Efficiency of Using Frozen-Thawed Testicular Sperm for Multiple Intracytoplasmic Sperm Injections

NORITAKA FUKUNAGA,[1,3] KOSUKE HAIGO,[1] KOICHI KYONO,[1] and YASUHISA ARAKI[2]

Submitted: February 13, 2001
Accepted: June 7, 2001

Purpose: *To compare fertilization and pregnancy rates of fresh and frozen-thawed testicular sperm injections (TESE-ICSI).*
Methods: *Sperm collected from the testes of 28 azoospermic patients by an open testicular biopsy technique was used for initial ICSI or cryopreserved.*
Results: *Fresh-sperm ICSI treatment (28 cycles) resulted in a 58.1% fertilization rate and a 32.1% clinical pregnancy rate per embryo transfer, while frozen-thawed sperm (24 subsequent cycles) had rates of 54.5 and 29.2%, respectively. The PR was lower using frozen-thawed sperm from nonobstructive azoospermia patients (9.1%) than from obstructive azoospermia patients (46.2%). PR declined to 0% upon the fourth ICSI attempt.*
Conclusions: *Fertilization, embryo cleavage, and pregnancy rates were unaffected by fresh or frozen-thawed sperm use. A 57.1% cumulative clinical PR was achieved using the latter. The PR was significantly lower using frozen-thawed sperm from nonobstructive azoospermia patients than from obstructive azoospermia patients.*

KEY WORDS: Frozen-thawed sperm; TESE-ICSI; testicular biopsy.

INTRODUCTION

Advancements in the field of assisted reproductive technology (ART) have included the development of intracytoplasmic sperm injection (ICSI) as an effective therapy for severe male subfertility. ICSI has resulted in high fertilization and pregnancy rates, with patients having obstructive and nonobstructive azoospermia in particular undergoing testicular sperm extraction, (TESE)-ICSI (1–3).

Patients with nonobstructive azoospermia often have small testes and an elevated FSH level. Clinically, these patients may be diagnosed as having testicular failure, but it is sometimes possible to extract sperm from testicular tissue despite the presence of Klinefelter syndrome. Multiple biopsies are difficult to perform in patients with nonobstructive azoospermia, because these patients typically have small testes and dysfunctional spermatogenesis. Therefore, it would be beneficial if the frozen-thawed sperm of such patients could be used for repeated cycles of ICSI therapy.

The use of frozen-thawed testicular sperm has been evaluated by several researchers (4–11), with conflicting results reported. Some investigators found that the fertilization rate (FR) and pregnancy rate (PR) are lowered when frozen-thawed sperm is used (5,6,12), while others have demonstrated that frozen-thawed sperm can function as well as fresh sperm (4,7,9,10).

In this report, we collected specimens from the testicular tissue of 41 patients and performed ICSI to compare the efficacies of fresh and frozen-thawed testicular sperm injections. We also present the cumulative results of repeated rounds of treatment using frozen-thawed testicular sperm.

MATERIALS AND METHODS

Patients

A total of 41 patients were diagnosed with azoospermia; 21 with nonobstructive azoospermia (NOA), and 20 with obstructive azoospermia (OA).

[1] Ladies Clinic Kyono, 3-8-6, Oomiya, Furukawa, Miyagi 989-6221, Japan.
[2] The Institute for ARMT, 2-39-3, Kamikoide, Maebashi, Gunma 371-0037, Japan.
[3] To whom correspondence should be addressed at Ladies Clinic Kyono, 3-8-6, Oomiya, Furukawa, Miyagi 989-6221, Japan; e-mail; lckyono@olive.ocn.ne.jp.

1058-0468/01/1200-0634/0

Biopsy results showed testicular sperm in 28 of the 41 patients. Of these 28 patients, the men were diagnosed as having either nonobstructive ($n = 9$), or obstructive ($n = 19$) azoospermia, based on the results of a clinical examination.

Retrieval of Sperm From Testicular Tissue

Biopsies were taken from an equatorial location and not from regions involving the rate testes. A small piece (5×5 mm) of extruding testicular tissue was excised and minced using sterile glass slides in a dish with 1 mL modified human tubal fluid (m-HTF) medium. An inverted microscope ($\times 400$) was used to check for motile sperm.

