A New Sperm Preparation Method for Testicular Sperm Extraction - Intracytoplasmic Sperm Injection (TESE-ICSI) Cycles: Simple, Effective and Rapid Method

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Abstract: *The aim of this study was to recover spermatozoa easily from fresh or frozen-thawed testicular sperm extraction (TESE) samples. We simply minced and washed the TESE samples in medium. Spermatozoa were recovered and washed in the bottom of a PVP droplet with an injection pipette and injected into oocytes. In all cycles 100% of spermatozoa (motile and/or immotile) could be recovered. Injected oocytes were fertilized (100% per cycle and in 41.9% of oocytes) and fertilized oocytes were cleaved (100% per cycle; 38.5% of injected oocytes; 91.8% of fertilized oocytes) after ICSI with fresh or frozen-thawed TESE spermatozoa. The rates of progression to the embryo stage (2–8 cell) and blastocyst formation and their quality were similar in both the fresh sample group and the frozen-thawed sample group. Both of embryo transfer (73.5%) and embryo-blastocyst transfer (26.5%) were performed successfully. Thirty-four cycles of ICSI with testicular spermatozoa resulted in 23.5% pregnancy rates (fresh sample group, 22.2%; frozen-thawed sample group, 24%) with both embryo transfer (8%) and embryo-blastocyst transfer (66.7%). This new sperm preparation method is very simple, easy, effective and rapid for recovering spermatozoa from TESE samples for ICSI.*

Key words: *Fresh or frozen-thawed TESE sample, Spermatozoa recovery, ICSI, Embryo and blastocyst, Pregnancy*

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The introduction of intracytoplasmic sperm injection (ICSI) [1] was originally considered for male factor infertility when spermatozoa could be found in the ejaculate. The possibility of achieving pregnancy with even one available spermatozoon has precipitated the search for a method to obtain spermatozoa from testicular tissue or epididymal sites, when seminal azoospermia is a constant finding. In patients with obstructive and nonobstructive azoospermia, fertilization and pregnancy can be accomplished by means of a combination of intracytoplasmic sperm injection with epididymal spermatozoa retrieved by microsurgical epididymal sperm aspiration or testicular sperm extraction [2–7]. Although ICSI is an effective clinical treatment strategy for male infertility patients, new types of technical difficulties have arisen, because of the extremely small number of spermatozoa handled in this procedure. The recovery of spermatozoa from extremely low quality sperm samples or TESE tissues for use in the ICSI procedure is very difficult [6–8].

The aim of this study was to recover the spermatozoa easily from fresh or frozen-thawed TESE samples in a 3% polyvinylpyrrolidone (PVP) droplet.

Materials and Methods

Female patients

Women under the age of 35 (mean: 28.7; range: 25–35 years) and having many oocytes retrieved (>10) were recruited for this study at Kyungpook National University Hospital.

Ovarian stimulation was performed with a concomitant gonadotropin-releasing hormone agonist (GnRHa)-menotropin regimen as described previously. Ovulation was induced by a 10,000 IU injection of human chorionic gonadotropin (hCG, IVF-C, LG, Korea) when two or more follicles >17 mm in diameter were present. Oocyte retrieval was performed at 34–36 hrs after hCG injection. The oocytes were obtained by ultrasonography-guided transvaginal follicular aspiration.

Male patients

The men in this study were characterized as having non-obstructive azoospermia based on the results of examination. An open testicular biopsy technique was used with local anesthesia. Retrieval was performed routinely at two sites on each testis.

Two-three days before or on the day of oocyte retrieval, scrotal exploration is performed through a median raphe incision under anesthesia, and sperm are retrieved by means of an open testicular biopsy. In order to confirm accurate identification of the testis and to avoid any injury to the epididymis, delivery of the testis is routinely performed. Testicular blood vessels in the tunica albuginea are identified with × 8–15 optical magnification. An avascular region near the midportion of the medial, lateral or anterior surface of the testis is chosen, and a generous incision in the tunica albuginea is created with a 150 ultrasharp knife, avoiding capsular vessels. With this approach, larger samples (450–500 mg) of testicular parenchyma can be harvested, instead of retrieving the usual diagnostic biopsy volume of 50–100 mg. The excised testicular biopsy specimen is placed in Ham's F-10 supplemented with 40% human follicular fluid (hFF). Isolation of individual tubules from the mass of coiled testicular tissue is achieved by initial dispersal of the testis biopsy specimen with two sterile glass slides, stretching the testicular parenchyme to isolate individual semniferous tubules. Additional dispersion of the tubules is achieved by passing the suspension of testicular tissue through a 24 gauge angiocatheter.

