Effect of *in vitro* testicular spermatozoa culture on pregnancy outcomes: an experience at a single university hospital

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Background: There are no guidelines for the optimal incubation time or temperature to improve pregnancy outcomes in testicular sperm extraction-intracytoplasmic sperm injection (TESE-ICSI) cycles. We aimed to evaluate whether a 24-hour *in vitro* culture of testicular spermatozoa affects pregnancy outcomes in TESE-ICSI cycles.

Methods: This was a retrospective study of 83 TESE-ICSI cycles using testicular spermatozoa in 46 couples with male partners suffering from nonobstructive or obstructive azoospermia. Sperm retrieval was performed either on the oocyte retrieval (OR) day (65 cycles in 33 couples; group A) or on the day before OR (18 cycles in 13 couples; group B) followed by *in vitro* culture for 24 hours. The clinical characteristics and pregnancy outcomes, including the number of retrieved oocytes, fertilization rates, embryo transfer rates, implantation and clinical pregnancy rates, were compared between the two groups.

Results: There were no differences in terms of clinical characteristics except for the levels of luteinizing hormone (LH) in males. Group B had higher LH levels than group A (4.56 ± 1.24 IU/L vs. 3.67 ± 1.07 IU/L, *p* = 0.017). Group B showed higher fertilization rate (72.4% ± 32.1% vs. 59.2% ± 21.7%, *p* = 0.045), implantation rate (35.0% ± 34.1% vs. 14.0% ± 21.5%, *p* = 0.010), pregnancy rate per cycle (80% vs. 39%, *p* = 0.033), and clinical pregnancy rate per cycle (80% vs. 37.5%, *p* = 0.024) than those of group A.

Conclusion: Testicular sperm retrieval performed on the day before OR followed by *in vitro* culture can potentially improve pregnancy outcomes.

Keywords: Azoospermia; Intracytoplasmic sperm injections; Testicular sperm retrieval

Introduction

Almost 20% of men with infertility suffer from azoospermia [1,2]. Assisted fertilization with testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) has been successfully ap-

plied for the treatment of obstructive and nonobstructive azoospermia. *In vivo*, spermatozoa are released from the seminiferous tubules and reach epididymal sites where they acquire progressive motility and fertilization potential [3]. However, in TESE cycles, it is difficult to find sufficient number of motile sperms because tes-

ticular spermatozoa show motility only for a short period of time after testicular biopsy and may become immotile due to depletion of metabolic resources [4].

Sperm motility refers to the movement of sperm and is often used as a clinical parameter to evaluate sperm viability in ICSI cycles. It has been reported that only 3% of testicular spermatozoa are motile after a biopsy [5]. *In vitro* culture of spermatozoa has been suggested as a way to improve sperm motility in TESE-ICSI cycles. The efficacy of cryopreserved spermatozoa was first evaluated by Oates et al. [6], who achieved a pregnancy rate of 40% per couple using cryopreserved spermatozoa. However, studies on *in vitro* maturation of sperm and its effects on clinical pregnancy outcomes show conflicting results.

Hu et al. [7] reported that sperms extracted on the day before oocyte retrieval (OR) showed increased motility, consequently resulting in a higher fertilization rate. On the contrary, Karacan et al. [8] reported that the timing of TESE does not affect the outcomes of ICSI-embryo transfer (ET) cycles. More importantly, there are no specific guidelines suggesting a suitable incubation time or temperature to improve sperm motility and pregnancy outcomes in TESE-ICSI cycles.

Hosseini and Khalili [9] studied the changes in sperm motility at different time intervals in an *in vitro* culture of spermatozoa and observed that sperm motility significantly increased after 24 hours but began to decline at day 2 of culture. Therefore, we aimed to evaluate whether a 24-hour *in vitro* culture of testicular spermatozoa affects outcomes of TESE-ICSI cycles. We compared the clinical characteristics and pregnancy outcomes between TESE-ICSI cycles with fresh testicular spermatozoa obtained on the day of OR and those with *in vitro* cultured spermatozoa retrieved on the day before OR.