The dissection medium was carefully aspirated to avoid aspirating any tissue and centrifuged for 5 min at 750 g. The pellet was resuspended in 1 mL of medium. The sperm suspension was kept in an incubator until the ICSI procedure, as incubating testicular sperm for several hours is known to improve sperm motility. When ICSI was initially unsuccessful, sperm from the original specimen was selected for further injection according to the continuous Percoll gradient.

Cryopreservation of Testicular Sperm and Thawing

The sperm suspensions were mixed with equal volumes of TEST-Yolk buffered with glycerol (Irvine Scientific, Irvine, CA), and exposed to liquid nitrogen (LN_2) vapor for 15 min before immersing in LN_2 for storage.

When the sperm needed to be thawed, the frozen sample tube was warmed at room temperature for 10 s and put directly into a 30°C water bath, where thawing was completed. We then centrifuged it with Percoll. The pellet was resuspended in 0.2 mL of serum containing 10% HTF, and then incubated at 37°C under 5% CO_2 in air for 2–3 h. Thereafter, an inverted microscope was used to check for motile sperm that could be used for ICSI. Any motile sperm was removed and transferred to new medium using an injection pipette.

Retrieval of Oocytes

Ovarian stimulation was achieved through the combined administration of gonadotrophin-releasing hormone (GnRHa), follicle stimulating hormone (FSH, Fertinorm P, Serono), and 10,000 units of human chorionic gonadotrophin (hCG, hCG-MOCHIDA, Mochida) when the leading follicle reached a mean diameter of 18–20 mm. Vaginal ultrasound-guided follicle puncture took place approximately 36 h after hCG injection. The cumulus corona cells were initially removed through exposure to 60 IU of hyaluronidase for up to 1 min. Only Metaphase II oocytes were injected.

RESULTS

Contents obtained from the biopsies of 41 azoospermic patients were analyzed for sperm under a microscope. Sperm identified in 68.3% (28/41) of patients was subsequently used for ICSI. For each pathological diagnosis, the number of patients in which sperm was found is shown Table I. Sperm was detected in 42.9% (9/21) of NOA patients and in 95% (19/20) of OA patients. Sperm detection was significantly lower for NOA patients than for OA patients ($P < 0.01$). Sperm was not identified in 31.7% (13/41) of the patient population. The histological findings included cell populations containing only Sertoli cells in 17.1%, maturation arrest in 46.3%, hypospermatogenesis in 4.9%, germinal cell aplasia 7.3%, and complete spermatogenesis 24.4% of the total 41 patients. Samples in which sperm was detected averaged a Johnsen's

Table I. Results of Histopathological Analysis and Pregnancy

Pathological diagnosis	No. of patients (%)	No. of patients in which sperm was detected (%)	No. of resulting pregnancies	
			Fresh sperm	Frozen sperm
Complete spermatogenesis	10 (24.4)	10 (35.7)	4	3
Maturation arrest	19 (46.3)	9 (32.1)	3	2
Hypospermatogenesis	2 (4.9)	1 (3.7)	1	0
Germinal cell aplasia	3 (7.3)	3 (10.7)	0	1
Sertoli cell only	7 (17.1)	5 (17.8)	1	1
Total	41 (100)	28 (100)	9	7

Table II. Results of ICSI Using Testicular Sperm From Specimens Having a Johnsen's Score <7

Variable	Value
No. of patients (No. of cycles)	10 (7)
No. of oocytes	67
No. of injected oocytes	51
No. of fertilized oocytes (%)	35/51 (68.6)
No. of cleaved oocytes (%)	32/35 (91.4)
No. of pregnancies (%)	2/7 (28.5)

score of 6, while those in which sperm was not detected had an average score of 2.5. Sperm was detected in 42.9% (10/28) of specimens having Johnsen's scores under 7. Moreover, specimens from 7 of these 10 patients resulted in successful ICSI and an overall pregnancy rate of 28.5% (2/7) (Table II).

Twenty-eight cycles of ICSI treatment were performed using fresh testicular sperm. After injection of 296 Metaphase II oocytes, a fertilization rate of 58.1% was obtained. Twenty-four ICSI cycles were attempted using frozen-thawed testicular sperm. Motile sperm was found in all testicular tissue samples that had been thawed and then incubated for approximately 2–3 h. Injection of 220 Metaphase II oocytes with frozen-thawed sperm resulted in a fertilization rate of 54.5% and a cleavage rate of 92.5% (Table III). The difference between the use of fresh and frozen-thawed sperm did not differ significantly between the NOA and OA patients, with PR rates of 33.9% (3/9) and 31.6% (6/19), respectively, when using fresh sperm. While PR from NOA patients was significantly lower than from OA patients when using frozen-thawed sperm: 9.1% (1/11) and 46.2% (6/13) ($P < 0.05$), respectively.

