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Influence of motility on the outcome of in vitro fertilization/intracytoplasmic sperm injection with fresh vs. frozen testicular sperm from men with obstructive azoospermia

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Objective: To assess the efficacy of fresh vs. frozen testicular sperm on fertilization and pregnancy using intracytoplasmic sperm injection.

Design: Retrospective study.

Setting: Hospital-based infertility research laboratory.

Patient(s): One hundred sixty patients with obstructive azoospermia undergoing testicular sperm extraction (TESE).

Intervention(s): Sections of seminiferous tubule were cryopreserved after TESE. Sperm motility and fertilizing ability were determined after thawing seminiferous tubule sections.

Main Outcome Measure(s): Sperm motility and optimal fertilization and pregnancy rate.

Result(s): Intracytoplasmic sperm injection was performed using fresh testicular sperm (fresh-sperm group; 84 cases) and thawed seminiferous tubules (thawed-sperm group; 177 cases). The overall fertilization rate was 65.4%, and the pregnancy rate was 34.0%. In the fresh-sperm group, the fertilization rate was 70.9%, and the pregnancy rate was 38.8%. In the thawed-sperm group, the fertilization rate was 62.7%, and the pregnancy rate was 21.7%. Fertilization rates were higher using fresh motile sperm vs. nonmotile sperm (77.0% vs. 29.3%). Pregnancy rates were higher using fresh motile sperm vs. nonmotile sperm (44.3% vs. 20.0%). The fertilization and pregnancy rates of motile vs. nonmotile sperm extracted from the thawed seminiferous tubule were 70.0% vs. 50.9% and 33.9% vs. 27.3%, respectively. Motile spermatozoa could be obtained several hours after thawing in most of the cases.

Conclusion(s): Optimal fertilization and pregnancy rates were achieved using fresh vs. frozen sperm obtained using TESE when motile sperm were identified. Motile spermatozoa provided superior results to nonmotile sperm and are necessary for optimal fertilization and pregnancy outcomes. (Fertil Steril® 2003;80:526–30. © 2003 by American Society for Reproductive Medicine.)

Key Words: TESE, testicular sperm, seminiferous tubule, cryopreservation, ICSI

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Testicular sperm extraction (TESE), in combination with intracytoplasmic sperm injection (ICSI), is an effective method for the treatment of obstructive and nonobstructive azoospermia (1–5). However, when pregnancy is not achieved, repeat TESE is required for the next cycle. To avoid further damage to the testis, testicular tissue may be frozen for subsequent ICSI trials (6). Some investigators reported that there were no differences in fertilization and pregnancy rates for fresh and frozen-thawed testicular sperm from men with obstructive and nonobstructive azoospermia (7–9). Recently, fertilization and pregnancies have been reported using thawed testicular spermatozoa (10, 11). Allan and Cotman (12) reported that freezing sections of seminiferous tubule–containing sperm is simple to perform, provides consistent sperm survival, and decreases the need for further TESE procedures. Also, Salzbrunn et al. (13) reported that the sperm frozen within a small section of the tubule could have satisfactory survival rates. With TESE, the testicular tissue can be divided into several

aliquots for cryopreservation and be used for multiple cycles of ICSI.

The purpose of this study was to assess the influence on fertilization and pregnancy of motility of fresh testicular sperm and sperm extracted from thawed seminiferous tubules in obstructive azoospermia.

MATERIALS AND METHODS

Patients

Men were evaluated with a physical examination, semen analyses, hormonal profile, and testicular biopsy. There were no specific abnormalities in physical examination and hormonal profile except azoospermia. Fresh testicular sperm was extracted at the time of TESE after failure of the surgery. Testicular sperm extraction for cryopreservation was done at the time of reconstruction (vasoepididymostomy) for the failure of the surgery or fresh TESE. All patients were candidates for the treatment of obstructive azoospermia by TESE. The mean age (mean ± SD) of the male partner was 37.5 ± 5.7 years, and that of the female partner was 34.0 ± 5.5 years. Cryopreservation of seminiferous tubule was performed in 261 IVF cycles from 160 patients.

Institutional review board approval was not obtained for this study because the data analyzed a well-established clinical therapeutic procedure that is not experimental and is not under institutional review board guidance.

