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Outcome of intracytoplasmic sperm injection with testicular spermatozoa in obstructive and non-obstructive azoospermia

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From 1 August 1993 until 30 September 1994, 69 couples suffering from azoospermia underwent testicular sperm extraction and intracytoplasmic sperm injection. In 50 couples with obstructive azoospermia a total of 631 metaphase-II oocytes were injected after testicular sperm extraction yielding a 2-PN fertilization rate of 57%. In female patients <40 years of age an ongoing pregnancy rate per transfer of 42% (14/33) was obtained. So far, eight healthy babies have been born, including two singletons and three twin gestations. In 19 couples with non-obstructive azoospermia a total of 264 metaphase-II oocytes were injected after testicular sperm extraction, yielding a 2-PN fertilization rate of 58%. An ongoing pregnancy rate per transfer of 31% (5/16) was established. So far, six healthy babies have been born including one singleton, one twin and one triplet gestation.

Key words: intracytoplasmic sperm injection/obstructive azoospermia/non-obstructive azoospermia/testicular sperm extraction

Introduction

Patients with obstructive azoospermia due to bilateral congenital absence of the vas deferens were considered untreatable until recently. Results with alloplastic spermatocoele had been disappointing and microsurgical sperm aspiration with in-vitro fertilization (IVF) had resulted in very low ongoing pregnancy rates (Belker *et al.*, 1986; Silber *et al.*, 1994). More recently, ongoing pregnancy and delivery rates using intracytoplasmic sperm injection (ICSI) in patients with impaired ejaculated semen samples have been achieved (Van Steirteghem *et al.*, 1993a,b). Subsequently, in patients with bilateral congenital absence of the vas deferens, improved fertilization, cleavage and pregnancy rates were reported using ICSI after microsurgical epididymal sperm aspiration (Tournaye *et al.*, 1994). Furthermore, in the absence of epididymis, fertilization has been reported using testicular sperm, extraction and ICSI (Schoysman *et al.*, 1993; Devroey *et al.*, 1994; Bourne *et al.*, 1995; Craft and Tsirigotis, 1995; Nagy *et al.*, 1995; Silber *et al.*, 1995). Fertilization has also been achieved with extraction of tiny numbers of testicular sperm in cases of non-obstructive azoospermia (Devroey *et al.*, 1995). The aim of this report is to compare the fertilization, cleavage and pregnancy rates using testicular spermatozoa for patients with obstructive azoospermia with those for patients with non-obstructive azoospermia who are identified as having testicular failure.

Materials and methods

Male patients

The mean age of the male patients with non-obstructive azoospermia was 35.2 years (ranging from 24 to 46) and with obstructive azoospermia was 40.6 years (ranging from 28 to 60). Testicular sperm extraction (TESE) was performed in 19 males with non-obstructive azoospermia and 50 with obstructive azoospermia for the purpose of sperm retrieval for ICSI. Non-obstructive azoospermia was defined by zero spermatozoa observed in several ejaculates, checked several times by careful examination of a centrifuged specimen. In addition clear proof of deficient spermatogenesis was demonstrated. All but one of the patients had severely deficient spermatogenesis, demonstrated histologically, including Sertoli-cell-only syndrome (n = 6), spermatogenic arrest (n = 3), tubular sclerosis (n = 3) and generalized reduction of spermatogenic activity in all seminiferous tubuli (n = 6). For one patient no histology was available (n = 1), but his testes were tiny (<1 cm diameter) and an elevated serum follicle stimulating hormone (FSH) of 40 IU/l was observed. On the other hand, obstructive azoospermia was confirmed when the semen consistently showed zero spermatozoa in a centrifuged specimen, but the testicular biopsy showed normal spermatogenesis. These cases included congenital bilateral absence of the vas deferens (n = 27), failed vasoepididymostomy (n = 20) and failed vasovasostomy (n = 3). All procedures were carried out between August 1, 1993 and September 30, 1994. Testicular sperm extraction was performed on the same day that oocytes were retrieved from the spouses after ovarian stimulation. The testicular biopsy tissue was transferred into a Petri dish with HEPES-buffered Earle's medium (1.5 ml). The testicular biopsy was performed under general anaesthesia and small pieces of tissue were taken until the wet preparation was revealed to be positive. The size of each biopsy was restricted to 0.5 cm (Silber, 1995). The biopsy specimen was shredded into small pieces with sterile glass microscope slides on the heated stage of a stereomicroscope. Using the inverted microscope (×200 or ×400 magnification) the presence of spermatozoa was assessed. If no spermatozoa were found in the first specimen, a second biopsy was taken. Sometimes many biopsies were needed. The effluent as well as the shredded biopsy tissue were transferred into a Falcon tube and transported in a 37°C thermobox to the microinjection laboratory. The pieces of biopsy tissue were then removed and the medium was centrifuged at 300 g for 5 min. The supernatant was saved and the sperm pellet was resuspended in 100 µl of Earle's medium for the intracytoplasmic injection.