The cumulative pregnancy rate achieved using frozen-thawed testicular sperm is shown in Table IV. The PR obtained following the first ICSI of frozen-thawed testicular sperm was 23.1% (3/13), and a PR of 50% (3/6) was obtained after a second injection. PRs following the third and fourth injections were found to be 33.3% (1/3) and 0% (0/2), respectively.

Table III. Pregnancy Outcomes After ICSI With Fresh/Frozen-Thawed Testicular Sperm

Variable	Value	
	Fresh sperm	Frozen-thawed
No. of patients (No. of cycles)	28 (28)	28 (24)
No. of oocytes	368	280
No. of injected oocytes	296	220
No. of fertilized oocytes (%)	172/296 (58.1)	120/220 (54.5)
No. of cleaved oocytes (%)	160/172 (93.0)	111/120 (92.5)
No. of pregnancies (%)	9/28 (32.1)	7/24 (29.2)

DISCUSSION

Testicular sperm extraction (TESE) retrieves sperm from men with nonobstructive azoospermia in around 70% of attempts (11). Numerous centers have reported using combination therapy of TESE and ICSI of frozen-thawed testicular sperm in patients with azoospermia (4–11). Kupker *et al.* (11) discussed a slightly lower overall fertilization rate (43–47%) was obtained with this regimen compared to that obtained using fresh ejaculated sperm. Average FR for fresh sperm was 52%. Clinical pregnancies were achieved in 20–50% of them.

In this study, 28 ICSI were attempted using fresh testicular sperm, followed by 24 ICSI multiple-treatment cycles using frozen-thawed testicular sperm. The final cumulative PR was 57.1%. Although adequate supplies of sperm for ICSI were stored for all patients, the use of frozen-thawed sperm after 3 ICSI cycles produced no pregnancies. This implies that some restriction may apply to multiple treatment, possibly due to limitations in the use of frozen-thawed testicular sperm itself, or by those involved in conventional IVF procedures, such as degraded oocyte quality from a frequently stimulated ovary. Further research should aim to elucidate whether multiple ICSI therapy is in fact limited to 4 cycles and if it is, to elucidate the causal factors.

In this study, sperm motility was present to some degree after all specimens of sperm were thawed and incubated in medium, and similar clinical PRs

Table IV. Cumulative Pregnancy Rates Obtained Using Frozen-Thawed Testicular Sperm

		Repeated trials of ICSI with frozen-thawed sperm			
	Fresh sperm	1	2	3	4
No. of patients	28	13	6	3	2
Successful pregnancy (%)	9 (32.1)	3 (23.1)	3 (50)	1 (33.3)	0 (0)
Cumulative rate of pregnancy (%)	9/28 (32.1)	12/28 (42.9)	15/28 (53.6)	16/28 (57.1)	16/28 (57.1)

were obtained for the use of frozen-thawed testicular sperm and fresh sperm for ICSI. This latter result is in good agreement with previously published reports (4,7,9,10). In addition, PR was not found to differ significantly between OA and NOA patients when using fresh sperm, although the rate was significantly lower in NOA patients when using frozen-thawed sperm. Our results indicate that the maximum number of repeated treatments to produce a viable clinical pregnancy could be four.

One advantage of using frozen-thawed testicular tissue for ICSI is that the pattern of spermatogenesis can be evaluated before ovarian manipulation of the female partner is attempted. No further operative intervention is required for the male partner if the couple has to undergo subsequent treatment cycles to achieve a pregnancy. We performed only one biopsy of tissue obtained from an avascular region as a side test to determine the status of spermatogenesis. We have found this to be an adequate indicator of such status, which causes minimal trauma. Repeated testicular biopsies may damage the testicles and result in a significant loss of testicular tissue in patients with small testes.

Of the patients described by Kupker *et al.* (11), 2.9% had azoospermia with a concomitant testicular neoplasm (11,13,14). Although carcinoma was absent in our patients, a precise histological evaluation of the biopsies by a pathologist as well as a sperm check is strongly advised.