Preparation of the TESE samples and recovery of spermatozoa

As shown in Fig. 1, the retrieved TESE sample was disrupted mechanically by mincing the extended tubules with a surgical blade (No. 15, Ailee, Korea) in a culture dish (3002, Falcon, USA) containing 0.5 ml of Ham's F-10 with 40% hFF and diluted in 10 ml of medium. Excess tissue and viscous mass were then removed and washed by centrifugation (at 1500 rpm for 5 min) to obtain the sample pellet in a 15 ml cornical tube (2099, Falcon, USA). The sample pellet was used for ICSI procedure and/or freezing.

For freezing, the pellet was resuspended in 5 ml of freezing medium (TEST Yolk Buffer; 9971, Irvine, USA). The mixture was divided into 5 parts for subsequent use in therapeutic ICSI cycles. The vials were placed horizontally 2 cm above liquid nitrogen (LN_2) for 10–30 min and then stored in LN_2. The frozen sample was thawed before ICSI. After oocyte retrieval, the frozen sample was plunged into a 37°C water-bath until thawed for about 3 min. Thawed samples were diluted with 10 ml of Ham's F-10 supplemented with 10% hFF and centrifuged (at 1500 rpm for 5 min) to obtain the sample pellet. The washed sample pellet (fresh or frozen-thawed) was suspended in 5–10 μl hFF. Percoll was not used for washing of the TESE samples (Fig. 1).

As shown in Fig. 2, sample pellets were well homogenized by repeated pipetting with a micro pipette (10 μl, Eppendorf, Hamburg, Germany). Spermatozoa were recovered from the bottom of the 3% PVP droplet (about 10 μl) placed at the center of a culture dish (3002, Falcon, USA). The PVP droplet was surrounded by two droplets (each 20–30 μl) as a reservoir for oocytes or a wash for holding and injection pipette (DMEM with 10% hFF). All droplets were covered with mineral oil (M-8410, Sigma, USA) (Fig. 2A). About 1–2 μl of sperm suspension was carefully placed in a 3% PVP droplet (right position) by using a glass pipette (inner diameter: about 20–30 μm) on the stage of a stereo microscope at × 10 magnification. The sperm suspension was expanded spontaneously in the PVP droplet (Fig. 2B). We discarded the excess cell mass, cell debris and red blood cells with a glass pipette in the PVP droplet (Fig. 2Cab). The expanded sample pellet was checked by means of an inverted microscope (Diaphot 300, Nikon, Japan) at × 200 magnification and spermatozoa were checked and recovered from the bottom of the PVP droplet.

As shown in Fig. 3, recovered spermatozoa in the dirty area (Fig. 3A) were transferred to the clean area of the PVP droplet and, immobilized and washed (Fig. 3B-C) for the ICSI procedure.

Oocyte preparation and ICSI

Retrieved oocytes were exposed briefly to 80 IU/ml hyaluronidase (H-3506, Sigma, USA) in Dulbecco's Phosphate Buffered Saline (PBS, 21300-025, Gibco, USA) and mechanically cleaned of their surrounding cumulus cells by repeated aspiration through a glass