Female patients

For the cases of non-obstructive azoospermia, the mean age of the female patients was 31.2 years (ranging from 24 to 39 years), for obstructive azoospermia it was 34.7 years (ranging from 26 to 44 years). The regimen of ovarian stimulation has been extensively described by our group. A desensitizing protocol of gonadotrophin-releasing hormone analogues (GnRHa) (Buserelin; Suprefact: Hoechst, Brussels, Belgium) in association with human menopausal gonadotrophins (HMG; Humegon: Organon, Oss, The Netherlands; Pergonal: Serono, Brussels, Belgium) was used; the protocol has been extensively described elsewhere (Smitz *et al.*, 1988). In the luteal phase, natural micronized progesterone (600 mg) was used intravaginally in three different doses (Utrogestan: Piette, Brussels, Belgium) (Smitz *et al.*, 1992, 1993).

Oocyte preparation

Thirty-six hours after the injection of human chorionic gonadotrophins (HCG; Pregnyl, Organon; Profasi, Serono), oocyte retrieval was carried out by vaginal ultrasound-guided puncture. The cumulus–corona cell complexes were put into 5 ml Falcon tubes with Earle's medium; the tubes were gassed (5% O_2, 5% CO_2, 90% N_2), closed and transported at 37°C to the micro-injection laboratory. The removal of the cells of the cumulus and the corona radiata, the assessment of the oocytes for the presence or absence of a germinal vesicle or a polar body and the preparation of the metaphase-II oocytes for injection have been described extensively elsewhere (Van Steirteghem *et al.*, 1993a,b).

ICSI procedure

A 5 µl droplet of the resuspended pellet was placed close to the central polyvinyl pyrrolidone (PVP) droplet. A single spermatozoon was aspirated into the injection pipette and placed in the PVP droplet. If the spermatozoon was imbedded in a Sertoli cell cytoplasm, careful extraction was needed. If the sperm cell was motile, it was rinsed in PVP solution and immobilized. A metaphase-II oocyte was aspirated with slight negative pressure by the holding pipette and the single spermatozoon was injected into the ooplasm (Devroey *et al.*, 1994, 1995).

Assessment of fertilization and embryonic development

About 18 h after the micro-injection, the oocytes were checked for the presence of two clearly distinct pronuclei under the inverted microscope with magnification of ×200 or ×400 (Nagy *et al.*, 1994). After 48 h the embryos were scored according to the size of the blastomeres and the number of anucleate fragments (Staessen *et al.*, 1994). Type A or *excellent* embryos were defined as embryos in which all blastomeres were of an equal size (1.0) or if of non-equal size were without anuclear fragments (2.0). Type B or *good* embryos had blastomeres of equal size (2.1) or if of non-equal size were without anuclear fragments (2.2). In type C or *fair* embryos anucleate fragments were present in 20–50% of the volume of the embryo (3.1) (Staessen *et al.*, 1994). Cleaved embryos with less than 50% of their volume filled with anucleate fragments were considered suitable for transfer. 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 approximately 48 h after injection. Supernumerary embryos were cryopreserved for later use (Van Steirteghem *et al.*, 1994).

Clinical follow-up of pregnancy

Clinical pregnancy was determined by visualization of at least one gestational sac by ultrasound at 7 weeks of pregnancy. Ongoing pregnancy was determined by visualization of at least one gestational sac with positive heart activity at 12 weeks of gestation. Prenatal diagnosis and a prospective follow-up study of the children were proposed to the patients (Bonduelle *et al.*, 1994, 1995; Wisanto *et al.*, 1995).