In summary, frozen-thawed testicular sperm for ICSI yields acceptable fertilization and pregnancy rates. Freezing the testicular tissue guarantees paternal gametes for subsequent cycles of ovarian manipulation and is advantageous in that only one combined diagnostic and therapeutic testicular biopsy is required. This study found no differences between using fresh or frozen-thawed TESE sperm with regard to rates of fertilization, embryo cleavage, or pregnancy. A clinical pregnancy rate of 29.2% was obtained per ICSI attempt using frozen-thawed testicular sperm extracted from patients with azoospermia, and the cumulative pregnancy rate was found to be 57.1%. However, the pregnancy rate was significantly lower following use of frozen-thawed sperm from NOA patients than from OA patients. Our findings suggest that multiple therapy using frozen-thawed testicular sperm is effective up to until a maximum of four trials. Thus, routine cryopreservation of testicular sperm taken at the time of diagnostic biopsies or during constructive surgery is a convenient and cost-effective option for patients requiring TESE for IVF treatment.

REFERENCES

1. Silber SJ: What forms of male infertility are there left to cure? Hum Reprod 1995;10:503–504
2. Silber SJ, Nagy Z, Liu J, Tournaye H, Lissens W, Ferec C, Liebaers I, Devroey P, van Steirteghem AC: The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: The genetic implications for male infertility. Hum Reprod 1995;10:2031–2043
3. Silber SJ, Nagy Z, Devroey P, Camus M, van Steirteghem AC: The effect of female age and ovarian reserve on pregnancy rate in male infertility: Treatment of azoospermia with sperm retrieval and intracytoplasmic sperm injection. Hum Reprod 1997;12:2693–2700
4. Gil-Salom M, Romero J, Minguer Y, Rubio C, de los Santos MJ, Remohi J, Pellicer A: Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Hum Reprod 1996;11:1309–1313
5. Romero J, Remohi J, Minguez Y, Rubio C, Pellicer A, Gil-Salom M: Fertilization after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Fertil Steril 1996;65:877–879
6. Verheyen G, Nagy Z, Joris H, DeCroo I, Tournaye H, van Steirteghem AC: Quality of frozen-thawed testicular sperm and its preclinical use for intracytoplasmic sperm injection into in vitro-matured germinal-vesicle stage oocytes. Fertil Steril 1997;67:74–80
7. Friedler S, Raziel A, Soffer Y, Strassburger D, Komarovsky D, Ron-El R: Intracytoplasmic injection of fresh and cryopreserved testicular spermatozoa in patients with nonobstructive azoospermia—a comparative study. Fertil Steril 1997;68:892–897
8. Perraguin-Jayot S, Audebert A, Emperaire JC, Parneix I: Ongoing pregnancies after intracytoplasmic injection using cryopreserved testicular spermatozoa. Hum Reprod 1997;12:2706–2709
9. Palermo GD, Schlegel PN, Hariprashad JJ, Ergun B, Mielnik A, Zaninovic N, Lucinda LV, Rosenwaks Z: Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men. Hum Reprod 1999;14: 741–748
10. Habermann H, Seo R, Cieslak J, Niederberger C, Prins GS, Ross L: In vitro fertilization outcomes after intracytoplasmic sperm injection with fresh or frozen-thawed testicular spermatozoa. Fertil Steril 2000;73:955–960
11. Kupker W, Schlegel PN, AI-Hasani S, Fornara P, Johannisson R, Sandmann I, Schill T, Bals-Pratsch M, Ludwig M, Diedrich K: Use of frozen-thawed testicular sperm for intracytoplasmic sperm injection. Fertil Steril 2000;73:453–458
12. Oates RD, Mulhall J, Burgess C, Canningham D, Carson R: Fertilization and pregnancy using intentionally cryopreserved testicular tissue as the sperm source for intracytoplasmic sperm injection in 10 men with non-obstructive azoospermia. Hum Reprod 1997;12:734–739
13. Giwercman A, Berthelsen JG, Muller J, von der Maase H, Skakkebaek NE: Screening for carcinoma in situ of the testis. Int J Androl 1987;10:173–180
14. Novero V, Goossens A, Tournaye H, Silber S, van Steirteghem AC, Devroey P: Seminoma discovered in two males undergoing successful testicular sperm extraction for intracytoplasmic sperm injection. Fertil Steril 1996;65: 1051–1054