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CASE REPORT

Pregnancy after intracytoplasmic sperm injection of spermatozoa extracted from frozen–thawed testicular biopsy

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The case report illustrates the successful application of a new method of sperm extraction from a frozen–thawed testicular biopsy specimen within an established programme of intracytoplasmic sperm injection.

Key words: intracytoplasmic sperm injection/ovarian stimulation/sperm preparation/testicular biopsy

Introduction

After the first report of Schoysman *et al.* (1993a) of successful in-vitro fertilization (IVF) of a human oocyte by subzonal insemination of spermatozoa prepared from a fresh testicular biopsy and a later report by the same group (Schoysman *et al.*, 1993b) on the establishment of a pregnancy after intracytoplasmic sperm injection (ICSI) of similarly obtained male gametes, the use of sperm extracted from testicular tissue (TESE) for ICSI has become a routine procedure in several IVF centres. Nagy *et al.* (1995) showed that comparable results can be obtained from performing ICSI with ejaculated, epididymal and testicular spermatozoa with regard to resulting pregnancy rates, although fertilization rates were significantly higher using ejaculated spermatozoa. Since then this highly specialized form of infertility treatment requiring additional operative intervention to obtain the male gametes has been restricted to couples where the male partner suffers from azoospermia due to obstruction of the epididymis, total blockage or absence of vasa deferentia, or to testicular failure (i.e. partial Sertoli cell-only syndrome). The organization of the biopsy and the oocyte retrieval on the same day can be problematic, especially in groups working on an outpatient basis. This case report illustrates the useful application of a new method of sperm extraction from a frozen–thawed testicular biopsy specimen within an established ICSI programme

Case report

The couple presented for the first time at the IVF Unit for IVF treatment early in 1995, with primary infertility and having tried to conceive for 5 years. The wife was 32 years of age and a uterus duplex had been diagnosed by her referring gynaecologist; hormonal parameters and general gynaecological findings were normal. The husband was 31 years old and presented with a fructose-positive normogonadotrophic azoospermia. Anamnestically recurrent inflammations of the epididymides were reported, leading to the suspicion of bilateral occlusion of the vasa deferentia.

Chromosomal analysis performed in June 1995 on lymphocytes of both partners revealed normal karyotypes. Diagnostic bilateral testes biopsies were carried out under local anaesthesia on July 17 at the University Hospital, Department of Andrology, Hamburg, with four extra tissue samples cryopreserved using a Nicoolbag 10 system (Air Liquid, Wiesbaden, Germany) as described earlier (Salzbrunn *et al.*, 1996). Briefly, freezing was carried out in a commercially available ready-to-use HEPES-buffered (18 mM) medium (SpermFreeze; MediCult, Copenhagen, Denmark) consisting of modified Earle's balanced salt solution with 0.4% human immunodeficiency virus (HIV)-negative and hepatitis-negative human serum albumin and 15% glycerol as cryoprotectant (Brotherton, 1990), as specified by the manufacturer. The biopsies were submerged in 0 5 ml of SpermFreeze, cooled to –60°C within 5 min followed by exponential cooling to –120°C over 55 min and storage in liquid nitrogen until use.

During a test preparation done immediately after the operation by enzymatic digestion of the tissue, several spermatozoa were isolated although some showed non-progressive motility.

Subsequent histological examination was performed by a semi-thin sectioning technique since this approach is superior to conventional paraffin section histology. With regard to normal testicular tissue this may not be important, but in cases of severe damage of the spermatogenic tissue (which is most commonly found in patients selected for TESE), this difference in quality can be decisive. With semi-thin sectioning (which also provides the option of performing electron microscopic investigation) it is even possible to identify relicts of germ cells in apparent Sertoli cell-only tubules, and occasionally even in lysosomes of Sertoli cells or macrophages. Therefore, valuable additional information is gained about the gametes in the cryopreserved tissue. Furthermore, in many cases the decision for further reproductive therapies is based on a thorough and competent morphological diagnosis which cannot be provided by paraffin histology.

In the case described here, semi-thin sectioning showed the presence of only slightly reduced spermatogenetic activity

