Comparative Results of Embryo Development and Clinical Pregnancy using Sperm Retrieved from Fresh and Frozen-thawed Testicular Tissue from Patients with Obstructive and Non-obstructive Azoospermia

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폐쇄성과 비폐쇄성 무정자증 환자에서 신선고환조직 정자와 동결고환조직 정자를 이용한 배발달률과 임신율의 비교 결과

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목 적: 폐쇄성과 비폐쇄성 무정자증 환자에서 신선고환조직 정자와 융해고환조직 정자를 이용한 배발달과 임신 결과를 비교 분석하였다.

연구방법: 폐쇄성과 비폐쇄성 무정자증 환자에서 총 222 주기의 TESE-ICSI를 시행하였다. 정자는 신선 또는 융해고환조직으로부터 회수하였다. 수정은 ICSI 후 16~18시간째 확인하였으며, 배발달률과 임신율을 분석하였다.

결 과: 폐쇄성 무정자증군과 비폐쇄성 무정자증군에서 수정률은 유의하게 차이가 났으나 (75.2% 대 56.7%, $p<0.05$), 배발달률에서는 차이가 나지 않았다 (96.9% 대 98.0%). 마찬가지로 임상적 임신율 (33.9% 대 36.0%)과 분만율 (28.1% 대 28.0%)에서도 차이가 나지 않았다. 신선고환조직 정자의 경우, 수정률은 폐쇄성 무정자증군과 비폐쇄성 무정자증군에서 유의하게 차이가 났으나 (76.4% 대 52.9%, $p<0.05$), 배발달률, 임상적 임신율, 분만율에서는 차이가 나지 않았다. 융해고환조직 정자의 경우에서도 수정률은 폐쇄성 무정자증군과 비폐쇄성 무정자증군에서 유의하게 차이가 났으나 (74.7% 대 65.6%, $p<0.05$), 배발달률, 임상적 임신율, 분만율에서는 차이가 나지 않았다.

결 론: 폐쇄성과 비폐쇄성 무정자증 환자에서 신선고환조직 정자와 융해고환조직 정자를 이용하였을 경우 수정률에서는 차이가 있으나, 배발달과 임상적 임신 결과에는 차이를 보이지 않았다. 그러므로, 폐쇄성 또는 비폐쇄성 여부와는 관계없이 무정자증 환자에서 신선고환조직 정자 그리고 융해고환조직 정자를 이용하여 ICSI를 시행하면 적절한 임신 성적을 거둘 수 있다. **[Korean. J. Reprod. Med. 2009; 36(4): 301-310.]**

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Testicular sperm extraction (TESE), in combination with intracytoplasmic sperm injection (ICSI), has proved an effective treatment in men with obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). TESE-ICSI has become a common procedure used in assisted reproduction programs for the treatment of azoospermia[1].

Successful sperm finding in TESE may occur in up to 50% of attempts in patients with OA.[2,3] In NOA cases, the successful recovery of mature sperm, establishment of pregnancy, and subsequent delivery have been reported.[4] However, when pregnancy is not achieved, a repeat TESE is required for the next cycle, requiring testicular biopsy. Such biopsies are invasive procedures that may be associated with significant complications.[5] In order to avoid repeat TESE, testicular tissue cryopreservation is necessary for subsequent ICSI trials.[6]

Previous reports showed there were no differences in fertilization and pregnancy rates for fresh and thawed testicular sperm from men with OA and NOA.[7,8] Also, fertilization and pregnancies have been reported using thawed testicular sperm and testicular tissue.[9,10] The process of freezing several sections of testicular tissue containing sperm is simple to perform, provides satisfactory survival rates, and avoids further TESE procedures.[11,12] The purpose of this study was to assess the results of fertilization, embryo development (cleavage embryo), clinical pregnancy and delivery using sperm retrieved from fresh and frozen-thawed testicular tissue from OA and NOA patients.

MATERIALS AND METHODS

1. Patients

A total of 222 cycles of TESE-ICSI with the patient's own sperm was performed in 158 azoospermic patients of which 124 had OA (184 cycles) and 38 had NOA (38 cycles). The mean age (mean ± SD) of the male partners was 36.9±5.7 years and was 33.5±4.8 years for the female partners. The obstructive azoospermic patients had congenital bilateral absence of the vas deferens, obstruction of the vas deferens, or a failed vasovasostomy. The non-obstructive azoospermic patients were diagnosed based upon a histological report and classified as hypospermatogenesis (HS), maturation arrest (MA), and Sertoli cell only syndrome (SCO).