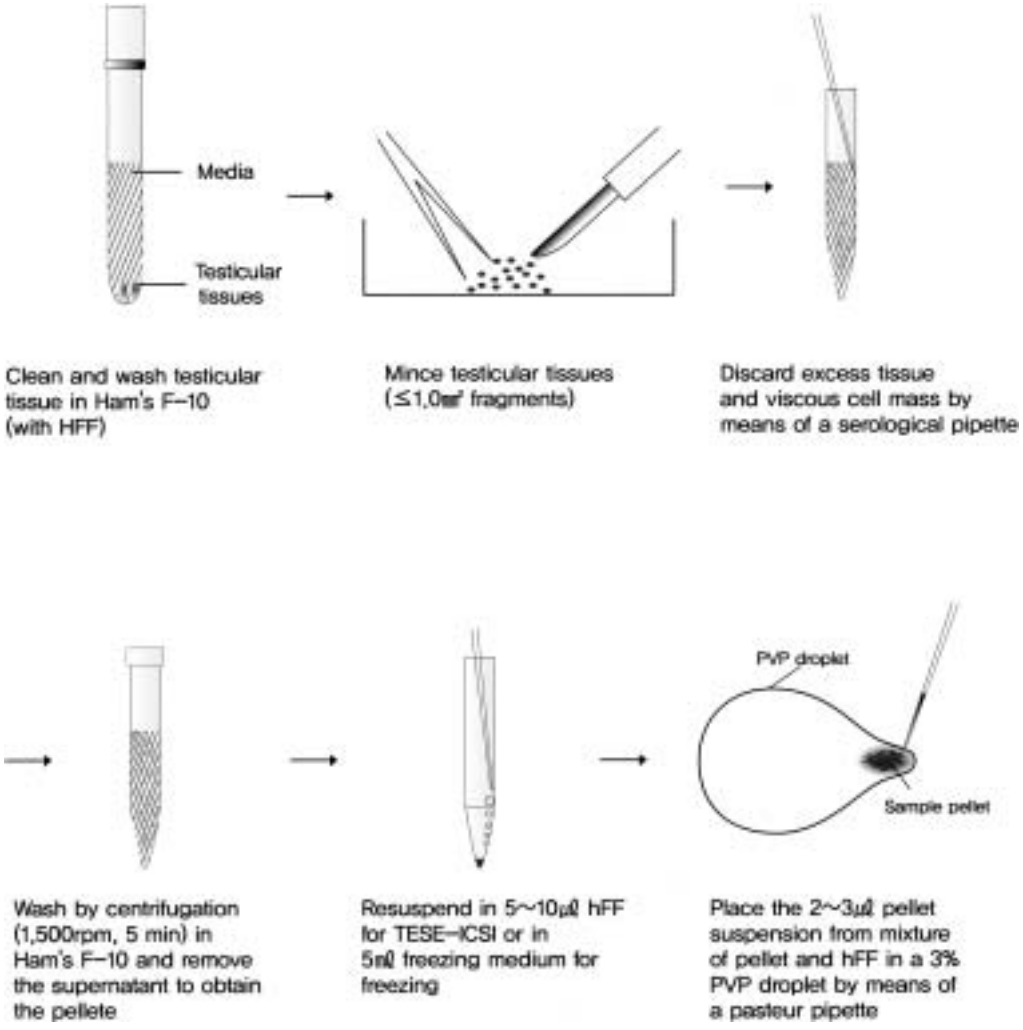

Fig. 1. Procedure for preparation of the TESE sample.

pipette (CO-7095B-5X, Corning, USA) with a 200 μm inner diameter. Denuded oocytes were washed five times in a culture medium (Dulbecco's Modified Eagle Medium, DMEM, 11966-025, Gibco, USA) supplemented with 10% hFF and 0.5% antibiotics. All oocytes were examined under an inverted microscope (Diaphote-300, Nikon, Japan) at $\times$ 200 magnification, and only oocytes in metaphase II were selected for the ICSI procedure.

The ICSI procedure was performed with an inverted-phase microscope equipped with differential interference contrast, and a micromanipulator set (TDU-500, RI, UK). The injection micropipette (ICSI micropipette, Humagen, USA) was lowered into the DPBS with 3% PVP (PVP360, Sigma, USA) solution, and a spermatozoon was chosen and immobilized by touching its tail near the end with the injection pipette. The immobilized spermatozoon was aspirated, tail first, into the injection pipette. After the oocyte was secured in position with the holding pipette, the injection pipette was introduced at the 3-o'clock position (polar body at the 6 or 12-o'clock position). Through the zona pellucida and the oolemma, until it reached two thirds of the cytoplasm, and then was withdrawn to the center. After this procedure, the spermatozoa was slowly injected into the oocyte after some of the cytoplasm was aspirated to verify that the oolemma had been broken (Fig. 3D). Fertilization was confirmed 16 hrs after injection by the presence of two pronuclei.

