A comparison of post-thaw results between embryos arising from intracytoplasmic sperm injection using surgically retrieved or ejaculated spermatozoa

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Objective: To study the effect of freeze–thaw on embryos derived from intracytoplasmic sperm injection (ICSI) using surgically retrieved and ejaculated spermatozoa.
Design: Retrospective study.
Setting: Private IVF center.
Patient(s): Three hundred eighty-three patients undergoing frozen–thawed ET cycles.
Intervention(s): Testicular sperm aspiration (TESA) or percutaneous epididymal sperm aspiration (PESA) were the sperm surgical retrieval methods used for ICSI. Embryos resulting from ICSI using surgically retrieved and ejaculated spermatozoa were frozen, thawed, and transferred.
Main Outcome Measure(s): Post-thaw survival, implantation, and pregnancy rates.
Result(s): No differences were found between the ejaculated sperm and TESA/PESA groups in terms of post-thaw survival rate (68.4% vs. 66.1%, respectively), pregnancy rate (20.1% vs. 16.1%), and implantation rate (10.6% vs. 12.7%). Similar results were found for those variables when comparing TESA and PESA groups.
Conclusion(s): Cleavage embryos arising from ICSI cycles using testicular and epididymal spermatozoa can be frozen with survival, pregnancy, and implantation rates comparable to those obtained with ejaculated spermatozoa. (Fertil Steril® 2009;91:727–32. ©2009 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, ICSI, azoospermia, testicular spermatozoa, epididymal spermatozoa

Approximately 10% of cases of male infertility involve azoospermia (1–3). It is well known that mature germ cells can be retrieved from the epididymis or the testis of azoospermic patients and used to perform intracytoplasmic sperm injection (ICSI). Surgical sperm retrieval followed by ICSI as an option to overcome azoospermia is a well-established method, showing high fertilization and good pregnancy rates, as reported in cases of testicular or epididymal sperm retrieval (4–6). However, the outcome of ICSI using non-ejaculated sperm may be influenced by various factors related to sperm quality and the source of surgically retrieved sperm, such as the rate of chromosomal aneuploidy and other genetic alterations related to spermatozoa DNA damage and genomic imprinting (7, 8). Indeed, the consequences of using sperm retrieved directly from the testis or epididymis are not fully known.

Assisted reproduction generally results in surplus embryos that can be cryopreserved for later use, which allows the storage of embryos for future treatment and consecutive, nonstimulated thaw cycles at low cost (9, 10). Embryo cryopreservation has become indispensable in assisted reproductive technology during the last 2 decades to increase the possibility of clinical pregnancy from a single ovarian stimulation attempt (11, 12). Freezing of surplus embryos also allows reduction of the number of replaced embryos in both fresh and frozen ETs, as well as the effective management of lower multiple gestation rates through the establishment of a successful cryopreservation program (13, 14). In addition, if the woman has a risk of developing ovarian hyperstimulation syndrome, cryopreservation could be applied to all embryos (15, 16).

This study was designed to compare the post-thaw survival, implantation, and pregnancy rates of embryos derived from ICSI using testicular or epididymal sperm with those generated using ejaculated spermatozoa obtained during the same period of study. The question arises as to whether freezing embryos obtained by ICSI using surgically retrieved spermatozoa may be considered generally useful.

MATERIALS AND METHODS
Patients

The study was performed retrospectively with frozen–thawed embryos generated using ejaculated spermatozoa (EJAC group) from 347 patients and frozen–thawed embryos

generated using surgically retrieved spermatozoa from 36 patients undergoing assisted reproductive treatment for azoospermia (testicular sperm aspiration [TESA]/percutaneous epididymal sperm aspiration [PESA] group) from January 2002 to December 2006. Seventeen patients who had frozen–thawed embryos derived from ICSI using testicular sperm were included in the TESA group, and 19 patients who underwent ICSI using epididymal sperm were included in the PESA group. Written informed consent regarding the procedures was obtained from all patients before inclusion in this study. Institutional review board approval was not required because all procedures are routinely performed.