Table I. Outcome of ICSI using testicular sperm extraction in obstructive and non-obstructive azoospermia

	Azoospermia	
	Obstructive (n = 50)	Non-obstructive (n = 19)
Cumulus–oocyte complexes	760	336
Germinal vesicle stage oocytes	73	34
Metaphase-I oocytes	26	12
Metaphase-II oocytes (%)	641 (86.6)	264 (85.1)
Oocytes injected	631	235
Oocytes intact (%)	579 (92)	209 (89)
2PN oocytes (%)	333 (57)	122 (58)
Cleaved embryos (%) (1.0–3.1)	230 (69.7)	81 (66.3)
Embryos (%)	223 (67)	74 (61)
Transferred	137	42
Cryopreserved	86	32

Statistical analysis

All statistical tests were carried out two-tailed at the 5% level of significance. The computations were performed using the SPSS/PC+ package on an IBM compatible microcomputer. The comparison of the fertilization rate, the cleavage rate of 2PN oocytes to transferable embryos, the percentages of excellent, good and fair embryos, and the implantation rate in cycles with obstructive azoospermia and in cycles with non-obstructive azoospermia was performed using the non-parametric Kruskal–Wallis test. The same test was used to compare these variables in cycles with obstructive azoospermia in patients ≤40 years of age, cycles with obstructive azoospermia in patients >40 years of age, and cycles with non-obstructive azoospermia (in which all patients were ≤40 years of age). This test was also done when the comparison was restricted to the two groups of patients ≤40 years of age. The comparison of the pregnancy and miscarriage rates in cycles with obstructive azoospermia and in cycles with non-obstructive azoospermia was performed using the Fisher Exact test. For the comparison of these variables in the three groups of cycles the χ^2 test was used.

Results

Overall, 69 cycles of ICSI were attempted using testicular spermatozoa. Fifty cycles were for obstructive azoospermia and 19 were for non-obstructive azoospermia. In one out of the 50 couples with obstructive azoospermia no testicular spermatozoa could be retrieved and in two out of 19 couples with non-obstructive azoospermia no testicular spermatozoa were available for injection. As demonstrated in Table I, a similar 2-PN fertilization rate was obtained in both groups. Similarly, no differences were observed in the cleavage rates or in the numbers of transferable embryos between ICSI cycles in cases of obstructive versus non-obstructive azoospermia. Embryos of sufficient morphological quality were either transferred freshly or cryopreserved for later use.

The percentages of excellent embryos (1.0, 2.0) without anucleate fragments, good embryos (2.1, 2.2) filled with 1–20% anucleate fragments and fair embryos (3.1) filled with

Table II. Embryo quality after testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) in obstructive and non-obstructive azoospermia

Embryo quality	Azoospermia	
	Obstructive	Non-obstructive
Excellent (%)	26 (7.8)	8 (6.6)
Good (%)	164 (49.2)	60 (49.2)
Fair (%)	40 (12)	13 (13)

Table III. Embryo quality of replaced embryos

Embryo quality of replaced embryos	Azoospermia		
	Obstructive		Non-obstructive
	Female partner		
	≤40 years	>40 years	
Number of excellent embryos/total (%)	17/102 (16.6)	4/35 (11.4)	8/42 (19)
Number of good embryos/total (%)	59/102 (57.8)	29/35 (82.8)	28/42 (66.6)
Number of fair embryos/total (%)	26/102 (25.4)	2/35 (5.7)	6/42 (14.2)

Table IV. Ongoing pregnancy rate in obstructive and non-obstructive azoospermia

	Azoospermia		
	Obstructive		Non-obstructive
Female age	≤40 years	>40 years	<40 years
Clinical pregnancy rate per TESE (%)	17/40 (42)	4/10 (40)	5/19 (26)
Clinical pregnancy rate per transfer (%)	17/33 (51)	4/8 (50)	5/16 (31)
Ongoing pregnancy rate per TESE (%)	14/40 (35)	0/10 (0)	5/19 (26)
Ongoing pregnancy rate per transfer (%)	14/33* (42)	0/8* (0)	5/16 (31)
Miscarriage rate (%)	3/17 (17.6)	4/4 (100)	0/5 (0)

*$P = 0.072$.

20–50% anucleate fragments were similar in both groups (Table II). In couples ≤40 years of age with obstructive azoospermia, a mean number of three embryos were replaced and in couples >40 years of age a mean number of 4.4 embryos were replaced. In couples suffering from non-obstructive azoospermia a mean number of 2.6 embryos were replaced. Table III demonstrates the embryo quality of the replaced embryos in obstructive and non-obstructive azoospermia.

Table IV demonstrates a similar ongoing pregnancy rate in obstructive and non-obstructive azoospermia in women ≤40 years of age. In women >40 years of age, there was a 100% miscarriage rate and no ongoing pregnancy. In contrast, only 17.6% of women under 40 miscarried.

The ongoing implantation rates (>12 weeks of gestation) in cases of obstructive azoospermia in women ≤40 years of age were 16.6% (17/102) and 0% (0/35) for women > 40 years ($P = 0.095$). In non-obstructive azoospermia (≤40 years) the ongoing implantation rate was 19% (8/42).