Testicular Sperm Preparation

After a small incision was made in the scrotal skin and tunica albuginea, a small piece (1 cm^3) of extruded testicular tissue was excised and placed directly in a Petri dish with 2 mL of Ham's F-10 medium supplemented with 0.4% (wt/vol) human serum albumin. The seminiferous tubule was rinsed two to three times in medium, and one piece of testicular tissue was gently dissected; the seminiferous tubule was squeezed with fine forceps under the dissecting microscope. Sperm were observed under the microscope at 200–400× magnification and placed in a 15-mL conical tube and centrifuged for 5 minutes at 1,500 rpm. The supernatant was discarded, and 0.2–0.3 mL of Ham's F-10 medium supplemented with 0.4% human serum albumin was added. The mixture was left in the incubator at 37°C, 5% CO_2 in air until the ICSI procedure (about 3–5 hours). The remaining seminiferous tubules were divided into multiple aliquots and cryopreserved immediately after confirmation of sperm presence.

Cryopreservation of Seminiferous Tubule

The sections of seminiferous tubule were diluted 1:1 with human semen preservation medium supplemented with 0.4% human serum albumin and drawn into 2-mL cryogenic vials (Corning Costar Co., Cambridge, MA) immediately after confirmation of sperm presence. Vials containing sections of seminiferous tubule were frozen with a computerized freezer (CryoMagic, version 1; Mirea Biotech., Seoul, Korea). The freezing program allowed initial cooling to 4°C at a rate of −0.5°C/min, followed by rapid cooling to −90°C at a rate of −10°C/min. At −90°C, the vials were placed in liquid nitrogen for storage.

Thawing and Preparation of Testicular Sperm

The testicular tissues were thawed by removing the cryogenic vials from liquid nitrogen and leaving them at room temperature for 10 minutes, followed by a 37°C water bath for 10 minutes. The tubules were washed free of preservation medium in fresh Ham's F-10 medium. The sperm extraction from thawed seminiferous tubule was tried before about 3–5 hours for ICSI, and the procedure was described as above.

Ovarian Stimulation and Oocyte Retrieval

Ovarian stimulation was performed using gonadotropin releasing hormone agonist, human menopausal gonadotropin, and human FSH. Human chorionic gonadotropin was administered when optimal follicle development was achieved, as evaluated by serial transvaginal ultrasound and estrogen determinations. Oocyte retrieval was performed via a transvaginal approach with sonographic guidance 34 hours after human chorionic gonadotropin injection. After oocyte retrieval, maturity of the oocytes was evaluated under the inverted microscope at ×400.

The oocytes were incubated in human tubal fluid (Irvine Scientific, Irvine, CA) medium supplemented with 10% synthetic serum supplement (SSS; Irvine Scientific) at 37°C, 5% CO_2 in air. Three to 5 hours after oocyte retrieval, cumulus cell mass and corona radiata of the oocytes were removed by incubation for 1 minute in medium with 0.1% hyaluronidase (Sigma, St. Louis, MO). The denuded oocytes were rinsed several times in fresh medium and observed again for oocyte maturity under the microscope at ×200 magnification.

Intracytoplasmic Sperm Injection Procedure

The ICSI procedure was carried out on the inverted microscope at ×200 magnification using a Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, NY). For ICSI, the suspensions of testicular cells were loaded into HEPES-buffered T6 medium supplemented with 10% SSS (synthetic serum substitute), and motile spermatozoa were selected. These injectable spermatozoa were transferred to droplet of 10% PVP (polyvinylpyrrolidone) for immobilization. A single spermatozoon was aspirated tail first into the injection pipette. The metaphase II oocyte was fixed in position with the holding pipette. The polar body was held at 12 o'clock position with the holding pipette, and the sperm-containing injection pipette was inserted through the zona pellucida. A single spermatozoon was injected with the smallest possible amount of medium. The injection pipette was withdrawn gently, and the injected oocyte was released from the holding pipette. After injection, the oocytes were washed and transferred to 20-μL microdrops of

TABLE 1

Outcome of ICSI using fresh testicular sperm and sperm extracted from thawed seminiferous tubule.

Variable	Total	Fresh	Thawed
No. of cycles	261	84	177
No. of retrieved oocytes	3,395	1,149	2,246
No. of injected oocytes	2,730 (80.4)	908 (79)	1,822 (81.1)
No. of fertilized oocytes	1,786 (65.4)	644 (70.9)	1,142 (62.7)
No. of transferred embryos	871 (48.8)	293 (45.5)	578 (50.6)
No. of transferred cycles	244 (93.4)	80 (95.2)	164 (92.7)
No. of pregnancies	83 (34.0)	31 (38.8)	52 (31.7)
No. of ongoing pregnancies	73 (29.9)	27 (33.8)	46 (28.0)

Note: Values in parentheses are percentages. Fresh = fresh testicular sperm; Thawed = sperm extracted from thawed seminiferous tubule.

