ cell aplasia or maturational arrest, with in most cases very few observed spermatozoa from focal production, and the outcome of renewed surgical spermatozoa collection would be uncertain. The possibility of conserving excess isolated spermatozoa for later use in further ICSI attempts should be considered. Fertilization and conception have been described using such isolated frozen–thawed spermatozoa (Gil-Salom et al., 1996; Podsadly et al., 1996; Romero et al., 1996; Khalifeh et al., 1997).

Apart from the feasibility of cryopreservation, the usefulness of exploration before an ICSI cycle is still open to discussion. This would avoid treatment of the wife in cases of absence of spermatozoa, especially in non-obstructive azoospermia, because ~30% of men possess no spermatozoa in their tissue (Oates et al., 1997); on the other hand, this strategy of initial exploration may alter the chance of success in cases of very poor sperm retrieval or focal production, which could be better suited to immediate sperm usage, because motility of freshly extracted spermatozoa is often very poor, and consequently cannot be guaranteed after cryopreservation. Moreover, repeated surgical spermatozoa retrieval will yield uncertain results.

These first ICSI results, using spermatozoa frozen–thawed within testicular tissue, must be confirmed in wider studies, especially in patients with very different serum FSH levels. It will be of interest to compare such a study with the recent observations of Novero et al. (1997), who found no relationship between serum FSH and fertilization, cleavage, pregnancy and implantation rates in ICSI using ejaculated spermatozoa.

This new method of semen cryopreservation, which will require later ICSI to obtain oocyte fertilization, has the advantage of eliminating the need for repeated testicular surgery to retrieve spermatozoa for successive ICSI cycles. Repeated surgery is especially harmful in these patients, because successive removal of parts of testicular tissue leads to a reduction of testicular mass and possible consecutive impaired testicular function due to post-sampling fibrosis or autoimmune reaction. This procedure is an important addition to the range of treatments which can be offered to patients whose ejaculates contain no spermatozoa or in whom the only source of motile spermatozoa is the testicle, as it increases the therapeutic efficacy of ICSI.

References


Pregnancies after injection of testicular cryopreserved spermatozoa


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