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Pregnancies after testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermia

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In this study (May 1 until August 31, 1994) a total of 15 azoospermic patients suffering from testicular failure were treated with a combination of testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI). Spermatozoa were available for ICSI in 13 of the patients. Out of 182 metaphase II injected oocytes, two-pronuclear fertilization was observed in 87 (47.80%); 57 embryos (65.51%) were obtained for either transfer or cryopreservation. Three ongoing pregnancies out of 12 replacements (25%) were established, including one singleton, one twin and one triplet gestation. The ongoing implantation rate was 18% (six fetal hearts out of 32 embryos replaced).

Key words: intracytoplasmic sperm injection/pregnancy /Sertoli cell/testicular spermatozoa

Introduction

In couples suffering from extreme oligoasthenoteratozoospermia, intracytoplasmic sperm injection (ICSI) has been established as a treatment which results in high fertilization and pregnancy rates (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993a,b). In couples with obstructive azoospermia such as in congenital absence of the vas deferens (CAVD), as well as in any case of irreparable obstruction, microsurgical epididymal sperm aspiration (MESA) and ICSI have been shown to result in surprisingly normal fertilization and high pregnancy rates (Silber *et al.*, 1994; Tournaye *et al.*, 1994). Furthermore, it has been well documented that even in the complete absence of epididymis, testicular sperm extraction (TESE) with ICSI will allow the development of viable embryos and establishment of viable pregnancies (Schoysman *et al.*, 1993; Devroey *et al.*, 1994; Silber *et al.*, 1995). In fact, fertilization and pregnancy rates with testicular spermatozoa and ICSI are quite normal. The aim of the present study was to assess the efficacy of ICSI with TESE in cases of non-obstructive azoospermia, to see if any spermatozoa could be retrieved from azoospermic patients with severe spermatogenic deficiency, or even an apparent complete absence of spermatogenesis.

Materials and methods

Male patients

A testicular biopsy was performed in 15 males with non-obstructive azoospermia [checked several times by careful examination of a centrifuged specimen and indicated by high follicle stimulating hormone (FSH) concentration and small testicular size]. All procedures were carried out between May 1 and August 31, 1994. On the same day, oocytes were retrieved from their spouses after routine ovarian stimulation. A testicular biopsy was taken and transferred into a Petri dish with HEPES-buffered Earle's medium. In the adjacent laboratory the biopsy specimen was fragmented on the heated stage of a stereomicroscope into small pieces with sterile glass microscope slides. The presence of motile free spermatozoa was then assessed on the inverted microscope at a ×200 or ×400 magnification. A second biopsy specimen was removed if no spermatozoa were present. The medium and fragmented biopsy tissue were transferred into the Falcon tube and transported in a 37°C thermobox to the microinjection laboratory. The testicular biopsy was sometimes further fragmented in a Petri dish if very few spermatozoa had been observed. The pieces of biopsy tissue were then removed and the medium was then centrifuged at 300 g for 5 min. The supernatant was discarded and the precipitated sperm suspension was used for the ICSI procedure. An extra biopsy specimen was sent for light microscopic evaluation. Hormonal determination for FSH and luteinizing hormone (LH) was performed.

Female patients

The mean age of the female patients was 32 years (range 24–39). Ovarian stimulation was carried out using a desensitizing protocol of gonadotrophin-releasing hormone (GnRH) analogues (buserelin; Suprefact; Hoechst, Brussels, Belgium) in association with human menopausal gonadotrophins (HMG; Humegon, Organon, Oss, The Netherlands; Pergonal, Serono, Brussels, Belgium). This regimen has been extensively described elsewhere (Smitz *et al.*, 1988). In the luteal phase, natural micronized progesterone (600 mg) was administered

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intravaginally in three doses (Utrogestan, Piette, Brussels, Belgium) (Smitz *et al.*, 1992, 1993).

Oocyte preparation

Oocyte retrieval was carried out by vaginal ultrasound-guided puncture 36 h after human chorionic gonadotrophin (HCG) injection. The number of cumulus–corona complexes was noted. The cumulus–corona cell complexes were placed in 5 ml Falcon tubes with Earle's medium; the tubes were gassed (5% O_2, 5% CO_2, 90% N_2), closed and transported in a thermobox at 37°C to the microinjection laboratory. The removal of the cells of the cumulus and the corona radiata has been described elsewhere. The oocytes were carefully assessed and examined for the presence or absence of a germinal vesicle or a polar body. All metaphase II oocytes were then prepared for microinjection as already described (Van Steirteghem *et al.*, 1993a,b).

Intracytoplasmic sperm injection procedure

The preparation of the holding and injection pipettes, as well as the injection procedure itself, have been extensively described (Van Steirteghem *et al.*, 1993a,b). A 5 µl droplet of the resuspended pellet was placed close to the central polyvinylpyrrolidone (PVP) droplet. For the injection, a morphologically normal spermatozoon with 'motility', if present, was aspirated into the injection pipette and placed in the PVP droplet. The spermatozoon was frequently embedded in Sertoli cell cytoplasm and needed to be carefully extracted. It was rinsed in PVP media solution and immobilized before injection into the oocyte. A metaphase II oocyte was immobilized by slight negative pressure on the holding pipette and the single spermatozoon was then injected into the ooplasm.