Park. Efficacy of sperm from thawed seminiferous tubule. Fertil Steril 2003.

human tubal fluid medium and incubated at 37°C, 5% CO_2 in air.

Assessment of Fertilization

Sixteen to 18 hours after ICSI, oocytes were observed for the presence of pronuclei and polar bodies under the inverted microscope (×200–400). The presence of two polar bodies together with two clearly visible pronuclei was considered as normal fertilization. The cleavage and quality of embryos were observed at 40–44 hours after ICSI.

Embryo Transfer and Establishment of Pregnancy

The embryos were transferred into the uterine cavity on the 3rd day after oocyte retrieval. Pregnancy was determined if serial serum β-human chorionic gonadotropin levels were 10 mIU/mL at 12 days after the oocyte retrieval. Ongoing pregnancy was defined as the presence of a fetal heartbeat using ultrasonography at approximately 6–7 weeks of pregnancy.

Statistical Analysis

All statistical tests were carried out using the χ^2 test. Differences were considered statistically significant at $P<.05$.

RESULTS

Two hundred sixty-one TESE cases were performed in 160 men with obstructive azoospermia. A total of 3,395 oocytes were retrieved, and ICSI was performed in 2,730 oocytes (80.4%). The overall fertilization rate was 65.4% (1,786/2,730), and 3.6 ± 1.2 embryos (mean ± SD) were transferred. The pregnancy and ongoing pregnancy rates were 34.0% (83 cycles) and 29.9% (73 cycles), respectively. In the fresh-sperm group (n = 84), 1,149 oocytes were retrieved, and ICSI was performed in 908 oocytes (79%). Fertilization rates were 70.9%, and pregnancy rates were 38.8%. In the thawed-sperm group (n = 177), 2,246 oocytes were retrieved, and ICSI was performed in 1,822 oocytes (81.1%). Fertilization rates were 62.7%, and pregnancy rates were 31.7%. There was no statistically significant difference between the two groups in fertilization and pregnancy rates (Table 1).

Table 2 shows comparative results of ICSI with fresh motile or nonmotile sperm (fresh-sperm group) and of ICSI with motile or nonmotile sperm extracted from thawed seminiferous tubules (thawed-sperm group). In the fresh-sperm group (n = 84), the motile sperm for ICSI were present 3–5 hours after retrieval in 73 cycles (86.9%), and nonmotile sperm were present 3–5 hours after retrieval in 11 cycles (13.1%). The optimal fertilization rate is 77.0% in ICSI with motile sperm, and 29.3% in ICSI with nonmotile sperm ($P<.05$). Pregnancy rates of motile and nonmotile sperm group were 44.3% and 20.0%, respectively ($P<.05$). In the thawed-sperm group (n = 177), nonmotile sperm were present immediately after thawing in almost all cycles. However, the number of motile sperm increased 3–5 hours after thawing. Motile sperm for ICSI were present in 114 cycles (64.4%). Thus, motile sperm could be obtained several hours after thawing in most of the cases. The fertilization rates of motile and nonmotile sperm extracted from the thawed seminiferous tubule were 70.0% and 50.9% ($P<.05$), whereas the pregnancy rates with motile and nonmotile sperm extracted from the thawed seminiferous tubule were 33.9% and 27.3% ($P<.001$), respectively.

Table 3 shows comparative results of ICSI with motile fresh or motile sperm extracted from thawed seminiferous tubule. The fertilization rate was not different between the fresh motile sperm group (77.0%) and the thawed motile sperm group (70.0%). The numbers of pregnancies and ongoing pregnancies were 31 cycles (44.3%) and 27 cycles (38.6%), respectively, in the fresh-sperm group and 37 cycles (33.9%) and 33 cycles (30.3%) in the thawed-sperm group, respectively. No differences in fertilization and pregnancy rates were observed using fresh or thawed sperm. The presence of sperm motility provided for optimal fertilization and pregnancy results.

TABLE 2

Outcome of ICSI using fresh motile or nonmotile testicular sperm and motile or nonmotile sperm extracted from thawed seminiferous tubule.