Materials and methods

1. Patients

This was a retrospective study of patients with successful TESE-ICSI cycles with TESE performed either on the day of OR or 24 hours before OR between January 2001 and December 2015 at Ajou University Hospital, Suwon, Korea. Clinical characteristics and pregnancy outcomes were evaluated by reviewing the medical records. This study was approved by the Institutional Review Board (IRB) of Ajou University Hospital (IRB No: AJIRB-MED-MDB-16-038). The informed consent from the patients was waived from the IRB. A total of 83 TESE-ICSI cycles in 46 couples were included in the study. All male partners with obstructive azoospermia or nonobstructive azoospermia, including chemotherapy exposure or varicocele, underwent repeated semen analyses; the absence of sperms

on at least two separate semen analyses confirmed the diagnosis of azoospermia.

Clinical characteristics, including male age, female age, type of azoospermia, follicle-stimulating hormone (FSH) level, type of ovulation induction, etiology of infertility, total gonadotropin dose (IU), and pregnancy outcomes, were included in the analysis. Clinical pregnancy was defined as the presence of a gestational sac with fetal heartbeat confirmed by ultrasonography. The implantation rate was analyzed as the number of gestational sacs with fetal heartbeat per embryo transferred.

2. Ovarian stimulation and oocyte retrieval

The ovarian stimulation protocol was individualized according to the patients' age, diagnosis, and ovarian reserve. Different stimulation protocols included long gonadotropin-releasing hormone (GnRH) agonist and short GnRH antagonist using recombinant FSH (Follitrope, LG Life Sciences Ltd., Seoul, Korea; Menopur, Ferring Pharmaceuticals, Lausanne, Switzerland), and/or human menopausal gonadotropin (IVF-C, LG Life Sciences Ltd). Transvaginal sonography was used to monitor follicle growth every 2 days until the dominant follicle grew to approximately 16–18 mm. Ovulation was triggered by injecting 10,000 IU of human chorionic gonadotropin (hCG; Pregnyl, Merck & Co. Inc., Haarlem, the Netherlands) intramuscularly when the mean diameter of the leading follicle reached ≥18 mm or when urine luteinizing hormone (LH) was positive.

Thirty-six hours after hCG administration, OR was performed through vaginal ultrasonography-guided punctures of the ovarian follicles. Following the OR, patients received either intramuscular injection of progesterone (Sugest, 50 mg; Sanzyme Ltd., Hyderabad, India) once a day or vaginal progesterone (Utrogestan, 200 mg; Besins Healthcare Ltd., London, UK) for luteal support until 2 weeks after the procedure when the pregnancy was confirmed by analyzing serum hCG, and continued to receive the injection thereafter.

3. Testicular sperm extraction and *in vitro* culture of spermatozoa

Testicular sperm extraction was performed by a urology specialist either on the day of OR or 24 hours before the day of OR. After sterilization of the scrotal skin, 1% lidocaine was injected locally into the underlying tunics, and an incision was made to remove a 3-mm segment of testicular tissues. The initial examination for the presence of spermatozoa was performed under an inverted phase-contrast microscope (Diaphot 300/N88, Nikon, Tokyo, Japan). Individual spermatozoa were observed carefully with a high magnification under the microscope to evaluate any sign of sperm

motility, such as twitching of the head or tail. The suspension was then transferred to a test tube if spermatozoa were noticed. The biopsy samples were then independently minced using sterile needles and dissected into small pieces in a sterile Petri dish.