Assessment of fertilization and embryonic development

Approximately 18 h after the microinjection, the oocytes were checked under the inverted microscope (×200, ×400) for the presence of two clearly distinct pronuclei (2PN). Embryonic development was further evaluated after 1 day. The embryos were scored for quality according to the size of blastomeres and the number of anucleate fragments (Staessen *et al.*, 1989, 1994; Nagy *et al.*, 1994). Cleaved embryos with <50% of their volume filled with anucleate fragments were considered suitable for transfer. A maximum of three embryos were loaded into 5 µl of Earle's medium and into a Frydman catheter (LG 4.5; Prodimed, Neuilly-en-Thelle, France) and replaced into the uterine cavity ~48 h later. If supplementary embryos (<20% anucleate fragments) were available, they were cryopreserved (Van Steirteghem *et al.*, 1994).

Establishment and follow-up of pregnancy

Clinical pregnancy was determined by visualization of a gestational sac at 7 weeks of pregnancy. Prenatal diagnosis was proposed either by chorionic villus sampling at ~10 weeks or by amniocentesis at 16 weeks. A prospective follow-up study of the children is planned (Bonduelle *et al.*, 1994).

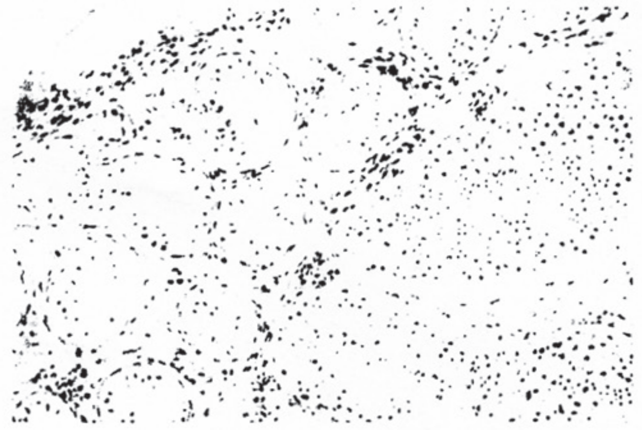

Figure 1. Sertoli-cell-only syndrome with occasional seminiferous tubule producing spermatozoa.

Results

The histology of 14 azoospermic patients revealed severe spermatogenic defects. For one patient, no histological report was available but the patient had small testes and FSH was 40 IU/l. None of these patients had any evidence of normal spermatogenesis or of obstruction as a cause of the azoospermia. In six of the patients diagnosed as Sertoli-cell-only, either an occasional single seminiferous tubule with spermatogenic activity was observed (n = 3) or no spermatogenic activity (n = 3) (Figure 1) was noted at all on the histological preparation. Three patients had maturation arrest and three patients had severe hypo-spermatogenesis defined as a non-focal generalized reduction in spermatogenetic activity in all seminiferous tubules. Two patients had severe tubular sclerosis, and for one patient, unfortunately, no histology was available (Table I).

FSH was elevated in 12 of the patients, was surprisingly normal in two and was not available in one.

Despite the severe histological defects indicating absence or near-absence of spermatogenesis associated with documented persistent azoospermia, 13 of these 15 patients had (extremely small) numbers of spermatozoa retrieved directly from the testicle and in only two of these 15 cases could no spermatozoa be retrieved for injection. In general, a few spermatozoa were observed attached to the free-floating Sertoli cells; some were immotile and some had only the barest occasional slight 'twitching' motion.

In these 15 cycles, 261 cumulus–corona oocyte complexes were retrieved, i.e. a mean of 17.4 cumuli/cycle. A total of 211 metaphase II oocytes (i.e. 80.8%) of the cumulus–corona complexes contained a fertilizable oocyte. A total of 29 metaphase II oocytes from two patients could not be injected because of the absence of spermatozoa in the testicular biopsy. Out of 182 injected metaphase II oocytes, 23 were damaged (12.6%). The percentage of 2PN oocytes was 47.8% and the cleavage rate was 78.16%. In all, 57 embryos were eligible for immediate replacement and 25 were cryopreserved for later use (Table II). Out of the 15 patients, 12 had transferable embryos. For two patients, no spermatozoa were available and in one patient's oocytes cleavage did not occur. Three ongoing

Table I. Histological analysis of testicular biopsy in non-obstructive azoospermic patients

Patient no.	Hormonal evaluation		Histological report	Testicular spermatozoa	Pregnancy
	FSH (IU/l)	LH (IU/l)			
1	19.0	12.0	Sertoli cell only	+	+
2	21.9	13.1	Sertoli cell only	+	–
3	-	-	maturation arrest	–	–
4	10.6	5.5	tubular fibrosis	+	–
5	4.8	3.1	maturation arrest	+	–
6	17.0	3.7	maturation arrest	–	–
7	14.2	5.4	hypospermatogenesis	+	+
8	22.8	5.9	Sertoli cell only	+	–
9	7.9	4.5	hypospermatogenesis	+	–
10	40.0	20.0	not available	+	–
11	17.5	6.7	Sertoli cell only	+	–
12	24.0	10.0	tubular fibrosis	+	+
13	3.0	1.5	hypospermatogenesis	+	–
14	9.0	5.9	Sertoli cell only	+	–
15	7.5	4.5	Sertoli cell only	+	–

FSH = follicle stimulating hormone; LH = luteinizing hormone.