TESA and PESA Procedures

For non-obstructive azoospermia, TESA was the procedure of choice. Percutaneous epididymal sperm aspiration was the first approach in obstructive patients, whereas TESA was used when the former was not effective. All procedures took place in our outpatient procedure room. Testicular sperm aspiration and PESA were both performed under local anesthesia in the spermatic cord. A negative pressure device was gently applied with a 21-gauge butterfly needle connected to a 20-mL plastic syringe for TESA. A similar procedure was performed for PESA, but a 27-gauge needle connected to a 1-mL syringe was used. If the aspiration was unsuccessful, the same procedure was performed on other sites of the testicle or epididymis (in TESA or PESA, respectively). Samples were then transferred to a dish containing human tubal fluid (HTF) buffered medium (HTF modified with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]; Irvine Scientific, Irvine, CA) and assessed for the presence of sperm cells under an inverted Nikon Diaphot microscope (Nikon, Tokyo, Japan) at ×400 magnification. Only motile sperm cells were used for injection.

Stimulation Protocol in Retrieval Cycle

Patients were stimulated with a standard long GnRH (Lupron Kit; Abbott S.A., Societé Française des Laboratoires, Paris, France) agonist protocol. Controlled ovarian stimulation was achieved with human recombinant FSH (Gonal-F; Serono Laboratories, Norwell, MA) in a step-down protocol, until a minimum of two follicles reached an average diameter of 18 mm. Transvaginal oocyte pick-up was performed 34–36 hours after recombinant hCG (Ovidrel; Serono Laboratories) administration. Oocytes were washed in HTF–HEPES medium supplemented with 10% human synthetic albumin (HSA; Irvine Scientific) and incubated in HTF cultured medium (Irvine Scientific) supplemented with 10% HSA under oil (Ovoil; Vitrolife, Kungsbacka, Sweden) for a maturation culture interval of 2 to 3 hours. Oocyte–corona–cumulus complexes were treated with HEPES-buffered medium containing hyaluronidase (80 IU/mL; Irvine Scientific) for 30 seconds. The remaining cumulus cells surrounding each oocyte were then removed with a fine hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, VA) to allow visualization of ooplasm and polar body.

ICSI Procedure

Oocytes in metaphase II were transferred into drops of HTF–HEPES medium covered with oil and placed on a warm plate of an inverted Nikon Diaphot microscope. Intracytoplasmic sperm injection was performed according to the technique described by Palermo et al. (17). Approximately 16 hours after ICSI, fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body. Embryos were kept in a 50-μL drop of HTF medium supplemented with 10% HSA under oil, in a humidified atmosphere of 5% CO_2 in air, at 37°C, until transfer. Excess embryos (6–8 cells) of good quality (blastomeres of equal size and anucleate fragments present in ≤20% of the volume of the embryo) were selected for freezing on post-insemination day 3.

Embryo Freezing and Thawing Procedures

Cryopreservation was carried out with a programmed biological freezing chamber (Cryochamber CL8800; Cryologic, Mulgrave, Victoria, Australia), following a slow-freeze protocol using propanediol (PROH) as a cryoprotectant. Freezing and thawing were performed using the Embryo Freeze and Embryo Thaw media kit as recommended by the manufacturer (Irvine Scientific).

Volumes of 0.5 mL of the freezing solutions 1 and 2 were dispensed into the corresponding wells of a four-well dish (Nunc, Roskilde, Denmark). The dish was allowed to equilibrate to room temperature (25°C). Embryos were first incubated in freezing solution 1 (1.5 mol/L PROH solution) for 10 minutes and then transferred to the freezing solution 2 (1.5 mol/L PROH solution containing 0.1 mol/L sucrose). One to three embryos were then immediately loaded into sterile plastic ministraws (Minitüb Abfüll- und Labortechnik, Tiefenbach, Germany), which were sealed with sterile plugs. Straws were transferred to the freezing machine at a temperature of 20°C. Embryos were then cooled to −7°C at 2°C per minute, at which temperature manual seeding was performed and confirmed by the appearance of a small white speck of ice crystal in the straw. Afterward, embryos were cooled to −35°C at 0.3°C/min, to −43°C at 2°C/min and rapidly frozen until a temperature of −180°C was reached. At this stage, straws were transferred to a marked canister and into a liquid nitrogen container to be kept until thawing.