without degenerative changes of the seminiferous tubules. Based on these positive andrological conditions, ICSI treatment was started on August 28, 1995, with down-regulation therapy by the gonadotrophin-releasing hormone analogue (GnRHa), triptorelin (Decapeptyl®; Ferring, Kiel, Germany), 0.1 mg s.c. per day. A blood test for steroid hormones and gonadotrophins as well as ultrasonographic examination of ovaries and endometrium performed on September 15 confirmed successful down-regulation. Ovarian stimulation began on September 19 with two ampoules per day of human menopausal gonadotrophin (Menogon; Ferring) and was continued concomitantly with 0.1 mg GnRHa per day until the first of October, corresponding to the 13th day of stimulation, 10 000 IU of human chorionic gonadotrophin (HCG, Choragon; Ferring) were administered at 23:45 hours on the same day and 36 h later oocyte retrieval was performed by ultrasonographically-controlled follicular puncture. Eight oocytes were obtained, all of them were in metaphase II stage as confirmed by the presence of the first polar body. Before starting the follicular puncture, one of the testes biopsies had already been processed as described previously (Salzbrunn *et al.*, 1996) Briefly, the specimen was thawed in a 37°C water bath, transferred immediately to 1 ml of prewarmed culture medium (IVF medium; MediCult), the tubuli pulled gently apart by means of anatomical tweezers and incubated for 30 min at 37°C in an atmosphere of 5% CO_2 in air. Collagenase (type Ia; Sigma, Deisendorf, Germany) was added at a final concentration of 400 IU/ml for a further incubation of 30 min followed by repeated dissecting and smoothing out of the remaining tubuli with tweezers. Larger tissue particles were then removed and the suspension centrifuged at 800 *g* for 10 min. The supernatant was discarded and a small portion of the sediment transferred into an IVF medium droplet under mineral oil and incubated at 37°C to allow motile spermatozoa to swim out of the remaining tissue. These motile spermatozoa were picked out of the culture medium and transferred into a 5 µl droplet of a 10% polyvinylpyrrolidone (PVP) solution (MediCult) where they were immobilized and subsequently injected into the oocytes according to the techniques described by Van Steirteghem *et al.* (1993). All eight ova survived the procedure and the following day fertilization was confirmed in three of them by the presence of two pronuclei. One day later, corresponding to the 17th day of treatment, embryo transfer was carried out with the three embryos all being in the 4-cell stage and showing fragmentation of about 30% of the blastomeres. On the 30th day of treatment a pregnancy test was performed after luteal support with daily vaginal micronized progesterone suppositories (Utrogestan; Laboratoire Golaz, Ecublens, South Africa; two tablets three times per day = 600 mg) and 1000 IU of HCG (Pregnesin; Serono, Unterschleissheim, Germany) five times every other day starting on the day of embryo transfer. This first test was clearly positive with 310 IU of β-HCG. One week later ultrasonographical examination revealed a twin pregnancy in the left horn of the uterus with positive heart beats in both gestational sacs. Amniocentesis revealed two normal female karyotypes but ultrasonographical examination in the 11th week of gestation showed a hygroma in one of the fetuses. At the 24th week of gestation this twin died *in utero* while the other developed normally until delivery The pregnancy ended in the birth of a healthy girl in June 1996.

Discussion

The use of frozen–thawed human spermatozoa is well established in insemination cycles and IVF procedures (Mahadevan *et al.*, 1983; Cohen *et al.*, 1985) using ejaculated spermatozoa. For epididymal gametes in combination with microinjection techniques (Tournaye *et al.*, 1994), the possibility of freezing excess spermatozoa obtained during the actual treatment for later use in additional ICSI cycles has been successfully applied (Nagy *et al.* 1995). The post-thaw survival rates and motility parameters of cryopreserved spermatozoa depend on several factors such as initial sperm quality, cryoprotectant, freezing and thawing protocol as well as dilution and washing procedures. The combination of glycerol as cryoprotectant with a slow computer-controlled freezing protocol and rapid thawing to room temperature of the samples proved a reliable method to obtain a high proportion of motile and morphologically-normal spermatozoa from ejaculates of normal donors (Verheyen *et al.*, 1993). The freezing method described by Salzbrunn *et al.* (1996) used a similar approach for whole testicular tissue of infertility patients and showed the applicability of this technique for the isolation of single motile spermatozoa which can be microinjected into the female partners' oocytes.

The clinical application of this newly described modification of sperm extraction from cryopreserved testicular biopsies proved a valuable supplement in the treatment of male factor infertility by the ICSI technique. The method compares well with other descriptions of sperm retrieval from fresh testicular tissue (Shrivastav *et al.*, 1994; Nagy *et al.*, 1995). The most evident advantage is that active spermatogenesis can be identified before the female partner undergoes any stimulation treatment, and no further operation is needed in the male for the treatment to proceed, or when subsequent cycles are needed until pregnancy is achieved. Conversely, all cases in which male gametes with fertilization potential are not produced can be detected quite reliably by the intensive andrological pretreatment diagnosis (semi-thin sectioning histology, test preparation for TESE), thus saving the female partners a frustrating IVF cycle.

By the end of 1995, a total of 13 cycles in 11 couples had been performed in our centre in the same way as described in this case report and eight pregnancies had been achieved (unpublished data). The fertilization rate of oocytes remaining intact after the ICSI procedure with spermatozoa extracted from frozen–thawed testicular tissue was 52% and compared well with the figure of 57% reported by Nagy *et al.* (1995) for spermatozoa from fresh testicular tissue.

In conclusion, we consider that the modifications in the preparation of spermatozoa from frozen–thawed testicular tissue described here are a real facilitation in cycle management for both the affected patients and the personnel involved in the ICSI programme, as well as being highly successful in terms of pregnancy results.

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