Table II. Two-pronuclear (2PN) fertilization and cleavage rate after testicular sperm extraction and ICSI in non-obstructive azoospermic patients

	No.	Percentage
Metaphase II oocytes injected		182
Oocytes intact	159	87.4
2PN oocytes	87	47.8
Transferable embryos	68	78.2
Embryos transferred or frozen	57	65.5

ICSI = intracytoplasmic sperm injection.

pregnancies have been established, including one triplet, one twin and one singleton. In all, 32 embryos were transferred and on six occasions positive fetal heart activity was confirmed by ultrasound. The ongoing implantation rate was 18.75%.

Discussion

The success with TESE and ICSI in cases of obstructive azoospermia allowed us to speculate whether the same approach could not be used in sterile men with a severe spermatogenic defect (Devroey *et al.*, 1994; Silber *et al.*, 1995). Our centre routinely performs ICSI with the tiniest numbers of spermatozoa found in a centrifuged portion of ejaculate from an apparently almost 'azoospermic' man and such patients were not considered candidates for this study. This study was limited to patients who on repeated examination were demonstrated to be completely azoospermic and whose histology verified severe spermatogenic defects.

The rationale for attempting this study is the observation that in patients with Sertoli-cell-only syndrome or tubular sclerosis there may be a single seminiferous tubule producing spermatozoa, despite the rest of the testicular biopsy showing complete lack of spermatogenesis and no spermatozoa being found even in the centrifuged specimen of the ejaculate. We wished to see whether we could possibly retrieve these rare numbers of spermatozoa directly from the testicle under the assumption that in such small quantities they could not find a way to the ejaculate. We were surprised to discover that we could indeed retrieve enough spermatozoa for successful ICSI procedures in patients with such severe spermatogenic defects. Therefore, even when the testicle biopsy specimen showed not a single tubule with spermatogenesis, the knowledge that there could be a tiny point of normal spermatogenesis somewhere in the testicle led us to attempt TESE and ICSI even with patients whose testicle biopsy revealed no spermatogenesis whatsoever. Again, we were surprised to observe that in 13 out of 15 cases we were able to find sufficient numbers of spermatozoa, however rare, with which to perform ICSI successfully for the couple.

If spermatozoa showed some motility, they were preferentially used. If all spermatozoa were immotile, ICSI was performed successfully and 2PN fertilization was obtained. The findings demonstrate that testicular spermatozoa have gained full maturity and initiate normal fertilization, normal embryo cleavage and implantation. The epididymis is not necessary for fertilization of human oocytes *in vitro* using ICSI in the presence of azoospermia due to testicular failure. It was also surprising to observe that the fertilization rate and cleavage rate were remarkably similar in patients with severe spermatogenic defects to what we observed using testicular spermatozoa from men with normal spermatogenesis (in cases of obstructive azoospermia) (Devroey *et al.*, 1994; Silber *et al.*, 1995). The resulting embryos had a normal implantation rate (18%), which is similar to the implantation rate with ejaculated spermatozoa.

In one twin pregnancy, amniocentesis was performed and two normal karyotypes were noted. The other two patients refused prenatal diagnosis. Although the spermatogenic defects in these patients were of such an extreme extent that they were all considered hopeless cases for ICSI with ejaculated spermatozoa, direct extraction of spermatozoa from the testicle was nonetheless highly successful.

The FSH concentration was elevated in 12 patients, as one would expect with severe spermatogenic deficiency. The two patients who had FSH at a normal concentration, had matura-

tion arrest or hypospermatogenesis rather than Sertoli cell-only syndrome. Since FSH inhibition is regulated by a complex interaction between all the spermatogenic elements in the testes, testicular size and Sertoli cell secretion of inhibin, it would not be surprising for such patients with severe spermatogenic defects occasionally to have a normal FSH concentration. In fact, this experience in the era of ICSI shows that the FSH concentration is not a predictive factor in the evaluation of treatment planning for male factor infertility patients. As expected in the two patients with maturation arrest, no spermatozoa were found for injection.

It may be postulated that the FSH concentration is not a predictive factor in patients whose testicular spermatozoa are to be used, since in obstructive azoospermia the FSH concentrations are normal and in non-obstructive azoospermia FSH concentrations are three times higher, and yet we have observed that the fertilization rates in both groups were similar.

In the past, males with a triad of non-obstructive azoospermia, elevated FSH and small testes were considered sterile. Our studies have demonstrated that these patients are potentially fertile, and by using the association of TESE and ICSI, they have a fertilizing potential comparable to that of patients with normal spermatogenesis.

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