Preparation of Vero cells and embryo co-culture

The technical aspects of Vero cells maintenance have

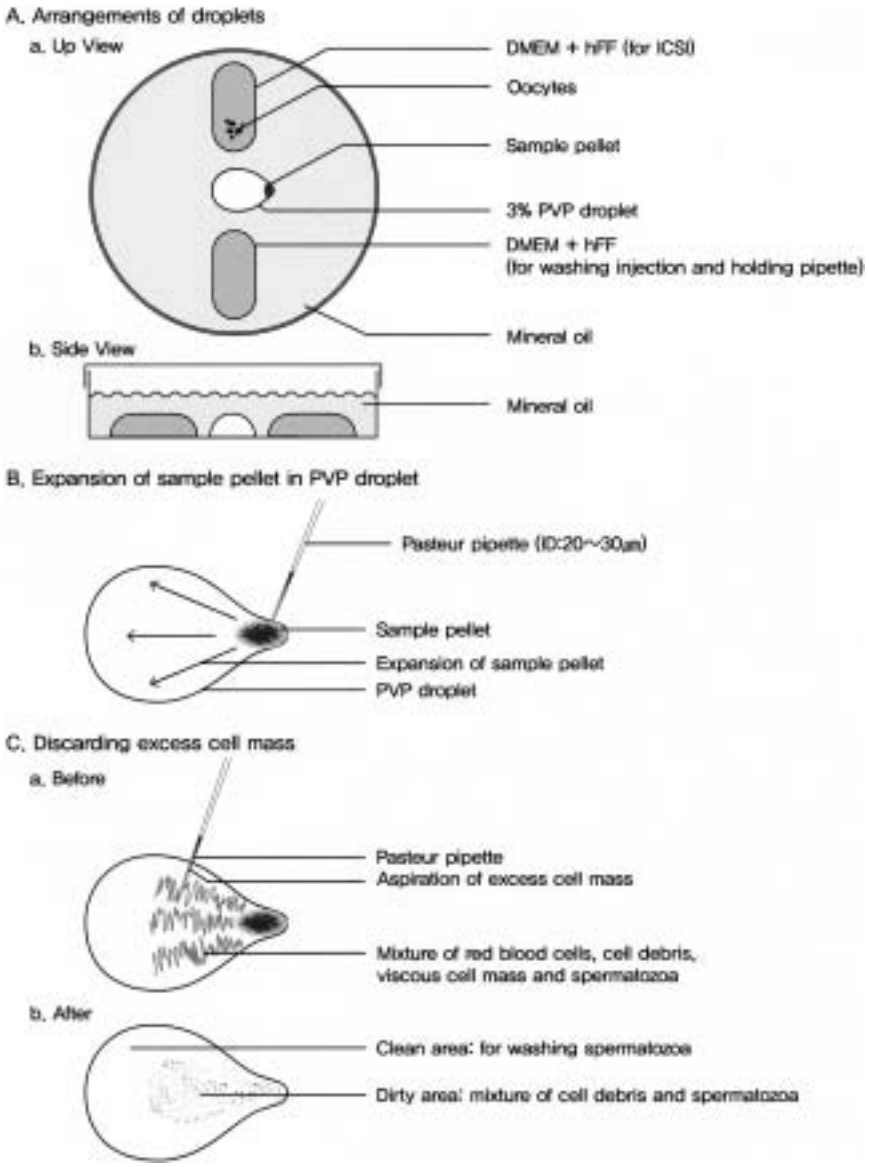

Fig. 2. Preparation of droplets for recovering spermatozoa from TESE sample pellet.

been described by Ouhibi *et al.* [9]. Briefly, from the frozen cells, flasks (3013, Falcon, USA) were seeded with 2–3 $\times$ 10^6 cells and reached confluence within 4 days (6–8 $\times$ 10^6 cells /flask). After trypsinization with Trypsin-EDTA (25300-054, Gibco, USA), the cell suspension was divided into three aliquots. One was used to seed in a new flask, another was frozen with a Cell Culture Freezing Medium-DMSO (11101-011, Gibco, USA), and the thirds was used to seed in culture dishes (3037, Falcon, USA) at 100,000 cells/well. Thus, confluence was reached in 3 days. The culture medium was Medium 199 (11150-059, Gibco, USA) containing 10% fetal bovine serum (FBS, 26140-079, Gibco, USA) and 0.5% antibiotics. The methods for Vero cell freezing and thawing are described by Ouhibi *et al.* [9].

Injected and fertilized oocytes were co-cultured in DMEM supplemented with 20% hFF until transferred.

Embryo and blastocyst grading

After cleavage of normally fertilized zygotes, the morphological grade of all embryos was assessed on day 2 and 5–7 post-oocyte retrieval as follows, embryo and blastocyst grading were based on the systems devised by Veeck [10] and Dokras *et al.* [11],

