Article

Crude cumulative delivery rate following ICSI using intentionally frozen–thawed testicular spermatozoa in 51 men with non-obstructive azoospermia

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Abstract

This prospective study evaluated the crude cumulative delivery rate following delayed intracytoplasmic sperm injection (ICSI) using spermatozoa recovered by testicular extraction (TESE) and intentionally frozen in men with non-obstructive azoospermia (NOA). This procedure can be termed 'cryoTESE-ICSI'. This study involved a series of 118 patients who underwent testicular biopsy for diagnosis of NOA in the period from January 1998 to December 2002. Testicular histology confirmed the diagnosis of NOA. Testicular parenchyma was obtained surgically from both testicles under general anaesthesia. Cryopreservation of spermatozoa was performed in 51 of 118 patients (43%). Ninety-nine delayed ICSI procedures were performed. Frozen–thawed suspensions were used in all cycles. Application of pentoxifylline was required to stimulate spermatozoa in 52% of cases. Fertilization, embryo transfer, and ongoing pregnancy rates were 60, 98 and 29% respectively. The crude cumulative delivery rate was 49% after two cycles and 57% after four cycles. A total of 39 healthy children were born in 29 deliveries. Thus, cryoTESE-ICSI is an effective procedure for routine use in patients with NOA. The main advantages of cryoTESE-ICSI are to (i) avoid repeated surgical biopsy, (ii) ensure the availability of spermatozoa when the ovarian stimulation cycle is begun, and (iii) allow programmed biopsy and therefore dissociate it from ICSI.

Keywords: *frozen–thawed spermatozoa, ICSI, non-obstructive azoospermia, pentoxifylline, pregnancy outcome, TESE*

Introduction

Testicular sperm extraction (TESE) has been used in combination with intracytoplasmic sperm injection (ICSI) to achieve fertilization (Craft *et al*., 1993) and pregnancy (Schoysman *et al*., 1993) for men with non-obstructive azoospermia (NOA). However, no spermatozoa are found in diagnostic testicular biopsy samples in about 50% of patients with NOA (Friedler *et al*., 1997a; Schlegel *et al*., 2004). As a result, simultaneous ovarian stimulation for ICSI procedures often fails, because injection cannot be completed due to non-productive TESE. Since successful ICSI procedures have been reported with frozen–thawed testicular spermatozoa (Gil-Salom *et al*., 1996), use of cryopreserved spermatozoa recovered prior to ovarian stimulation has been proposed as a means of avoiding not only unnecessary egg retrieval (Wurfel *et al*., 1998), but also damage to the testis due to repeated testicular surgery. Several studies have confirmed that similar results can be obtained using fresh and frozen–thawed sperm (Friedler *et al*., 1997b; Liu *et al*., 1997; De Croo *et al*., 1998; Ben Rhouma *et al*., 2003; Dirnfeld *et al*., 2003; Thompson-Cree *et al*., 2003). Histopathology must be performed for diagnosis of NOA. It has also been shown to be the only reliable predictor of successful TESE (Tournaye *et al*., 1997; Schwarzer *et al*., 2003). As a result, biopsy procedures are usually performed at some time prior to ICSI. Since 1998, spermatozoa obtained during prior testicular diagnostic biopsy for delayed ICSI have been intentionally frozen. The purpose of this study carried out for the period up to May 31, 2004 was to evaluate the

crude cumulative delivery rate (CCDR) following delayed ICSI using testicular spermatozoa recovered and intentionally frozen between 1998 and 2002 in men with NOA. Previous series have reported CCDR following ICSI using freshly retrieved testicular spermatozoa (Osmanagaoglu *et al.*, 2003), but this is the first series involving ICSI using intentionally cryopreserved extracted testicular spermatozoa.

Materials and methods

Patients

This prospective series includes a total of 118 patients who underwent only one testicular biopsy for diagnosis of NOA between January 1998 and December 2002. In all cases, azoospermia was suspected on the basis of complete inverted-microscope examination of pellets obtained after centrifugation of two semen samples at 800 *g* for 20 min. All patients underwent physical examination, hormonal (FSH, LH) and biochemical marker assessment. Scrotal ultrasound and karyotype analysis were also performed. Klinefelter patients were excluded from the study. Testicular histology performed on the same day as TESE with a mean of 37 tubules (range, 10–70) confirmed diagnosis of NOA. The aetiology of NOA was classified into three groups: group 1, Sertoli cell-only syndrome (SCOS) in 51 cases; group 2, maturation arrest (MA) in 43; and group 3, hypo-spermatogenesis (HS) in 24. In patients with HS, histology demonstrated numerous tubules without spermatogenesis associated with a few tubules showing limited spermatogenesis. Patients with normal spermatogenesis or discrete hypo-spermatogenesis, i.e. a few tubules without spermatozoa and normal or slightly reduced spermatogenesis in most tubules were excluded from study. The mean age of the NOA patients included in this study was 35.2 years (range, 25–47) on the day of TESE. Female age was recorded on the day of ICSI cycle treatment.

