Efficacy of cryopreservation of embryos generated by intracytoplasmic sperm injection with spermatozoa from frozen testicular tissue

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Abstract

Purpose To evaluate the viability of frozen embryos generated by intracytoplasmic sperm injection (ICSI) with frozen testicular spermatozoa.

Methods A total of 68 fresh embryo transfer (ET) cycles and 85 subsequent frozen-thawed ET (FET) cycles were grouped according to the source of spermatozoa: fresh testicular spermatozoa (TESE) or frozen-thawed testicular spermatozoa (t-TESE). **Results** There were no significant differences in the age of female patients, number of oocytes, or fertilization rates in fresh ET cycles with TESE (TESE-fresh ET) versus t-TESE (t-TESE-fresh ET). The rate of embryo survival after thawing

(95.7 % vs. 94.0 %) was similar in frozen ET cycles (FET) with TESE (TESE-FET) and with t-TESE (t-TESE-FET). While there were significant differences in the proportion of good quality embryos, no statistical differences were found in the pregnancy or clinical abortion rates between the two groups. Moreover, delivery rates were not significantly different.

Conclusions Although the proportion of good quality embryos was affected by cryopreservation of testicular tissue, embryo survival rate was not. As well, subsequent pregnancy could be achieved successfully via t-TESE-FET cycles. Therefore, FET is not affected by the cryopreservation of testicular tissue, and avoids further oocyte retrieval and TESE procedures.

Capsule The use of frozen-thawed embryos generated by intracytoplasmic sperm injection (ICSI) using frozen-thawed testicular spermatozoa could provide satisfactory clinical outcomes and avoid further oocyte retrieval and testicular sperm extraction (TESE) procedures.

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(t-TESE) · Testicular sperm extraction (TESE)

Introduction

Recent years have witnessed the successful use of assisted reproductive technology (ART) in many difficult cases. For obstructive or non-obstructive azoospermic patients, intracytoplasmic sperm injection (ICSI) using spermatozoa obtained from testicular tissue is well established, and has acceptable fertilization and pregnancy rates [1–4].

Since testicular biopsy is an invasive procedure that may be associated with significant complications, it is advantageous to avoid damage to the testis stemming from repeated biopsy. Therefore, cryopreservation of testicular sperm and/or tissue cryopreservation can be performed at the time of testicular sperm extraction (TESE) or diagnostic testicular biopsy to avoid repeated TESE. The frozen testicular sperm or tissue can be used to subsequent ICSI trials if pregnancy is not initially achieved [5, 6]. Freezing and in vitro culture of

testicular spermatozoa or tissue are reliable approaches for the management of azoospermic patients and could all for the possibility of multiple IVF-ICSI procedures [7]. Previously Park et al. [8] reported that acceptable fertilization and pregnancy rates were achieved using frozen testicular spermatozoa as opposed to fresh testicular spermatozoa in obstructive azoospermia (OA). Although the use of cryopreserved testicular sperm is not easy because of their low numbers and motility [3, 9], there were no differences in fertilization and pregnancy rates for fresh and thawed testicular sperm from men with OA and non-obstructive azoospermia (NOA) [10, 11]. Also, fertilization and pregnancies have been reported using thawed testicular sperm and testicular tissue [12, 13].

Embryo cryopreservation and frozen embryo transfer (FET) is now a well-established technique that allows the storage of supernumerary embryos created by ART [14]. It has several advantages which lower risks such as multiple pregnancies and ovarian hyperstimulation syndrome (OHSS) while providing benefits, e.g. cost effectiveness, increased cumulative pregnancy rates, and emotional stability for infertile couples [15, 16]. Several factors have been shown to affect the outcome of FET cycles, e.g. maternal age, number of transferred embryos, possible differences in freezing-thawing protocols and culture conditions [17, 18], number of retrieved oocytes, quality of embryos available for cryopreservation in fresh cycle [19, 20].