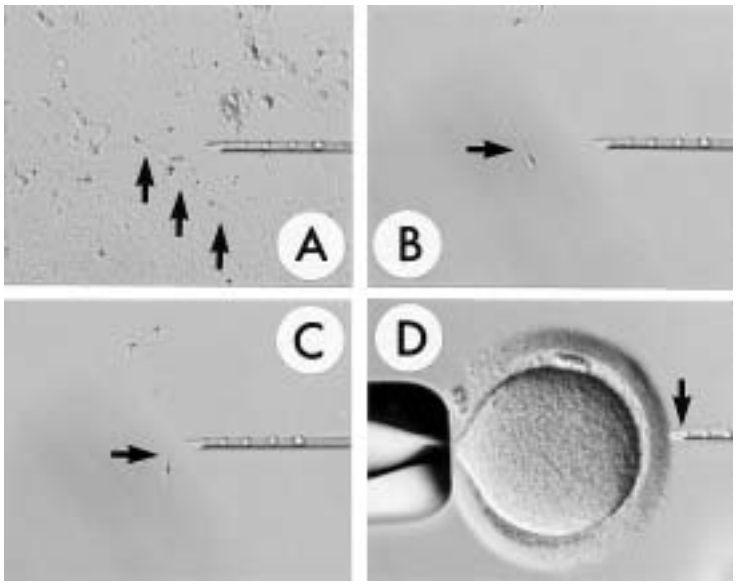

Fig. 3. Procedure for recovery of spermatozoa (Arrows) from TESE samples for lCSI (× 200). Spermatozoa in the dirty area of a PVP droplet (A). Recover the spermatozoon (covered with small cell debris) (B) and immobilize, clean and wash the spermatozoon (C) by means of injection pipette in the clean area in the PVP droplet. ICSl procedure in DMEM droplet (D).

respectively.

Embryos were classified as follows: grade 1 (G1: blastomeres of equal size and with no cytoplasmic fragments), G2 (blastomeres of equal size and with minor cytoplasmic fragments or blebs), G3 (blastomeres of equal or unequal size and with few cytoplasmic fragments or none), G4 (blastomeres of equal or unequal size and with significant cytoplasmic fragmentation) or G5 (blastomeres of any size and with severe or complete cytoplasmic fragmentation).

Blastocyst grades 1 (BG1) demonstrated typical development with early cavitation occurring on day 5 or before day 6 of development resulted in an expanded single cavity lined with clear trophectoderm (TE) and a distinct inner cell mass region (ICM). BG2 had the same appearance as BG1 but their formation was delayed for 24–48 hrs after initial cavitation had begun. BG3 had dark degenerative area within the ICM and/or TE. An additional category of cavitating embryos was also identified. These were not considered to be blastocysts at all since they lacked a clear morula stage where the cells had undergone compaction and neither a distinct TE layer nor an ICM region was identifiable. These were often embryos of 6 to 8 cells that subsequently developed single or multiple cavities of different sizes and which were also unusual since they had well defined smooth edges.

Embryo transfer and pregnancy monitoring

After assessment of embryo or blastocyst morphology, a maximum of four selected embryos and/or blastocysts showing the best morphological development were transferred to the uterine cavity by means of a Tom Cat catheter (8890-703021, Sherwood, USA) on day 2 and/or 5 post-oocyte retrieval.

Pregnancy was defined as a serum β-hCG level of >10 mIU/ml on day 10 after embryo transfer, and a clinical pregnancy was determined by the presence of a gestational sac on vaginal ultrasound examination 3 weeks after oocyte retrieval.

Statistical analysis

The Microsoft Excel 97 was used to compare spermatozoa recovery rates, fertilization rates, cleavage rates, embryo and blastocyst grading and pregnancy rates in the groups. For statistical analysis, Student's t-test was used. Results were considered statistically significant when the p value was less than 0.05.

Table 1. Laboratory variables in ICSI with fresh or frozen-thawed TESE sperm samples

Variable	TESE samples			*P*-value*
	Fresh	Frozen-thawed	Total	
	Group I	Group II		
No. of cycles reaching oocyte retrieval	9	25	34	–
Mean female age (years)	28.7 (25–34)	28.6 (25–35)	28.7 (25–35)	NS
No. of oocytes (/cycle)				
Retrieved	158 (17.6)	470 (18.8)	628 (18.5)	NS
MII	152 (16.9)	408 (16.3)	560 (16.5)	NS
Injected	121 (13.4)	375 (15.0)	496 (14.6)	NS
Spermatozoa recovery rate (/cycles)	9 (100)	25 (100)	34 (100)	NS
Fertilization rate (2 PN)				
/cycles	9/9 (100)	25/25 (100)	34 (100)	NS
/injected oocytes	68/121 (56.2)	140/375 (37.3)	208/496 (41.9)	0.00005
No. of cultured zygotes	68	140	208	–
Cleavage rate				
/cycles	9/9 (100)	25/25 (100)	34/34 (100)	NS
/injected oocytes	62/121 (51.2)	129/375 (34.4)	191/496 (38.5)	0.00032
/fertilized oocytes	62/68 (91.2)	129/140 (92.1)	191/208 (91.8)	NS

*P-value: group I vs. II. NS = not significant.