Volumes of 0.5 mL of the thawing solutions 1, 2, and 3 were dispensed into the corresponding wells of a four-well dish (Nunc) and allowed to equilibrate to room temperature (25°C). Then the straws were quickly removed from liquid nitrogen, exposed to room temperature for 30 seconds, and immersed in a 30°C water bath for approximately 30 seconds until ice crystals in the medium disappeared. After the straw was removed from the bath it was carefully wiped, the plug removed, and the other end cut. Embryos were retraced, placed into the center of the four-well dish, and then transferred immediately into thawing solution 1 (1 mol/L PROH solution containing 0.2 mol/L sucrose). After 5 minutes, embryos

were transferred into thawing solution 2 (0.5 mol/L PROH solution containing 0.2 mol/L sucrose) for 5 minutes and then into thawing solution 3 (0.2 mol/L sucrose) for 10 minutes. After the thawing procedure the embryos were transferred into equilibrated medium, and the state of embryo cleavage and quality were carefully recorded with an inverted Nikon Diaphot microscope. Frozen–thawed embryo survival and integrity were considered when ≥50% or all of the blastomeres showed morphologically normal membranes and clear cytoplasm, respectively. Embryos were cultured in a 50-μL drop of HTF medium supplemented with 10% HSA under oil, in a humidified atmosphere of 5% CO_2 in air, at 37°C, for 2–4 hours before transfer. Assisted hatching was performed with an OCTAX laser (Fertilase; OCTAX Microscience, Herborn, Germany), on all embryos chosen for transfer.

Replacement Preparation

Transfer of frozen–thawed embryos was performed either in a natural or in a down-regulated hormone replacement cycle. The luteal phase was routinely supported with micronized P. Embryos were thawed, cultured for 2–4 hours, and subsequently transferred into the uterus under ultrasound guidance with the use of a soft transfer catheter (Wallace; Smiths Medical International, Hythe, Kent, United Kingdom). One to four embryos were replaced into the uterine cavity.

Serum β-hCG levels were assessed for the first time 12 days after replacement of the embryos. Clinical pregnancy was confirmed by the presence of a gestational sac(s) with or without fetal heartbeat on transvaginal ultrasonography. The clinical pregnancy rate was determined by dividing the number of clinical pregnancies by the total number of thaw cycles transferred. To calculate the implantation rate, the number of gestational sacs was divided by the number of embryos transferred. Miscarriage was defined as spontaneous abortion before 20 weeks' gestation. The number of implantations was underestimated because all the early abortions were counted as single implantations.

Statistical Analysis

Data were expressed as mean $\pm$ SD. Continuous variables were analyzed using Student's t-test. Post-thaw survival, incidence of embryonic loss, embryo implantation, and clinical pregnancy rates were evaluated and group comparison performed using the χ^2 test. Fisher's exact test was used when expected frequency was 5 or less. Differences were considered statistically significant at $P<.05$.

RESULTS
Outcomes of Frozen–Thawed Transfer Cycles After ICSI Using Surgical Retrieval or Ejaculated Spermatozoa

A total of 1,838 frozen embryos from 347 patients in the EJAC group and 186 frozen embryos from 36 patients in the TESA/PESA group were thawed and transferred from January 2002 to December 2006.

No differences were found between the EJAC and TESA/PESA groups in terms of mean maternal age at freezing (32.8 $\pm$ 5.0 years and 32.1 $\pm$ 4.5 years, respectively; $P>.05$) and at freezing–thawing cycle (33.8 $\pm$ 6.2 years and 33.0 $\pm$ 5.7 years, respectively; $P>.05$) or mean number of embryos transferred (2.7 $\pm$ 1.3 and 2.1 $\pm$ 1.3 embryos, respectively; $P>.05$; Table 1).

The comparison of post-thaw results between the EJAC and TESA/PESA groups is shown in Figure 1. Similar results between EJAC and TESA/PESA groups were found for the embryo survival rate (68.4% vs. 66.1%, respectively; $P=.3163$) and rate of intact embryos after thawing (20.1% vs. 15.1%; $P=.1004$). No significant differences were noted between the two groups in terms of clinical pregnancy (20.1% vs. 16.1%; $P=.6241$), miscarriage (24.6% vs. 20.0%; $P=.8570$), and implantation rates (10.6% vs. 12.7%; $P= .8036$) (Fig. 1).