Variable	Fresh sperm (n = 84)		Thawed sperm (n = 177)	
	Motility (+)	Motility (−)	Motility (+)	Motility (−)
No. of cycles	73	11	114	63
No. of retrieved oocytes	958	191	1,370	876
No. of injected oocytes	792 (82.7)	116 (60.7)	1,128 (82.3)	694 (79.2)
No. of fertilized oocytes	610 (77.0)[a]	34 (29.3)[a]	789 (70.0)[a]	353 (50.9)[a]
No. of transferred embryos	264 (43.3)	29 (85.3)	395 (50.1)	183 (51.8)
No. of transferred cycles	70 (95.9)	10 (90.9)	109 (95.6)	55 (87.3)
No. of pregnancies	31 (44.3)[a]	2 (20.0)[a]	37 (33.9)[b]	15 (27.3)[b]
No. of ongoing pregnancies	27 (38.6)	2 (20.0)	33 (30.3)	13 (23.6)

Note: Values in parentheses are percentages. Motility (+) = ICSI with motile spermatozoa; Motility (−) = ICSI with nonmotile spermatozoa.

[a] $P<.05$.

[b] $P<.001$.

Park. Efficacy of sperm from thawed seminiferous tubule. Fertil Steril 2003.

DISCUSSION

Most men with obstructive azoospermia can be treated successfully with TESE and ICSI. However, if pregnancy is not achieved in the first TESE-ICSI cycle, repeat TESE may be necessary. Recently, interest in cryopreservation of testicular sperm has increased, but use of cryopreserved testicular sperm is not easy because of their low numbers and motility (14, 15). In our study, optimal quality of sperm could be obtained for ICSI and cryopreservation for next IVF cycle by open TESE.

Several reports of fertilization and pregnancy rates after ICSI with sperm from frozen-thawed seminiferous tubules have been published. Gianaroli et al. (11) reported that sperm recovered from the cryopreserved testicular tissue showed fertilization, embryo development, and pregnancy rates of 64%, 84%, and 33%, respectively. Perraguin-Jayot et al. (16) reported better sperm survival and motility after thawing when whole testicular tissue was frozen than when sperm were extracted before freezing. Our preliminary report (17) compared the results between frozen-thawed testicular sperm and sperm extracted from the thawed seminiferous tubule section. Although there was no statistically significant difference between the groups, the usable sperm for ICSI were high in frozen-thawed seminiferous tubule sections. Therefore, we decided to cryopreserve seminiferous tubules rather than extracted testicular sperm.

TABLE 3

Comparative result of fresh motile testicular sperm and motile sperm extracted from thawed seminiferous tubule.

Variable	Fresh	Thawed
No. of cycles	73	114
No. of retrieved oocytes	958	1,370
No. of injected oocytes	792 (82.7)	1,128 (82.3)
No. of fertilized oocytes	610 (77.0)	789 (70.0)
No. of transferred embryos	264 (43.3)	395 (50.1)
No. of transferred cycles	70 (95.9)	109 (95.6)
No. of pregnancies	31 (44.3)[a]	37 (33.9)[a]
No. of ongoing pregnancies	27 (38.6)	33 (30.3)

Note: Values in parentheses are percentages. Fresh = fresh testicular sperm; Thawed = sperm extracted from thawed seminiferous tubule.

[a] $P<.001$.

Park. Efficacy of sperm from thawed seminiferous tubule. Fertil Steril 2003.

In our study, because testicular sperm are of poor quality, seminiferous tubules were cryopreserved using a computer-programmed freezing method. Verheyen et al. (18) also reported that the computer-programmed freezing method is better than the vapor-freezing method in cases of extremely poor sperm parameters. Because several studies reported pregnancies were successfully established after using glycerol as a cryoprotectant for testicular sperm (19, 20), we used glycerol as a cryoprotectant. Using this methodology, we demonstrate that ICSI using sperm extracted from frozen-thawed seminiferous tubule has favorable results.

Initially after thawing, we could not find motile sperm in most cycles. However, about 3–5 hours after thawing and sperm retrieval, we could get motile sperm. Viability testing of testicular sperm after thawing was not performed, because we regarded spermatozoa with whipping or sluggish pattern and feeble motility as viable spermatozoa, and any kind of movement can impair the viability test. The question is whether to use TESE for recovery of fresh motile sperm or to use nonmotile sperm recovered from the cryopreserved

testicular tissue. By characterizing sperm motility after thawing, we have shown that the quality of testicular sperm motility is adequate for ICSI for most men. In cases of absence of motile sperm after thawing, spermatozoa were selected for ICSI based on normal morphology. These results suggest that successful pregnancy in TESE-ICSI treatment is influenced by the motility of fresh testicular sperm and sperm extracted from thawed seminiferous tubule in obstructive azoospermic patients and that presence of sperm motility is necessary for an optimal fertilization and pregnancy result.

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