Results

Stimulation and laboratory variables and clinical outcomes after ICSI with fresh or frozen-thawed TESE samples are shown in Table 1, 2 and 3.

Mean female age was similar in both groups. In all cycles, spermatozoa (motile and/or immotile) can be harvested completely, and fertilization was established (100% per cycles; 41.9% per oocytes), fertilized oocytes were cleaved (100% per cycles; 38.5% per injected oocytes; 91.8% per fertilized oocytes). Fertilization rates (56.2% vs. 37.3%) and cleavage rates (51.2% vs. 34.4%) per injected oocytes were significantly higher (p=0.0005 and 0.00032) in group I than group II, but no difference between groups was found in the cleavage rates (91.2% vs. 92.1%) per fertilized oocytes (Table 1).

Of the fertilized oocytes in group I (68) and II (140), 62 (91.2%) and 129 (92.1%) were cleaved at embryo stage of 2–3 cell (32.3% vs. 30%), 4–6 cell (55.8% vs. 56.4%) and 8 cell (2.9% vs. 5.7%) on day 2, respectively. No differences were found in the number of embryos showing signs of blastulation (50% vs. 49%) and their grades following as early (16.7vs. 24.5%), BG1 (16.7 vs. 16.3%), BG2 (8.3 vs. 4.1%) and BG3 (8.3 vs. 4.1%) and on day 5–7, in group I and II. The rate of progression to the embryo (2-8 cell) and the blastocyst stage and their quality were similar in bothe groups (Table 2).

Embryo transfer (73.5%) or embryo-blastocyst transfer (26.5%) was performed successfully. Of the 34 initiated cycles, 9 (26.5%) culminated in blastocysts available for transfer group I (22.2%) and II (28%). Rates of embryo (77.8% vs. 72%) and embryo-blastocyst transfer (22.2% vs. 28%) and the mean number of transferred embryos (and/or blastocysts) were similar in both group I and II. Clinical pregnancy rates (22.2% vs. 24%) per embryo transfer (14.3% vs. 5.6%) and embryo-blastocyst transfer (50% vs. 71.4%) were similar in both group I and II. There was a tendency for pregnancy rates to be higher in embryo-blastocyst transfer than that in embryo transfer in all cycles (Group I: 14.3% vs. 50%; Group II: 5.6% vs. 71.4%; Total: 8% vs. 66.7%) (Table 3).

Discussion

Testicular sperm extraction can be used to retrieve spermatozoa from men with nonobstructive azoospermia in up to 70% of attempts. The use of a combination of TESE and ICSI with fresh or frozen-thawed testicular spermatozoa from azoospermic men has been reported at many centers. The overall fertilization rate has been slightly lower than that obtained with the use of fresh ejaculated spermatozoa. Clinical pregnancies were achieved in 16.7-50% of sperm retrieval attempts [8, 12–15].

We recovered spermatozoa from all samples (fresh or frozen-thawed TESE samples) having extremely low quality characteristics. Their motility is almost absent. Sometimes we recovered motile spermatozoa, but

Table 2. Characteristics of embryos and blastocysts obtained after TESE-ICSI with fresh or frozen-thawed TESE sperm samples

Variable	TESE samples			*P*-value*
	Fresh	Frozen-thawed	Total	
	Group I	Group II		
Embryo grading on day 2				
No. of cycles	9	25	34	–
No. of cultured zygotes	68	140	208	–
No. (%) of cleaved embryos	62 (91.2)	129 (92.1)	191 (91.8)	NS
2~3 cell	22 (32.3)	42 (30.0)	64 (30.8)	NS
G1**	3 (4.4)	4 (2.9)	7 (3.3)	NS
2	7 (10.3)	6 (4.3)	13 (6.3)	NS
3	6 (8.8)	19 (13.6)	25 (12.0)	NS
4	6 (8.8)	13 (9.3)	17 (8.2)	NS
5	0 (0)	0 (0)	0 (0)	NS
4~6 cell	38 (55.8)	79 (56.4)	117 (56.3)	NS
G1	17 (25.0)	22 (15.7)	39 (18.8)	NS
2	10 (14.7)	18 (12.9)	28 (13.5)	NS
3	9 (13.2)	23 (16.4)	32 (15.4)	NS
4	1 (1.5)	15 (10.7)	16 (7.7)	0.02090
5	1 (1.5)	1 (0.7)	2 (1.0)	NS
8 cell	2 (2.9)	8 (5.7)	10 (4.8)	NS
G1	0 (0)	2 (1.4)	2 (1.0)	NS
2	1 (1.5)	1 (0.7)	2 (1.0)	NS
3	1 (1.5)	4 (2.9)	5 (2.4)	NS
4	0 (0)	1 (0.7)	1 (0.5)	NS
5	0 (0)	0 (0)	0 (0)	NS
Blastocyst grading on day 5–7				
No. of cycles	2	7	9	–
No. of cultured embryos	12	49	61	–
Blastocyst formation rate	6 (50.0)	24 (49.0)	30 (49.2)	NS
Early	2 (16.7)	12 (24.5)	14 (22.9)	NS
BG1†	2 (16.7)	8 (16.3)	10 (16.4)	NS
BG2	1 (8.3)	2 (4.1)	3 (4.9)	NS
BG3	1 (8.3)	2 (4.1)	3 (4.9)	NS