Only limited data are available concerning the efficacy of frozen embryos fertilized with frozen-thawed testicular spermatozoa. While considerable attention has been paid to the efficacy of cryopreservation of embryos generated by ICSI with spermatozoa from frozen testicular tissue, few studies have addressed this topic. Al-Hasani et al. [21] reported successful pregnancies using the transfer of frozen pronuclear-stage oocytes obtained by ICSI with spermatozoa extracted from frozen testicular tissues in patients with NOA, and embryos generated following ICSI using surgically retrieved spermatozoa from azoospermic men have also been reported [14].

Therefore, to test the efficacy of cryopreservation of embryos generated by ICSI with spermatozoa from frozen testicular tissue, we analyzed the pregnancy outcomes of fresh embryos generated by fertilization with fresh testicular spermatozoa (TESE) versus those from frozen-thawed testicular spermatozoa (t-TESE) as well as frozen-thawed embryos generated by fertilization with TESE versus t-TESE.

Materials and methods

Patients

Fresh IVF-ET cycles ($n=324$) after ICSI using fresh testicular spermatozoa (TESE-fresh ET) and frozen-thawed testicular spermatozoa (t-TESE-fresh ET) from azoospermic patients

were performed. To evaluate the effects of fresh versus frozen-thawed testicular spermatozoa and of the cleavage stage of embryos on pregnancy and delivery rates, TESE-fresh ET and t-TESE-fresh ET cycles with frozen embryos (zygote, cleavage embryo or blastocyst, $n=68$) were selected, and subsequent frozen-thawed ET (FET, $n=85$) cycles were analyzed. Oocyte donation, preimplantation genetic diagnosis (PGD) and frozen-thawed oocyte cycles were excluded.

The selected 68 cycles were divided into TESE-fresh ET ($n=27$) and t-TESE-fresh ET ($n=41$). The rates of transfer of good quality embryos, pregnancy, and delivery among these fresh ET cycles were compared. Additionally, the delivery rates of subsequent FET ($n=85$) cycles were compared between TESE-FET ($n=35$) and t-TESE-FET ($n=50$). Institutional review board approval was obtained for this study.

Testicular sperm preparation and freezing & thawing

The testicular sperm extraction and preparation procedure was performed as described previously [8, 22]. Briefly, a small piece (1 cm^3) of extruded testicular tissue was excised and placed in Ham's F-10 medium (Sigma, St. Louis, MO) supplemented with 0.4 % (w/v) human serum albumin (HSA; Sigma). Testicular tissues were rinsed two to three times with the Ham's F-10 medium and squeezed with fine forceps to determine the presence of spermatozoa under a microscope ($\times 200\sim\times 400$). Spermatozoa were kept in an incubator at 37°C , 6 % CO_2 in air, until the time of the ICSI procedure (about 3–5 h). Spermatozoa-containing tissue was frozen by adding Cryosperm (Medicult, Jyllinge, Denmark) and drawn into 2-mL cryogenic vials (Corning Costar Co., Cambridge, MA). Vials were frozen using a computerized freezer (CryoMagic-I; Mirae Biotech., Seoul, Korea). For thawing, vials were removed from liquid nitrogen and kept at room temperature for 5 min. The procedure of spermatozoa extraction from frozen-thawed testicular tissue was as described above.

Ovarian stimulation/oocyte retrieval and ICSI

Controlled ovarian hyperstimulation (COH) was performed using a GnRH analogue with hMG or recombinant FSH (rFSH). Oocyte retrieval was performed via a transvaginal approach with sonographic guidance 36 h after the administration of 10,000 IU of hCG (Pregnyl, Organon, the Netherlands). ICSI was performed on metaphase II oocytes using fresh or frozen-thawed testicular spermatozoa. Recovered spermatozoa were loaded in $10\text{ }\mu\text{L}$ drops of Gamete medium to observe their movement. Immotile testicular spermatozoa were then treated with 5 mM pentoxifylline (PF) to assess the viability. Spermatozoa with slight tail movement were considered motile.

Assessment of fertilization and embryo grading

Normal fertilization was considered to be the presence of two clearly visible pronuclei at 16–18 h after ICSI. Fertilized embryos were transferred to G-I.III medium. Embryos were scored according to the number of blastomeres and percentage of enucleate fragments [22, 23]. 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 %. Good quality embryos were considered to be embryos of grade I, I-1, II.