Institutional Review Board (IRB) approval was not obtained for this study because the data analyzes a well-established clinical therapeutic procedure that is not experimental and is not under IRB guidance.

2. Testicular sperm preparation, cryopreservation, and thawing

The methods for testicular sperm preparation, cryopreservation, and thawing of testicular tissue were described previously.[12] A small piece (0.3~0.5 cm^3) of extruded testicular tissue was excised. The tubules were gently dissected using two fine forceps. With the fine forceps, mingled tissue masses were dissolved and all of the connective tissues, blood vessels, and tissue remnants were removed carefully. Tubules were squeezed with fine forceps and sperms were extracted. For cryopreservation, informed consent was obtained from each patient and the remaining tubules that contained sperm were cryopreserved with a computerized freezer (CryoMagic-I, MIRAE BIOTECH., Seoul, Korea). The thawing procedure was performed about 3~5 hours before the ICSI and sperm preparation was described as outlined above.

3. Ovarian stimulation, oocyte retrieval, and ICSI procedure

Ovarian stimulation was carried out by administering GnRH agonist or hMG antagonist, and human recombinant FSH. Human chorionic gonadotropin (hCG) was administered when optimal follicle development was achieved, as evaluated by serial transvaginal ultrasound

(TVS) and estrogen determination. Oocyte retrieval was performed 34 hrs after the hCG injection. The oocytes were incubated in G-I.III (Vitrolife, Sweden) medium supplemented with 10% HSA solution (Vitrolife, Sweden) at 37℃, 6% CO_2 in air. Three to five hours after oocyte retrieval, cumulus cell masses were removed by incubation for 1 min in G-Fert medium with 0.05% hyaluronidase (Sigma, St. Louis, MO).

For ICSI, the suspensions of testicular cells were loaded to 10 μl drops of Gamete medium (Vitrolife) and sperm motility was evaluated. When motile sperm were not present, sperm activation was induced by the addition of a 5 mM pentoxifylline solution in the sperm-containing drop. The progressive motile sperm were transferred to a droplet of SpermCatch (Nidacon International AB, Sweden) medium or 7% PVP (polyvinylpyrrolidone) in HEPES-HTF for immobilization. A single spermatozoon was injected with the smallest possible amount of medium. After injection, the oocytes were washed and transferred to 20 μl microdrops of G-I.III medium and incubated at 37℃, 6% CO_2 in air.

4. Assessment of fertilization, embryo grading, embryo transfer, and establishment of pregnancy

Normal fertilization was defined as the presence of two clearly visible pronuclei (PN) at sixteen to eighteen hours after ICSI (13, 14). Fertilized embryos were transferred to G-I.III medium. Embryos were scored according to the number of blastomeres and the percentage of enucleate fragments. Embryo grading was classified into five groups as follows. Grade I, even blastomeres, no fragmentation; grade I-1, even blastomeres, fragmentation < 25%; grade II, uneven blastomeres, fragmentation < 25%; grade II-1, uneven blastomeres, fragmentation 25~50%; grade III, even or uneven blastomeres, fragmentation ≥ 50%. The arrested and degenerated embryos were excluded in total cleaved embryos. The good quality embryos were considered embryos that had the grades I, I-1, or II. The embryos were transferred into the uterine cavity on day 3~5 after oocyte retrieval. Pregnancy was determined when serum β-hCG level was ≥ 5 mIU/ml on day 12 after the oocyte retrieval. Clinical pregnancy was defined as the presence of a G-sac using ultrasonography at approximately 6~7 weeks of pregnancy.

5. Statistical analysis

Statistical tests were carried out using a Student's t-test and chi square test. Differences were considered significant at $p<0.05$.