TESE spermatozoa were accompanied by red blood cells (RBCs), debris, testicular cells, and a large number of dead or immotile spermatozoa that have detrimental effects on existing motile spermatozoa. The biopsy tissues were then centrifuged with an RBC lysis buffer for 5 minutes at $300 \times g$ to eliminate these cells. In group A (TESE performed on the day of OR), the tissue was centrifuged twice at $300 \times g$ for 5 minutes, and the pellet was placed in the Sydney IVF fertilization medium (Cook Medical, Bloomington, IN, USA) containing 10% human serum albumin. In group B (TESE performed on the day before OR), the extracted tissues were incubated in the same IVF fertilization medium and cultured for 24 hours at 37°C in 6% CO_2 and 5% O_2 balance. Centrifugation of the biopsy samples in group B was performed on the day of the OR. The pH and temperature of the culture medium and incubator were controlled daily and maintained throughout the study.

4. Intracytoplasmic sperm injection procedure

Using sterile 23-gauge needles, the testicular spermatozoa were prepared for oocyte insemination by squeezing the biopsy tissues to identify the tubules and release spermatozoa. The tubules were placed in Quinn's Sperm Washing Medium (SAGE In Vitro Fertilization, Trumbull, CT, USA) containing gentamicin and 5.0 mg/mL human serum albumin. One drop of sperm washing medium was replaced with a 7% polyvinylpyrrolidone medium, and five drops were replaced with the sperm washing medium. Motile spermatozoa with forward progression were transferred from the sperm suspension drop to the polyvinylpyrrolidone drop using an injection needle with an inside diameter of 12 to 15 μm. The spermatozoa were then immobilized and stored in the polyvinylpyrrolidone drop. After this, assisted fertilization by ICSI was performed as previously described [10].

5. Evaluation of the outcomes of intracytoplasmic sperm injection

Fertilization was assessed 17 to 19 hours after ICSI, and embryo cleavage and quality were evaluated 2 to 5 days after OR. The criteria for embryo grading were based on the gross morphological appearance on light microscopy [11]. The zygotes were cultured in the media (cleavage medium 10% human serum albumin; Cook Medical) under oil in a 6% CO_2 and 5% O_2 incubator. The embryos were transferred into Pronase solution (Sigma-Aldrich, St. Louis, MO, USA) under oil for 60 seconds to soften the zona pellucida and then transferred to fresh cleavage medium. Depending on the

status of the embryo, ET was performed 3 to 5 days following OR. The number of embryos transferred was determined by the Korean Ministry of Health and Welfare, depending on the female partner's age and embryo development. Embryos with multiple nuclei or cytoplasmic abnormalities were discarded. Progesterone was administered either intramuscularly (Sugest, 50 mg) once a day or vaginally (Utrogestan, 200 mg) for luteal support until 2 weeks after the procedure when the pregnancy was confirmed by analyzing serum hCG and continued thereafter.

Clinical pregnancy was defined as the presence of a gestational sac with a fetal heartbeat confirmed by ultrasonography. The implantation rate was analyzed as the ratio between the number of gestational sacs and the number of transferred embryos. The delivery rate was defined as the ratio between the number of deliveries and the number of ETs.

6. Statistics

Quantitative variables are described using means and standard deviations, and qualitative variables are shown as percentages. The data were first tested for normality, and then the differences between groups were compared using an independent t-test or Mann-Whitney U test depending on the distribution. Pearson chi-square test or Fisher exact test was used to determine the association between two categorical variables. Data were analyzed using IBM SPSS version 25.0 (IBM Corp., Armonk, NY, USA). A p-value of less than 0.05 was considered statistically significant.

Results

Group A consisted of 65 cycles in 33 patients and group B consisted of 18 cycles in 13 patients. The clinical characteristics of both groups are shown in Table 1. The two groups were comparable in terms of age of male and female partners, basal FSH and anti-Müllerian hormone levels, and the number of stimulation days. The distribution of type of azoospermia, etiology of infertility, and type of ovulation induction also showed no difference between the two groups. Although statistically insignificant, the total dose of gonadotropin used for group B was slightly higher than that used for group A (2,387.5 ± 500.3 IU vs. 2,130 ± 490.4 IU, $p = 0.053$).