Embryo freezing and thawing

Embryo freezing and thawing procedures were described previously [24]. Supernumerary embryos generated by fertilization with fresh testicular spermatozoa or frozen-thawed testicular spermatozoa were frozen at the pronuclear (PN) or cleavage embryo stage (on day 3) by the slow-freezing method, and some cleavage stage embryos (on day 3) and blastocysts were vitrified. Cryopreserved pronuclear stage embryos were excluded from total cleaved embryos. For slow-freezing, 1.5 M PROH (1,2-Propanediol; Sigma, St. Louis, MO) and 0.1 M sucrose was used as a cryoprotectant. The embryos were loaded into a 0.25-mL straw (Bicef, L'Aigle, France) which was loaded into a computerized freezer (CryoMagic-I; Mirae Biotech., Seoul, Korea). Frozen embryos were thawed by the rapid thawing method. To thaw frozen human embryos, the straws were warmed by holding them in air for 40 s before plunging them into a water bath at 37 °C for 1 min. The cryoprotectant was then removed by reverse stepwise dilution. For vitrification, a pull and cut (PNC) straw was made by pulling and cutting a 0.25-ml straw. The embryos were equilibrated in 7.5 % ethylene glycol (EG) and 7.5 % dimethyl sulfoxide (DMSO) for 15–25 min depending on the embryo

stage, and exposed to 15 % EG and 15 % DMSO in PBS, adding 0.5 M sucrose within 1 min and then immediately plunging them into liquid nitrogen. To thaw vitrified embryos, they were warmed in 1 M sucrose for 1 min, then in 0.5 M sucrose for 3 min, and were then washed twice in PBS.

Assessment of pregnancy

Fresh or frozen-thawed embryos were transferred on day 3–5. Clinical and ongoing pregnancy was defined as the presence of a gestational sac by transvaginal ultrasound in the 5th to 7th gestational week and the existence of a fetal heart beat at 12 weeks. Patient characteristics and pregnancy outcomes were compared between the two groups.

Statistical analysis

The Fisher's exact test, Chi-square test or *t*-test was used for statistical analysis. Data is shown as mean ± SD and differences were considered statistically significant at <0.05.

Results

Sixty-eight cycles were divided into two groups according to whether they were fertilized with fresh testicular spermatozoa (TESE-fresh ET cycle; *n*=27) or frozen-thawed testicular spermatozoa (t-TESE-fresh ET cycle; *n*=41) (Table 1).

In the TESE-fresh ET cycles, fertilized embryos were evaluated as follows: 322 fertilized embryos with normal 2-PN, 24 embryos with 1-PN and 21 embryos with delayed developmental status. The fertilization rate was 76.1 % (2-PN rate; 66.8 %). In the t-TESE-fresh ET cycles, 461 embryos were normal 2-PN, 29 embryos were 1-PN and 30 embryos were of delayed developmental status. The fertilization rate was 76.7 % (2-PN rate; 67.9 %). The fertilization rate was not

Table 1 Characteristics of fresh IVF-ET cycles after ICSI using fresh- or frozen-thawed testicular spermatozoa

	TESE	t-TESE	<i>P</i> value
No. of cycles	27	41	
Mean age of female patients (yr)	33.2±3.6	31.8±3.9	NS
No. of matured oocytes	482/594 (81.1)	690/835 (82.6)	NS
No. of fertilized embryos*	367/482 (76.1)	520/678 (76.7)	NS
No. of 2-PN zygotes	322/482 (66.8)	461/678 (67.9)	NS
Causes of male infertility			
NOA (<i>n</i> =17)	8/26 (30.8)	9/36 (25.0)	NS
OA (<i>n</i> =45)	18/26 (69.2)	27/36 (75.0)	NS

*Fertilized embryos included 2-pronuclear, 1-pronuclear and delay-developed embryos, TESE fresh testicular spermatozoa, t-TESE frozen-thawed testicular spermatozoa, NOA non-obstructive azoospermia, OA obstructive azoospermia, NS not significant. Values in parentheses are percentages