RESULTS

In total, 3,313 stimulated oocytes were retrieved in 222 cycles and 79.9% of the matured oocytes were used to perform ICSI with testicular sperm. One thousand nine hundred two oocytes were fertilized (71.9%) and the embryo development (cleavage embryos) rate was 97.0%. In OA cases (n=184), the percentage of fertilization and embryo development was 75.2% and 96.9%, respectively. In NOA cases (n=38), the percentage of fertilization and embryo development was 56.7% and 98.0%, respectively. The difference in fertilization rate was statistically significant between the OA and NOA groups ($p<0.05$), however, the embryo development rate was not significantly different between the two groups. The total mean number of transferred embryos was 3.1 ±1.0, which also did not differ significantly between the two groups (3.2±0.9 vs. 3.0±1.2). The percentage of clinical pregnancies and deliveries were 34.2% and 28.1%, respectively. Furthermore, OA and NOA groups had no differences in their clinical pregnancy and delivery rates (33.9% vs. 36.0% and 28.1% vs. 28.0%), respectively. Therefore, patients with azoospermia can contribute to producing an acceptable embryo development, clinical pregnancy, and delivery (Table 1).

Table 1. Comparative results of fertilization, embryonic development and clinical pregnancy using testicular sperm in obstructive and non-obstructive azoospermia

	Total (n=222)	Obstructive (n=184)	Non-obstructive (n=38)
Mean age of males	36.9±5.7	37.2±5.9	35.8±4.5
Mean age of females	33.5±4.8	33.8±5.0	32.3±3.6
No. of retrieved oocytes	3313	2624	689
No. of injected oocytes	2647 (79.9)	2155 (82.1)	496 (72.0)
No. of fertilized oocytes	1902 (71.9)	1621 (75.2)*	281 (56.7)*
No. of cleavage embryos	1197/1234 (97.0)	1052/1086 (96.9)	145/148 (98.0)
No. of good embryos	731/1197 (61.1)	649/1052 (61.7)	82/145 (56.6)
Mean No. of transferred embryos	3.1±1.0	3.2±0.9	3.0±1.2
No. of E.T. cycles	196 (88.3)	171 (92.9)	25 (65.8)
No. of pregnancies	86 (43.9)	75 (43.9)	11 (44.0)
No. of clinical pregnancies	67 (34.2)	58 (33.9)	9 (36.0)
No. of deliveries	55 (28.1)	48 (28.1)	7 (28.0)

Values in parentheses are percentages
*$p<0.05$

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Next, we analyzed whether embryo quality and clinical pregnancy outcome was different between OA and NOA groups using sperm retrieved from fresh testicular tissue (Table 2). The fertilization and cleavage embryo rate was 76.4% vs. 52.9% and 94.3% vs. 98.9% between the OA and NOA groups, respectively; also, clinical pregnancy and delivery rates were 37.3% vs. 35.3% and 29.4% vs. 29.4% between the OA and NOA groups, respectively. In this result, we detected a statistically significant difference in the fertilization rate between OA and NOA groups ($p<0.05$); however, embryo development, clinical pregnancy, and delivery rates were not significantly different between the two groups. Therefore, an acceptable embryo quality and clinical pregnancy outcome can be produced from OA and NOA groups using sperm retrieved from fresh testicular tissue. Table 3 shows results of embryo quality and clinical pregnancy between OA and NOA groups using sperm retrieved from frozen-thawed testicular tissue. The fertilization and cleavage embryo rates were 74.7% vs. 65.6% and 98.0% vs. 96.3% between the OA and NOA groups, respectively, while clinical pregnancy and delivery rates were 32.5% vs. 37.5% and 27.5% vs. 25.0% between the OA and NOA groups, respectively. As in Table 2, we found statistically significant differences in the fertilization rate between OA and NOA groups ($p<0.05$), however, embryo development, clinical pregnancy, and delivery rates were not different between the two groups. Therefore, sperm retrieved from frozen-thawed testicular tissue can produce an acceptable embryo quality and clinical pregnancy outcome in both of the OA and NOA groups.

