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Normal fertilization of human oocytes after testicular sperm extraction and intracytoplasmic sperm injection*

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Intracytoplasmic sperm injection in cases of extreme oligoasthenoteratozoospermia allows the development of embryos and the establishment of ongoing pregnancies and deliveries (1–3). Similar results have been obtained with the association of intracytoplasmic sperm injection and microsurgical epididymal aspiration of sperm in cases of bilateral congenital absence of the vas deferens (4). On some occasions, both epididymides are absent and the only source of spermatozoa is the testis. The aim of this study was to assess the efficacy of intracytoplasmic sperm injection with testicular sperm.

MATERIALS AND METHODS

In the males of three infertile couples with obstructive azoospermia, a scrotal exploration was performed in July, 1993. The epididymis was found to be absent after previous surgery. No sperm was found in the rete testis and therefore a testicular biopsy was performed on the same day that oocytes were retrieved from their wives after controlled ovarian superovulation. The testicular tissue was placed in a Petri dish (Falcon type 1006; Becton Dickinson, Erembodegem, Belgium) containing HEPES-buffered Earle's medium. The testicular tissue was dispersed mechanically by the help of blunt-ended plastic rods and an injection needle. After the dispersion, the testicular tissue was checked under an inverted microscope at 200× and 400× magnification for the presence of sperm cells. Many spermatids were noted to be attached to free floating Sertoli cells. Sporadic free spermatozoa (±20) were seen. Those spermatozoa had only the barest slightly occasional "twitching" motion. The findings were in accordance with previously published data (5). The technique was to isolate a single spermatozoon. Without any further treatment, the homogenized testicular tissue solution was kept in an incubator at 37°C until the moment of the injection procedure. The time lapse between the testicular tissue extraction and intracytoplasmic sperm injection was never >4 hours. Ovarian superovulation was obtained by hMG (Humegon, Organon, Oss, The Netherlands; Pergonal, Serono, Brussels, Belgium) in association with GnRH agonists (GnRH-a) (Suprefact; Hoechst, Frankfurt, Germany). Thirty-six hours after the injection of hCG (Pregnyl, Organon; Profasi, Serono) the cumulus-corona-oocyte complexes were retrieved by vaginal ultrasound. After transfer to the microinjection laboratory, the cumulus and corona cells were removed by incubation for <1 minute in HEPES-buffered Earle's medium containing 80 IU/mL hyaluronidase (type VIII; Sigma Chemical Co., St. Louis, MO) and by mechanical action.

Afterwards, the oocytes were observed under the inverted microscope and nuclear maturity was recorded. Until the moment of the injection procedure, metaphase II oocytes were kept in 25-μL droplets of B_2 medium (bio-Mérieux; Montalieu, Vercieu, France) covered by light-weight paraffin

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oil (British Drughouse; Pasture, Brussels, Belgium) in an incubator at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. The intracytoplasmic sperm injection procedure was carried out on the heated stage (37°C) of an inverted microscope (Diaphot; Nikon Corporation, Tokyo, Japan) at 400× magnification using a Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, NY). The microscope was equipped with two coarse positioning manipulators (3D Motor Driven Coarse Control Manipulator MM-18; Narishige, Tokyo, Japan) and with two three-dimensional hydraulic remote-control micromanipulators (Joystick Hydraulic Micromanipulator MO-188; Narishige). The holding and injection pipettes had an outer diameter of 60 to 80 μm and 8 to 10 μm and an inner diameter of 10 to 15 μm and 5 to 7 μm, respectively. They were fixed to a tool holder and were connected to a micrometer-type microinjector (IM-6; Narishige). The injection dish contained a central droplet of 5 μL HEPES-buffered Earle's medium with 10% polyvinyl pyrrolidone (PVP) solution. This central droplet was surrounded by eight droplets of HEPES-buffered Earle's medium each containing a single oocyte. Just before the injection procedure, testicular tissue suspension was centrifuged at 300 × *g* for 5 minutes. Five microliters of the resuspended pellet also was put into the injection dish next to the central PVP droplet. For the injection, a morphological normal spermatozoon with some motility was looked for. The sperm cell was aspirated, tail first, into the injection pipette. The oocyte was held to the holding pipette by slight negative pressure, the injection pipette was introduced deeply into the cytoplasm, and the sperm was deposited together with the smallest possible amount of medium (±1 pL). Only metaphase II oocytes were injected and after washing were returned to Petri dishes containing B_2 medium to be stored in the incubator. About 16 hours after microinjection, oocytes were checked for intactness and for fertilization, and 40 hours later normally fertilized oocytes were inspected for cleavage and morphological appearance.