Table 2 Survival rates of frozen-thawed embryos fertilized with fresh- or frozen-thawed testicular spermatozoa

	TESE	t-TESE	P value
No. of cycles	35	50	
Mean age of female patients (yr)	33.6±3.8	32.1±3.7	NS
No. of survived/thawed embryos	157/164 (95.7)	204/217 (94.0)	NS
pronuclear stage (PN, <i>n</i> =27)	126/130 (96.9)	157/163 (96.3)	NS
cleavage stage (CL, <i>n</i> =3)	10/10 (100)	10/11 (90.9)	NS
blastocyst stage (BL, <i>n</i> =2)	3/4 (75.0)	13/14 (92.9)	NS
mixed (PN+BL, <i>n</i> =3)	18/20 (90.0)	24/29 (82.8)	NS
Cleavage embryos (%)	148/157 (94.3)	195/204 (95.6)	NS

Values in parentheses are percentages

TESE fresh testicular spermatozoa, t-TESE frozen-thawed testicular spermatozoa, NS not significant.

statistically significantly different between the TESE and t-TESE cycles.

TESE and t-TESE cycles had tried for embryo transfer in fresh cycles, and cryopreserved supernumerary embryos were thawed (Table 2). The survival rates of frozen-thawed embryos in the two groups did not significantly differ (95.7 vs. 94.0 %). Cryopreserved embryo stage did not influence the survival rate. Although blastocyst stage and mixed stage embryos showed different survival rates, statistical significance was not observed.

Table 3 shows the comparative clinical outcomes of TESE-FET cycles and t-TESE-FET cycles. Good quality embryos were found at rates of 82.4 % and 71.9 %, respectively ($p<0.05$). Positive beta-hCG and clinical pregnancy rates in TESE-FET and t-TESE-FET did not differ. Moreover, the delivery rates were not significantly different between the TESE-FET (34.3 %) and t-TESE-FET (44.0 %) groups.

Discussion

Fertilization by azoospermic patients can be achieved by the application of ART [4]. When pregnancy is not achieved, repeated testicular biopsy may be required. In the clinical application of ART, freezing of testicular spermatozoa or tissue is an effective method to avoid repeated biopsy procedures that carry the potential risk of testicular damage. While much controversy of fertilization and pregnancy rate remains

over the cryopreservation of testicular spermatozoa [25–27], these results indicate that there are no adverse effects on IVF outcomes [28–30]. Previously, Park et al. [8] reported that t-TESE had a lower fertilization rate than fresh TESE, but they did not find significantly different ongoing pregnancy rates in OA. Recently, similar data showed that IVF-ICSI outcomes with t-TESE suggest that cryopreservation does not adversely affect this procedure [31]. Testicular tissue could be cryopreserved successfully without markedly reducing subsequent fertilization and implantation rates [32]. Cryopreservation of testicular sperm is controversial. Several studies have reported that the lower number and motility of frozen sperm might affect fertilization and pregnancy rates compared with fresh sperm [25–27]. However, no differences in fertilization, embryo cleavage, pregnancy, delivery, and spontaneous abortion rates were reported between fresh and frozen-thawed testicular sperm from men with OA and NOA patients [10, 11, 30, 33]. On the other hand, Palermo et al. [34] 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.

With respect to spermatozoa quality, the outcome of ICSI using extracted spermatozoa may be affected by various factors, including the method of spermatozoa retrieval, spermatozoa maturity, motility status of the retrieved spermatozoa, spermatozoa status (fresh or after freezing-thawing) and the timing of spermatozoa retrieval relative to oocyte collection [10, 32]. According to Lee et al. [24], the clinical outcomes of