Table 2. Comparison of embryo quality and clinical pregnancy outcomes from obstructive and non-obstructive azoospermic patients with sperm retrieved from fresh testicular tissue

	Obstructive (n=55)	Non-obstructive (n=27)
No. of retrieved oocytes	812	474
No. of fertilized oocytes	504/660 (76.4)*	174/329 (52.9)*
No. of cleavage embryos	315/334 (94.3)	93/94 (98.9)
No. of good embryos	205/315 (65.1)	52/93 (55.9)
Mean No. of transferred embryos	3.1±1.0	2.9±1.2
No. of E.T. cycles	51 (92.7)	17 (63.0)
No. of pregnancies	24 (47.1)	6 (35.3)
No. of clinical pregnancies	19 (37.3)	6 (35.3)
No. of deliveries	15 (29.4)	5 (29.4)

Values in parentheses are percentages
*$p<0.05$

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Table 3. Comparison of embryo quality and clinical pregnancy outcomes from obstructive and non-obstructive azoospermic patients with sperm retrieved from frozen-thawed testicular tissue

	Obstructive (n=129)	Non-obstructive (n=11)
No. of retrieved oocytes	1812	215
No. of fertilized oocytes	1117/1495 (74.7)*	107/163 (65.6)*
No. of cleavage embryos	737/752 (98.0)	52/54 (96.3)
No. of good embryos	444/737 (60.2)	30/52 (57.7)
Mean No. of transferred embryos	3.2±0.9	3.0±1.3
No. of E.T. cycles	120 (93.0)	8 (72.7)
No. of pregnancies	51 (42.5)	5 (62.5)
No. of clinical pregnancies	39 (32.5)	3 (37.5)
No. of deliveries	33 (27.5)	2 (25.0)

Values in parentheses are percentages
*$p<0.05$

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DISCUSSION

A number of studies of TESE from azoospermia patients have reported acceptable fertilization and pregnancy outcomes. Using TESE reports have found that the sperm recovery rate is about 90% in OA patients and 40 to 60% in NOA patients,[2,15,16] fertilization rate 57% to 68%, and pregnancy rate 29% to 49%.[17~20]

However, the majority of reports show lower fertilization or pregnancy outcomes in NOA cases[21~23] and this may be affected by the degree of spermatogenic defects.[24] In cases with NOA, testicular size, testicular biopsy, and molecular markers before assisted reproductive technique have been evaluated to determine predictive parameters for successful sperm retrieval,[25~27] however, serum FSH concentration is not a highly predictive factor.[28] These different reports may be due to differences in the assisted reproductive techniques used in each center, and the protocols for ovarian stimulation of the female partner, testicular biopsy, and the method of testicular sperm preparation may also influence the result.[29]

As described in the materials and methods, we used a fine forcep method for testicular sperm retrieval. The seminiferous tubules were excised and one piece of tubule was gently dissected using two fine forceps. With the fine forceps, mingled tissue masses were dissolved and all of connective tissues, blood vessels, and tissue remnants were carefully removed. With this procedure we could produce intact and clear tubules. Dissolved tubules were moved to fresh medium, the tubule was squeezed with fine forceps, and spermatogenic cells were extracted. In contrast to the slide mincing method, the benefit of fine forcep method is that damage can be preventing germ cells mixed with blood, tissues particles, and various remnants as well as exposure to the harmful environment of spermatogenic cells during handling of the seminiferous tubule. The fine forcep method does not need more time for total preparation compared with the slide mincing method, furthermore, using this method we could gain more intact tubules and avoid enzyme treatment. From the intact tubule, we did not need to perform another additional preparation method to obtain pure sperm retrieval and thus could reduce the loss of sperms during preparation.

In OA and NOA patients, testicular sperm and/or tissue cryopreservation was performed at the time of TESE or diagnostic testicular biopsy. In OA patients, testicular tissues can be divided into multiple aliquots for cryopreservation, and each aliquot can be used for the next IVF cycle.[12,30,31] According to the report by Dafopoulos *et al*.,[32] sperms were not extracted before freezing, and the tissue that was frozen was the size of a rice grain. This method can reduce the freezing shock to spermatozoa and advocates the freezing of testicular tissue. In addition, the freezing protocol had produced a good post-thaw sperm recovery with only about a 25% reduction in motility.[33] The fertilization and pregnancy rates are lower with frozen sperm compared with fresh sperm[23,34,35] and cryopreservation of testicular sperm is controversial because of the lower number and motility. In our previous report,[12] we noted that sperm motility was an important factor for normal fertilization and pregnancy rates in ICSI with testicular sperm, and this finding is consistent with the results of other reports.[3032,36] Verheyen *et al*.,[8] reported that both motility and vitality are equally affected by freezing and thawing, and that the ratio of motile-to-vital sperm cells is maintained after thawing and preparation of testicular sperm. However, Kone *et al*.,[37] reported that a difference was observed in the fertilization rate of motile vs. non-motile sperm (72% vs. 62%), but no difference was obtained in embryo development and implantation rates. Therefore, these authors concluded that motility is not an essential prerequisite for fertilization and embryo development. Also, no significant differences were found in the fertilization rate when fresh and frozen-thawed TESE was used with or without motile sperm.[38]