Table 2 describes the ICSI outcomes and pregnancy outcomes of groups A and B. The numbers of retrieved oocytes and good-quality oocytes were not significantly different between the two groups (16.8 ± 11.3 vs. 14.5 ± 7.6, $p = 0.608$; 14.7 ± 9.7 vs. 12.4 ± 6.7, $p = 0.235$). However, the fertilization rate was significantly higher in group B than in group A (72.4% ± 32.1% vs. 59.2% ± 21.7%, $p = 0.045$). Group B also showed higher implantation rate (35.0% ± 34.1% vs. 14.0% ± 21.5%, $p = 0.010$), pregnancy rate per

Table 1. Clinical characteristics in groups A and B

Variable	Group A	Group B	*p*-value
No. of cycle/couple	65/33	18/13	
Age (yr)			
Female	31.1 ± 3.4	31.1 ± 3.2	0.925[b]
Male	34.3 ± 3.8	35.6 ± 6.4	0.284
Basal FSH (IU/L)			
Female	5.3 ± 1.7	4.2 ± 2.2	0.304
Male	4.8 ± 2.9	4.3 ± 1.6	0.966
AMH in females (ng/mL)[a]	4.2 ± 2.5	4.7 ± 2.7	0.657
Basal LH in males (IU/L)	3.6 ± 1.1	4.6 ± 1.2	0.017
Type of azoospermia			0.658
Obstructive	58 (89.2)	16 (88.9)	
Nonobstructive	7 (10.8)	1 (5.6)	
Etiology of infertility			
Only male factor	42 (64.6)	11 (61.1)	1.000
Male factor+anovulation	1 (1.5)	1 (5.6)	0.389[c]
Male factor+tubal factor	4 (6.1)	0	0.572
Male factor+endometriosis	13 (20.0)	5 (27.8)	0.524
Type of ovulation induction			
FSH+GnRH antagonist	14 (21.5)	2 (11.1)	0.502
FSH+GnRH agonist	15 (23.1)	8 (44.4)	0.084
Pure FSH	8 (12.3)	3 (16.7)	0.697
FSH+hMG	28 (43.1)	5 (27.8)	0.286
Total gonadotropin dose (IU)	2,130.0 ± 490.4	2,387.5 ± 500.3	0.053
Duration of stimulation (day)	8.6 ± 4.1	9.0 ± 1.2	0.336

Values are presented as number only, mean±standard deviation, or number (%).
Group A, testicular sperm extraction (TESE) performed on the day of oocyte retrieval (OR); group B, TESE performed on the day before OR.
FSH, follicle-stimulating hormone; LH, luteinizing hormone; AMH, anti-Müllerian hormone; GnRH, gonadotropin-releasing hormone; hMG, human menopausal gonadotropin.
[a]The number of patients with available AMH levels was 9 for each group. [b]Mann-Whitney test. [c]Fisher exact test.

cycle (80% vs. 39%, *p* = 0.033), and clinical pregnancy rate per cycle (80% vs. 37.5%, *p* = 0.024) than those of group A. The incidence of miscarriage, ectopic pregnancy, and live birth and cryopreservation rates were comparable between the two groups.

Discussion

In the present study, we compared the outcomes of TESE-ICSI cycles with fresh spermatozoa obtained on the day of OR and TESE-ICSI cycles with spermatozoa obtained 24 hours before OR. The number of retrieved oocytes, fertilized eggs, and cleaved embryos were comparable between the two groups. On the other hand, TESE-ICSI cycles with spermatozoa obtained 24 hours before OR showed higher fertilization rate, implantation rate, pregnancy rate, and clinical pregnancy rate than those of TESE-ICSI cycles with fresh spermatozoa. These results indicate that *in vitro* maturation of spermatozoa for 24 hours resulted in improved pregnancy outcomes compared to fresh spermatozoa.