Table 3 Comparison of clinical outcomes between TESE-FET and t-TESE-FET cycles

	TESE	t-TESE	P value
No. of surviving embryos	157/164 (95.7)	204/217 (94.0)	
Mean no. of transferred embryos	2.9±0.8	3.2±0.9	
No. of good quality embryos	84/102 (82.4)	115/160 (71.9)	<0.05
No. of Beta-hCG positive patients	21/35 (60.0)	30/50 (60.0)	NS
No. of clinical pregnancies	19/35 (54.3)	26/50 (52.0)	NS
No. of ectopic pregnancies	1 (2.9)	0	
No. of clinical abortions	5 (14.3)	3 (6.0)	
No. of 2nd trimester losses	1 (2.9)	1 (2.0)	
No. of deliveries/ET	12/35 (34.3)	22/50 (44.0)	NS

TESE fresh testicular spermatozoa, t-TESE frozen-thawed testicular spermatozoa, FET frozen-thawed embryo transfer, NS not significant, Values in parentheses are percentages.

IVF using testicular spermatozoa may be influenced by extracted spermatozoa quality, which affects embryo quality and development. Factors related to the female partner, such as age, the number of oocytes available for ICSI, oocyte status (fresh or after freezing-thawing), and in vitro matured oocytes, have been shown to affect IVF outcomes [35].

When spermatozoa derived from testicular tissue are immotile, it is difficult to select viable spermatozoa. Even if fertilization occurs after injection of immotile spermatozoa into the oocyte, a high incidence of mitotic spindle defects can result in impaired, retarded or arrested embryonic development [31, 36]. Park et al. [8] reported that motile thawed testicular spermatozoa provided fertilization rates superior to those of immotile spermatozoa. Therefore, spermatozoa motility after t-TESE is an important factor that influences fertilization and pregnancy rates.

To evaluate whether clinical IVF outcomes were affected by the status (fresh vs. frozen-thawed) of testicular spermatozoa, we retrospectively studied the subsequent FET cycles with embryos that were generated by fertilization with fresh or frozen-thawed testicular spermatozoa. All of the fertilized embryos in 12 fresh IVF cycles were frozen without embryo transfer (owing to the risk of OHSS and individual complications, etc.). All other cycles had the opportunity for embryo transfer in fresh cycles, and had cryopreserved supernumerary zygotes for further frozen-thawed cycles. The cryopreservation of zygotes allows for repeated attempts at pregnancy without repeat ovarian stimulation [15]. Nicopoullos et al. [14] reported significantly lower implantation rates in FET cycles using embryos generated from surgically retrieved spermatozoa as opposed to those generated from ejaculated spermatozoa (0 % vs. 11.5 %). Both the clinical pregnancy rate (CPR) (5 % vs. 21 %) and the live birth rate (LBR) (0 % vs. 21 %) were lower for embryos generated with surgically retrieved spermatozoa than for those generated with ejaculated spermatozoa. The outcome of fresh ET and FET cycles from patients with NOA has been reported [21], and, in that report, the clinical pregnancy and implantation rates were 36 % and 13 %, respectively, in fresh ET cycles, while the clinical pregnancy and implantation rates were 24 % and 8 %, respectively, in FET cycles. Our results show that the embryo survival and cleavage rates after thawing did not differ between the TESE-FET and t-TESE-FET groups (95.7 % vs. 94.0 % and 94.3 % vs. 95.6 %, respectively). In addition, the clinical outcomes of FET cycles were not detrimentally affected by t-TESE compared with TESE (Table 3). Therefore, both testicular spermatozoa freezing and embryo freezing may increase chances of pregnancy after a single stimulation procedure, and allow for additional IVF attempts. In the present study, approximately 50 cycles would be required in each group to conduct a comparative study. However, the sample size of the study was small, and this might have a limited clinical usefulness. Our findings should be interpreted with caution and

larger studies may be needed. Although, we had a limited number of cycles, our rates were similar in patients with TESE and t-TESE.

In conclusion, the present study shows that acceptable pregnancy rates can be achieved with frozen-thawed embryos generated by ICSI with TESE or t-TESE, and that FET is not affected by the use of spermatozoa from cryopreserved testicular tissue. Freezing of testicular tissue and of embryos derived therefrom is beneficial to azoospermic patients by reducing the need for additional testicular biopsy procedures and eliminates the requirement for additional ovarian stimulation of female partners while increasing cumulative pregnancy rates in couples with an azoospermic partner.

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