*P-value: group I vs. II. NS = not significant. **G1: Grade 1. †BG1: Blastocyst grade 1.

forward progression was extremely weak. The sperm quality recovered by our method was sufficient in terms of fertilization, embryonic development and pregnancy rates. TESE-ICSI cycles were performed with fresh or frozen-thawed testicular spermatozoa. In 34 TESE-ICSI cycles, 560 metaphase II oocytes were injected, resulting in a fertilization rate of 41.9%, cleavage rate of 38.5% and a clinical pregnancy rate of 23.5%. These clinical pregnancy rates were comparable to the pregnancy rate obtained at our institution when regular ICSI is performed with fresh ejaculated spermatozoa (20%) [16, 17].

Several methods have been described for aspiration of the testis tissue with a biopsy gun [18, 19] or 19- or 21-gauge needle [2, 4, 20]. When 21-gauge or finer needles are used, aspiration may be performed without any anaesthesia, but the biopsy gun and 19-gauge needles require local or loco-regional anaesthesia. The aspiration technique is simple, quicker and more non-invasive than biopsy gun systems or analogues [21]. In patients with normal spermatogenesis, a sperm recovery rate of 96% may be obtained by 21-gauge needle aspiration (2). Nevertheless, 21-gauge needle aspiration may provide material suitable only for cytological assessment, but larger needles may provide tissue cylinders which allow an accurate histopathological examination [4, 22]. In this study, we have performed a retrospective controlled comparison of open biopsy and fine needle aspiration. There were no differences in sperm recovery, fertilization, cleavage or pregnancy rates after ICSI.

In ICSI procedures, PVP has been successfully used

Table 3. Embryo transfer and clinical outcomes of ICSI with the fresh or frozen-thawed TESE sperm samples

Variable	TESE samples		
	Fresh	Frozen-thawed	Total
	Group I	Group II	
No. of cycles reaching oocyte retrieval	9	25	34
Mean no. of embryos and/or blastocysts transferred	3.6 ± 0.4	3.5 ± 0.6	3.5 ± 0.5
No. (%) of ET cycles	9 (100)	25 (100)	34 (100)
Embryos transfers	7 (77.8)	18 (72)	25 (73.5)
Day 2	7 (77.8)	16 (64)	23 (67.6)
Day 3	0	2 (8)	2 (5.9)
Embryo-blastocyst transfers	2 (22.2)	7 (28)	9 (26.5)
Day 5	0	2 (8)	2 (5.9)
Day 2–5	1 (11.1)	4 (16)	5 (14.7)
Day 3–5	1 (11.1)	1 (4)	2 (5.9)
No. (%) of clinical pregnancies per transfers	2/9 (22.2)	6/25 (24.0)	8/34 (23.5)
Embryo transfers	1/7 (14.3)	1/18 (5.6)	2/25 (8.0)
Day 2	1/7 (14.3)	1/16 (6.3)	2/23 (8.7)
Day 3	–	0/2 (0)	0/2 (0)
Embryo-blastocyst transfers	1/2 (50)	5/7 (71.4)	6/9 (66.7)
Day 5	–	1/2 (50)	1/2 (50)
Day 2–5	1/1 (100)	3/4 (75)	4/5 (80)
Day 3–5	0/1 (0)	1/1 (100)	1/2 (50)

The differences between two groups were not statistically significant (P>0.05). Values are the mean ± SEM.