It is possible to achieve satisfactory fertilization and pregnancy rates from patients with OA or NOA:[39,40] fertilization rates with fresh vs. frozen testicular sperm were 69% vs. 61.7%, respectively, pregnancy rates per embryo transfer were 29.1% vs. 25.0%, respectively. One previous report found slightly lower fertilization rates with frozen-thawed sperm as compared with fresh

sperm (70.9% vs. 62.7%) as well as lower pregnancy outcomes (38.8% vs. 21.7%), however, our study did not find a statistically significant difference.[12] No difference between fresh and frozen-thawed TESE sperm was found with regards to fertilization, embryo cleavage, pregnancy, delivery, and spontaneous abortion rates in OA and NOA patients,[41] however, a difference was observed in embryo development (83% vs. 75%, respectively). On the other hand, Palermo *et al*.,[17] reported a lower fertilization rate for NOA patients (57%) compared to OA patients (80%); however, the clinical pregnancy rate was similar for both patient groups. These results are accordance with our results that found a statistically significant difference in the fertilization rate between OA (75.2%) and NOA (56.7%) groups, however, the embryo development, clinical pregnancy, and delivery rates were not different between the two groups. In humans, although the mechanism is not well known, paternal factors (e.g., source of sperm) have been shown to affect the PN stage embryos, embryo development, morphology, the rate of chromosomally abnormal embryos, blastocyst formation, and implantation.[42] However, no correlation was found between the period of ovarian stimulation, oocyte morphology, PN morphology, the total dose of gonadotrophin ampoules that were used, body mass index (BMI), and peak estradiol levels.[43]

In conclusion, our study showed that although fertilization rates were different result, embryo quality, clinical pregnancy, and delivery rates were not different between patients with OA and NOA, nor when the sperm used for the fertilization were retrieved from fresh vs. frozen-thawed testicular tissue. Therefore, ICSI with sperm that were retrieved from fresh and frozen-thawed testicular tissue could achieve a relevant clinical result in patients with azoospermia.

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= **Abstract** =

Objective: To compare the embryonic development and pregnancy results using sperms retrieved from fresh and frozen-thawed testicular tissue in patients with obstructive (OA) and non-obstructive azoospermia (NOA).

Methods: A total two hundred twenty-two cycles of TESE-ICSI were performed in OA and NOA. Sperms were retrieved from fresh and frozen-thawed testicular tissue. ICSI was performed patient's own sperm. Fertilization was assessed 16~18 hrs after ICSI. Embryo development and pregnancy rates were analysed.

Results: The fertilization rates were significantly different between OA and NOA patients (75.2% vs. 56.7%, $p<0.05$), however, embryo development did not differ between the groups (96.9% vs. 98.0%). Likewise, OA and NOA groups had no differences in their clinical pregnancy and delivery rates, 33.9% vs. 36.0% and 28.1% vs. 28.0%, respectively. With regard to sperm retrieved from fresh testicular tissue, fertilization rates were significantly different between the OA and NOA groups (76.4% vs. 52.9%, $p<0.05$); however, embryo development, clinical pregnancy and delivery rates were not different. For sperm retrieved from thawed testicular tissue, the fertilization rates were significantly different between the two groups (74.7% OA group vs. 65.6% NOA group, $p<0.05$); however, embryo development, clinical pregnancy and delivery rates were not different.

Conclusions: Embryo development and clinical pregnancy did not differ in patients with obstructive and non-obstructive azoospermia, whether sperm retrieved from fresh and thawed testicular tissue were used, although the fertilization rates were different. Therefore, ICSI with sperm retrieved from fresh and thawed testicular tissue could achieve relevant clinical pregnancy results in patients with azoospermia.

Key Words: Obstructive azoospermia, Non-obstructive azoospermia, Cryopreservation, Testicular sperm, TESE-ICSI, Embryo development