Testicular sperm extraction

Testicular tissue was obtained surgically under general anaesthesia. An incision was made in the scrotal skin, tunica vaginalis and tunica albuginea (1–1.5 cm). Testicular parenchyma was extruded by gentle pressure on the testis. A large sample from both testicles (mean: 575 mg per testicle) was excised with curved scissors. After rinsing in BM1 medium (Ellios; Eurobio, Les Ulis, France) to remove red blood cells, specimens were sent to the laboratory in a closed tube containing 3 ml of medium. A small piece of the biopsy was randomly sent for histopathological examination. In the laboratory, testicular tissue was vigorously shredded in a Petri dish containing 4 ml of medium using two glass microscope slides. Mechanical shredding lasted at least 5–10 min so as to obtain a thick suspension containing no particles larger than 1 mm. After repeated aspiration and spilling out, the testicular suspension was placed in a 5 ml tube with a conical bottom and allowed to settle for 1–2 min so as to obtain a sedimentation pellet containing the largest particles. Leaving the pellet on the bottom of the tube, most of the supernatant (around 4 ml) was recovered, deposited in two conical-bottom tubes containing 1 ml of SupraSperm monolayer (80%) (MediCult, Limonest, France) and centrifuged at 800 *g* for 20 min. The supernatants were discarded and the interfaces (0.5 ml) between the monolayer and supernatant were recovered and set aside for treatment if needed. The two 80% phases were pooled and washed once with an equal volume of medium. The resulting centrifugation pellet was resuspended in 30 µl of medium (total testicular extract) and an initial examination of one 2 µl microdrop was performed under an inverted microscope to identify spermatozoa. If no spermatozoa were found the whole testicular extract was placed in a Petri dish and covered with paraffin mineral oil (Mineral Oil; Medicult). After searching for 15–20 min, if no spermatozoa were found, the undiscarded interfaces were washed once with an equal volume of medium and the centrifugation pellet was examined under the same conditions as previously described. At each step, as soon as motile or immotile spermatozoa were observed, the testicular extract (30 µl) was recovered, resuspended in 0.250 ml of BM1 medium and frozen. No vitality testing was carried out prior to freezing.

Cryopresevation and thawing of testicular spermatozoa

After slow dilution by adding an equal volume of cryopreservation medium (Sperm-Freeze; FertiPro, Beernem, Belgium) drop by drop for 5 min, the testicular extract was sealed in 0.150 ml straws (CBS 06433; CryoBioSystem, Paris, France). After 10-min incubation at 20°C, the straws were frozen according to a simple two-step freezing protocol using a controlled freezer (Nicool LM10; Air Liquide, Paris, France). During the first step of freezing from +20°C to –130°C, the cooling rate was –10°C/min. The second step comprised plunging the straws into liquid nitrogen for storage.

A thawing test was performed on the day of cryopreservation. One straw was thawed for 10 min at 37°C and washed with 2 ml of BM1 medium added drop by drop for 5 min. After centrifugation at 300 *g* for 15 min, the supernatant was discarded and the pellet was resuspended in 20 µl of BM1 medium. After placing this sperm preparation covered with paraffin mineral oil in a Petri dish and allowing it to settle for 5 min, motility was assessed under an inverted microscope. Examination of the whole preparation lasted for 15–20 min depending on the number of spermatozoa and amount of debris present. If total immotility was observed, a pentoxifylline (Torental; Aventis Pharma GmbH, Frankfurt, Germany) test was carried out (Terriou *et al.*, 2000). An equal volume of a solution of pentoxifylline diluted at 10% in BM1 medium was added to the whole sperm preparation. The pharmaceutical form (solution for intravenous injection) contains 100 mg of synthetic pentoxifylline in ampoules of 5 ml and the concentration in the 10% solution was 7.2 mmol/l. After allowing settling for 5 min, motility was reassessed for 15–20 min. Movement of the flagella (slight in extreme cases) was detected in all cases and no patient was excluded from study. This test allowed us to adapt the conditions of ICSI.

Ovarian stimulation and ICSI procedure

Ovarian stimulation was performed using recombinant follicular stimulating hormone (Gonal-F; Serono, Boulogne, France or Puregon; Organon, Eragny sur Epte, France) after pituitary desensitization using gonadotrophin-releasing hormone agonist (Decapeptyl 3 mg; Ipsen Biotech, Paris, France). Transvaginal