to increase the viscosity of the sperm solution, thus facilitating the handling of spermatozoa [23, 24]. Moreover, it has been shown to stabilize the sperm plasma membrane [25], so delaying calcium oscillations in the sperm-penetrated oocyte, as well as preventing nuclear decondensation [26] and DNA lesions [27]. The effect of *in vitro* treatment by PVP on the ultra structure of human spermatozoa has been tested by statistical analysis [28, 29]. PVP had a primary detrimental action on the plasma membrane, as well as on acrosomal and mitochondrial membranes. Furthermore, membrane damage induces deterioration of the chromatin, axonemal tubules, fibrous sheath and accessory fibers [29]. Nevertheless, concerning the safety of using PVP, some reports have suggested that PVP solutions reduced bull sperm membrane integrity [30] and are toxic to mouse embryo development [31–33]. On the other hand, it has been suggested that the toxicity of PVP is related to its impurities. Van Steirteghem *et al.* [34] and Fujii *et al.* [6] have used dialyzed PVP for human ICSI procedures and high fertilization and pregnancy rates have been achieved. Ray *et al.* [27] have tested the mutagenic potential of dialyzed PVP by sister chromatid exchange analysis and no increase in the basal frequency of sister chromatid exchanges was observed, but we obtained high fertilization and normal live birth rates with non-purified PVP in ICSI cycles (data not shown). We have used non-purified PVP.

The phenomenon of sperm 'swimming across' is usually seen in the 8-10% PVP droplet during the regular ICSI procedure. Fujii *et al.* [6] reported that 8% PVP could clearly separate motile spermatozoa but was not useful for weakly progressive motile spermatozoa, and tried to use a droplet intermediate between a 3% PVP droplet and 8% PVP. He concluded that the 3% PVP droplet limited the dispersion of immotile cells and debris to a small extent but it allowed the progression of motile spermatozoa to the edge of the droplet. But we recovered motile or immotile spermatozoa with 3% PVP in ICSI by using motile or immotile spermatozoa after TESE. In our preliminary experiment, we tried sperm preparations with 10, 8, 5 and 3% PVP droplets. When sperm pellets were placed in the upper the 3% PVP droplets, the sperm suspension never went down to the bottom of the droplet (data not shown).

The HOS test has been used to select viable ejaculated and testicular spermatozoa to perform ICSI in patients with a total absence of sperm motility [15]. We did not attempt to use the HOS test for selection of motile spermatozoa, due to the high fertilization and embryonic development rates.

Before ICSI procedures, oocytes were exposed to

hyaluronidase, intense light and fluctuations in temperature and are subjected to the creation of an artificial breach in the zona pellucida and oolemma. This opening may increase the risk of introduction of toxins and debris into the perivitelline space and oolemma, all of which may affect oocyte quality [35]. Van de Veld *et al.* [36] suggested that a concentration as low as 10 IU/ml hyaluronidase in combination with a pipette of at least 1000 μm inner diameter can be used successfully to denude oocytes for microinjection. And different timing of cumulus-corona cell removal has no effect on the outcome of ICSI. Our previous study [17] showed that the degree of oocyte denudation (complete or partial) did not affect the outcome of ICSI, but denuded oocytes with an incubation period of more than 2 hr resulted in better ICSI outcomes than with an incubation period less than 2 hr.

Most TESE methods use gentle mincing of testicular tissue with sterile needles, thus achieving the mechanical isolation of spermatozoa [37–39]. We prepared a TESE samples by mincing testicular tissue with a surgical blade, not needles. Some have used Percoll [40–42] and a combined enzymatic and mechanical approach [3, 43]. We have not used Percoll and/or enzymes in washing TESE samples for the preparation of spermatozoa due to the 100% recovery rate of spermatozoa.

Balaban *et al.* [7] demonstrated that embryo development and implantation are under paternal influence and the severity of the spermatogenic disorder is associated with the success of ICSI. Blastocyst formation is commonly used as an indicator of embryo quality and viability is affected by the source of spermatozoa utilized for ICSI. When spermatozoa are retrieved from the testes as in the case of non-obstructive azoospermia, a lower rate of blastocyst formation and implantation per blastocyst should be anticipated [7].

In conclusion, we can recover spermatozoa from all TESE-samples by our simple method for ICSI which yields an acceptable fertilization, cleavage and pregnancy rate. Freezing the testicular tissue guarantees paternal gametes for subsequent ovarian stimulation cycles and needs only one combined diagnostic and therapeutic testicular biopsy procedure. And this sperm preparation method is very simple, easy, effective and rapid in recovering spermatozoa from fresh or frozen-thawed TESE-samples for ICSI.

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