There are various factors known to influence pregnancy outcomes following TESE-ICSI cycles, such as paternal age, maternal age, male testosterone, sperm motility, and/or type of azoospermia [12-14]. Among these factors, the motility of spermatozoa is emphasized as an important predictive factor for successful fertilization by ICSI [15]. *In vitro* maturation of spermatozoa has been suggested as a way to improve sperm motility, thereby improving pregnancy outcomes in TESE-ICSI cycles [16-19]. In a study by Balaban et al. [20], the fertilization rate increased from 42.1% to 68.8% after *in vitro* culture of spermatozoa with improved sperm motility. Similarly, in our study, group B showed a higher fertilization rate than group A (72.4% vs. 59.2%, *p* = 0.045) due to *in vitro* maturation of spermatozoa. The exact mechanism behind improved testicular sperm motility after *in vitro* culture is not yet clear. Some studies have suggested that co-culture with all testicular cells, including Sertoli, Leydig, and germ cells, may enhance sperm maturation [4]. In addition, the washing procedure before the culture also eliminates inhibitory factors, which activates the

Table 2. Pregnancy outcomes of TESE-ET cycles in groups A and B

Variable	Group A	Group B	p-value
No. of oocytes retrieved	14.5 ± 7.6	16.8 ± 11.3	0.608[a)]
No. of good oocytes	12.4 ± 6.7	14.7 ± 9.7	0.235
No. of fertilized eggs	7.0 ± 3.7	10.7 ± 8.8	0.211[a)]
Fertilization rate (%)	59.2 ± 21.7	72.4 ± 32.1	0.045
No. of embryos cleaved	5.1 ± 2.1	8.1 ± 7.0	0.102[a)]
No. of good embryos	2.8 ± 1.5	2.1 ± 1.3	0.097
No. of embryos transferred	3.8 ± 1.5	2.5 ± 1.4	0.001
Implantation rate (%)	14.0 ± 21.5	35.0 ± 34.1	0.010[a)]
Pregnancy rate per cycle	25 (38.5)	12 (66.7)	0.033
Clinical pregnancy rate per cycle	24 (36.9)	12 (66.7)	0.024
No. of miscarriages	4 (6.2)	1 (5.6)	1.000[b)]
No. of ectopic pregnancies	1 (1.5)	0	1.000[b)]
No. of of live births	13 (20.0)	2 (11.1)	0.040
Cryopreservation rate	18 (27.7)	9 (50.0)	0.074

Values are presented as mean±standard deviation or number (%).
Group A, testicular sperm extraction (TESE) performed on the day of oocyte retrieval (OR); group B, TESE performed on the day before OR.
[a)]Mann-Whitney test. [b)]Fisher exact test.

essential factors required for enhanced motility [12]. However, a long-term culturing may decrease sperm motility because of the gradual degeneration of testicular somatic cells.

Despite collective evidence to support sperm retrieval before OR and have sperm cultured before ICSI, there are no guidelines to suggest suitable incubation times. Determination of appropriate sperm retrieval timing and incubation times before the ICSI procedure is critical. Various incubation time periods have been suggested as an optimal time to improve sperm motility and pregnancy outcomes. Wu et al. [21] observed that after a 24-hour *in vitro* culture, the number of motile sperm showed a remarkable increase and reached a maximum motility rate between 48 and 72 hours. Hu et al. [7] also investigated the feasibility of performing testicular biopsies 24 hours prior to OR and observed that after *in vitro* culture, the overall fertilization rate was 58%, the implantation rate was 20%, and the clinical pregnancy rate was 45%. In another study, Hosseini and Khalili [9] observed that sperm motility changed from 13% immediately after biopsy to 76% at 24 hours and 15% at 72 hours of culture, suggesting that 1 day of culture is an ideal *in vitro* culture time to optimize sperm motility in azoospermic TESE samples. However, the results of these studies only implied that *in vitro* culture may enhance sperm motility but failed to provide evidence for the effect on pregnancy outcomes.