RESULTS

In total, 68 cumulus-oocyte complexes were retrieved. Forty-five metaphase II oocytes were injected and 44 oocytes were intact after injection. Twenty oocytes showed two distinct pronuclei 18 hours after injection, and 17 embryos cleaved normally. As demonstrated in Table 1, respectively, nine, five, and three four-celled embryos were obtained. In the first patient, three of nine embryos were replaced and six were cryopreserved for later use. In the second patient, all five embryos were transferred because the patient was >40 years of age. In the third patient, all three embryos were replaced. So far, no pregnancy has occurred. At this writing, of the six frozen embryos, four were thawed and three were replaced without the establishment of a pregnancy; two embryos are still frozen.

Table 1 Fertilization of Human Oocytes by Testicular Sperm Extraction Using Intracytoplasmic Sperm Injection

Patient	Cumulus-oocyte complexes	Metaphase II oocytes injected	No. of intact oocytes	No. of two pronuclei fertilization	No. of embryos obtained
1	34	22	21	10	9
2	22	14	14	6	5
3	12	9	9	4	3
Total	68	45*	44†	20‡	17§

* 62.2% of the cumulus-oocyte complexes contained a metaphase II oocyte that was injected with a single spermatozoon.
† 97.8% of the injected oocytes were intact after the injection.
‡ 45.5% of the successfully injected oocytes had 2 pronuclei 18 hours after intracytoplasmic sperm injection.
§ 85.0% of the normally fertilized oocytes developed in vitro to embryos that were transferred into the uterus or cryopreserved.

DISCUSSION

In obstructive azoospermia, traditional IVF and especially intracytoplasmic sperm injection have proven to be efficient using epididymal sperm (4, 6). A remaining group are patients with absent epididymis and where the only source of spermatozoa is located in the testis. It has been well documented that motile spermatozoa are present in human testicular tissue especially when the entire epididymis is destroyed and that histologic examination by light microscopy demonstrates its complete spermatogenesis (5). It must be mentioned that all three testicular specimens were remarkably similar. Our observation indicates that testicular sperm from a peripheral biopsy can induce normal fertilization (45%) and normal embryo cleavage (85%). Using testicular tissue as a source of sperm cells for IVF is a totally new approach. Microinjection techniques have made it possible for difficult procedures such as testicular sperm aspirations to provide a fair hope for fertilization. In our opinion, the use of testicular sperm requires intracytoplasmic sperm injection because a limited number (±20) of spermatozoa were observed. Of the different steps of assisted fertilization techniques, intracytoplasmic

sperm injection seems to be the best procedure for testicular sperm insemination because intracytoplasmic sperm injection provides the highest fertilization rate compared with other types of assisted fertilization techniques (2). Furthermore, intracytoplasmic sperm injection requires only one sperm cell for one oocyte, which is the most economical use, especially in cases of testicular sperm. Although several mechanisms, including passage through the epididymis, are needed for IVF, surprisingly the epididymis is not needed for use of intracytoplasmic sperm injection. This indicates that the exact role of the epididymis has to be reconsidered as related to in vivo and in vitro conditions. Normal fertilization after intracytoplasmic sperm injection in extreme oligoasthenoteratozoospermia after microsurgical epididymal aspiration and after testicular sperm extraction demonstrates that almost all infertile men can be helped. Only in the less common conditions such as Sertoli cell-only syndrome, spermatogenetic arrest, and genetic reasons (Klinefelter syndrome, carrier of autosomal recessive disease) would this approach be of no use. Thus, with the advent of intracytoplasmic sperm injection, the demand for donor sperm will be reduced to a certain extent, if financial resources are available.

An important advantage of testicular sperm is its repeatability. Many testicular biopsies can be taken without major inconvenience. If our data are confirmed, the use of a simple testicular biopsy could alleviate the failure of microsurgical epididymal sperm aspiration, vasoepididymostomy, or vasovasostomy. An interesting observation was that embryos originating from testicular sperm can be cryopreserved and thawed. Morphological appearance being comparable with cryopreserved and thawed embryos after traditional IVF made them suitable for replacement. For sure, more experience is needed to estimate definite fertilization, cleavage, implantation, pregnancy, and delivery rates.

In summary, the association of testicular sperm extraction and intracytoplasmic sperm injection yield high fertilization rates in males with absent epididymis. The need for donor sperm banks will be reduced if our observations are confirmed.

Key Words: Azoospermia, intracytoplasmic sperm injection, fertilization, testicular sperm extraction

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