In obstructive azoospermia 11 ongoing singleton pregnancies were obtained. Two patients delivered healthy babies. Furthermore, three ongoing twin pregnancies were established, with delivery of six healthy babies. In non-obstructive azoospermia five ongoing pregnancies were obtained, including three singletons, one twin and one triplet. So far, six healthy babies have been born (one singleton, one twin and one triplet).

Discussion

Our data indicate that the fertilization rate after ICSI with testicular spermatozoa is similar in cases of obstructive and non-obstructive azoospermia. One might have speculated that spermatozoa retrieved from a testicle in cases of obstructive azoospermia with normal spermatogenesis would yield better fertilization and better pregnancy rates than spermatozoa retrieved from an essentially pathological testicle with non-obstructive azoospermia. Non-obstructive azoospermia included Sertoli-cell-only syndrome and severe testicular hypoplasia; the testicles in patients with obstructive azoospermia always revealed completely normal spermatogenesis. Nonetheless, spermatozoa retrieved from either of these two extremely different testicular conditions resulted in similar fertilization rates.

Furthermore, in women ≤40 years of age there was no difference in the ongoing pregnancy rate whether their husbands had normal spermatogenesis with obstructive azoospermia or deficient spermatogenesis with non-obstructive azoospermia. These findings appear to indicate that the source of the spermatozoa, whether from severely deficient testicles or from testicles with normal spermatogenesis, did not relate to the successful ongoing pregnancy rate.

In couples with obstructive azoospermia, our analysis has demonstrated an ongoing pregnancy rate (>12 weeks) per retrieval of 35% (14/40) and per transfer of 42.42% (14/33) in women ≤40 years of age. A zero ongoing pregnancy rate was obtained in women >40 years of age. This observation supports generally accepted data on reduced ongoing implantation rates in women >40 years of age. For the sake of clarity, the initial pregnancy rate per transfer in women ≤40 years of age was 51.5% (17/33) and 50% (4/8) in women >40 years of age. Although the initial pregnancy rates were similar, among the younger patients (≤40 years) only three out of 17 (17.6%) aborted but among the older women four out of the four aborted. Comparing the ongoing implantation and pregnancy rates per transfer in women ≤40 years of age and women >40 years of age, no significant differences were found ($P = 0.095$; $P = 0.072$). Significance was probably not reached, due to the small number of patients. Women >40 years of age should be counselled carefully about their reduced chances. Surprisingly, the 2-PN fertilization rate of women ≤40 years of age and women >40 years of age was very similar, i.e. 52.79% (274/519) versus 52.6% (59/112). These data support the hypothesis that the problem is not located at the level of fertilization. Therefore the potential to become fertilized with testicular spermatozoa is independent of

women's age. This observation is highlighted even more by the 16.66% (17/102) ongoing implantations in women <40 years of age and 0% (0/35 embryos) in women ≥40 years. The mean age of the male partner in women >40 years of age was 42.2 (range 40–45 years) and 39.1 (range 28–60 years) in women ≤40 years of age. These data suggest that the absence of ongoing implantation rates in women ≤40 years of age is not related to the male age. In fact, the only factor which effected the ongoing pregnancy rate was the age of the female partner.

In couples with obstructive azoospermia (≤40 years of age) seven patients did not have an embryo replacement for various reasons, namely absence of testicular spermatozoa in one, immotile spermatozoa in one, and only a reduced number of metaphase-II eggs in three (respectively one, two and three eggs). Only in two patients with a sufficient number of eggs (10 and 11) was the absence of fertilization not understood. These data suggest that if ovarian stimulation is not adequate, the oocyte retrieval should be cancelled, avoiding surgery in the male partner, and a subsequent cycle should be advised. In two couples with non-obstructive azoospermia demonstrating spermatogenic arrest, no spermatozoa were available for injection.

It must be emphasized that even in cases of the most severe testicular failure such as Sertoli-cell-only syndrome, patients now must be counselled that if a few spermatozoa are found, the chances of pregnancy for the couple are no different from those for a couple with normal spermatogenesis. However, couples in whom the age of the female is advanced should be warned that, although this technique allows a normal fertilization rate, the miscarriage rate is extremely high and the chance of the delivery of living offspring is extremely low. The combination of ICSI with testicular sperm extraction is a new solution to problems of severe male-factor infertility involving azoospermia, whether obstructive or non-obstructive. However, it is not an adequate solution for couples in whom the female is >40 years.

In conclusion, testicular spermatozoa yield high fertilization and implantation rates both in cases of normal spermatogenesis and in cases with severe spermatogenic failure. The limiting factor for such couples will be the age of the female partner.

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