Levran et al. [18] first reported the outcomes of TESE-ICSI using testicular spermatozoa obtained on the day of OR or the day before OR. They obtained similar fertilization rates (61.7% vs. 58.9%) and clinical pregnancy rates (34.8% vs. 29.2%) in their analysis of 47 IVF-ICSI cycles using testicular spermatozoa retrieved on the day of or on the day before oocyte aspiration [18].

More recently, Karacan et al. [8] compared the fertilization rates between ICSI-ET cycles with fresh testicular spermatozoa obtained on the same day or the day before OR with frozen-thawed spermatozoa. They observed no difference between the groups in terms of pregnancy outcomes with a fertilization rate of 68.7%–70%, implantation rate of 13.4%–16.5%, and clinical pregnancy rate of 30.9%–31.3%.

Contrary to the results of Karacan et al. [8], our study showed significantly higher implantation rate, pregnancy rate per cycle, and clinical pregnancy rate per cycle in ICSI cycles using *in vitro* cultured spermatozoa than in those with fresh spermatozoa (35.0% vs. 14.0%, $p = 0.010$; 80% vs. 39%, $p = 0.033$; 80% vs. 37.5%, $p = 0.024$, respectively). In the study by Karacan et al. [8], the baseline characteristics between the two groups were not different. On the contrary, group A in our study included a higher proportion of female infertility patients along with azoospermic male partners than group B. This may have influenced group B to have a higher number of retrieved oocytes and fertilized eggs, consequently leading to improved pregnancy outcomes compared to those of group A.

Another possible explanation may be the different hormone levels of male partners between the two groups. Although statistically insignificant, group A had higher basal FSH levels than group B ($4.8 ± 2.9$ IU/L vs. $4.3 ± 1.67$ IU/L, $p = 0.966$). The baseline LH levels in group B, however, were higher than those in group A ($4.56 ± 1.24$ IU/L vs. $3.67 ± 1.07$ IU/L, $p = 0.017$). There have been conflicting reports on the association between hormonal levels and the chance of sperm retrieval. Salehi et al. [22] reported that high levels of FSH are significantly associated with lower chances of sperm retrieval in azoospermic men. On the other

hand, some studies have shown that serum FSH concentration is not an independent predictive factor for sperm retrieval because FSH concentration is controlled by various endocrine and paracrine factors [23,24]. The discrepancies in male FSH and LH levels observed in our study might have resulted from the small number of the study population in group B. If the number of cycles were equivalent between the two groups, the baseline hormonal levels could have different values. Nonetheless, our data suggest that the baseline male hormone levels might have influenced the pregnancy outcomes in TESE-ICSI cycles.

Our study has several limitations. The uneven sample size of groups A and B makes it difficult to extrapolate significant statistical importance. Moreover, although we have shown that fertilization rate, implantation rate, and pregnancy rates were significantly improved with *in vitro* cultured spermatozoa, we are unable to provide data for any change in sperm motility before and after *in vitro* culture. Any changes in sperm motility were only observed by the laboratory technician, and the recorded data were not provided. Another limitation is the effect of different controlled ovarian hyperstimulation (COH) protocols on pregnancy outcomes. It is known that different COH protocols may influence pregnancy outcomes. Because we aimed to evaluate the effect of *in vitro* culture of spermatozoa on pregnancy outcomes and the number of each COH protocol for groups A and B was too small, we did not compare the pregnancy outcomes according to the COH protocol. Nevertheless, our study provides enough evidence to support the previous findings that *in vitro* culture of spermatozoa in TESE-ICSI cycles may significantly improve pregnancy outcomes. Further studies with large sample sizes are needed to make definitive statements in this regard.

In conclusion, a sperm extraction procedure can be performed on the day before egg retrieval without compromising the risk of pregnancy. Although the mechanism is yet unknown, the *in vitro* culture of sperm before OR may improve clinical outcomes, especially for patients undergoing TESE-ICSI for azoospermia.

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