Outcomes of Frozen–Thawed Transfer Cycles After ICSI Using Testicular or Epididymal Sperm Retrieval

Data for 17 patients who had frozen–thawed embryos derived from ICSI using testicular sperm (TESA group) and 19 patients who had used epididymal sperm (PESA group) were evaluated. Mean maternal age, cryopreservation period, and the number of embryos transferred in the TESA and PESA groups are presented in Table 2.

Mean maternal age at freezing (TESA 33.3 $\pm$ 4.9 years, PESA 30.8 $\pm$ 3.9 years; $P>.05$) and at freezing–thawing cycle (34.4 $\pm$ 4.5 years and 31.7 $\pm$ 6.4 years; $P>.05$) did not differ between the TESA and PESA groups. Similarly, no statistically significant differences were observed in the mean number of embryos transferred (TESA 2.3 $\pm$ 1.2, PESA 1.9 $\pm$ 1.5).

The comparison of post-thaw results between TESA and PESA group is shown in Table 3.

Frozen–thawed ET cycles from both TESA and PESA groups showed relatively high results, with similar embryo survival (68.6% vs. 64.0%, respectively; $P>.05$), intact embryos after thawing (11.6% vs. 18.0%, $P>.05$), clinical pregnancy (13.3% vs. 18.8%; $P>.05$), and implantation rates (12.1% vs. 13.3%; $P>.05$). Miscarriage was observed only in the PESA group (33.3%).

DISCUSSION
Frozen–thawed embryo implantation depends on several factors. The age of the patient, the freezing and thawing procedure, the criteria used for cryopreservation, and the cleavage stage of the embryo at the time of freezing may influence the overall results of an embryo freezing program (18, 19). In the present study, no differences in those variables could be observed among the thaw cycles of the analyzed groups.

TABLE 1

Demographic profile of EJAC and TESA/PESA frozen-thawed ET cycles.

| Variable | Study group | | P value |
	EJAC	TESA/PESA	
No. of transfer cycles/no. of frozen-thawed cycles (%)	94.0	86.1	.8307[a]
Maternal age at freezing (y)	32.8 ± 5.0	32.1 ± 4.5	.4431[b]
Maternal age at thaw (y)	33.8 ± 6.2	33.0 ± 5.7	.5401[b]
Cryopreservation period (mo)	8.0 ± 11.9	6.9 ± 11.7	.5611[b]
No. of embryos transferred	2.7 ± 1.3	2.1 ± 1.3	.0772[b]

Note: Values are mean ± SD, unless otherwise noted.
[a] Statistical χ^2 test.
[b] Student's *t*-test.

Figueira. Post-thaw results from ICSI. Fertil Steril 2009.

To our knowledge, the only study that reported the outcome of freeze–thaw cycles from embryos generated after ICSI using surgically retrieved sperm from azoospermic men was performed by Nicopoullos et al. (20), who used a limited number of freeze–thaw cycles (19 cycles) performed in 10 couples. In this experiment the investigators observed only one ectopic pregnancy, demonstrating that clinical pregnancy (5% and 21%, respectively), implantation (0 and 11.5%, respectively), and live birth rates (0 and 21%) were lower in the surgically retrieved group when compared with the ejaculated group.

In the present experiment, with a larger data set, results showed that frozen–thawed embryos arising from ICSI cycles using ejaculated or surgically retrieved spermatozoa derived from azoospermic patients presented similar values for the embryo survival, clinical pregnancy, and implantation rates. A further important consideration in the present study is that, although the quality of surgically collected spermatozoa may differ depending on the origin of sperm, the post-thaw results were similar when the TESA/PESA and EJAC groups were compared.

During cryopreservation, cells are exposed to mechanical, thermal, and chemical stresses, which in turn can lead to compromised cell function and cell death (21). Data from the present study may suggest that deleterious effects of cryopreservation on embryonic cellular functions, which compromise the ability to develop normally after the freeze–thaw process, were independent of the sperm origin.

Viability of each embryo transferred depends on the biological quality of the spermatozoon and oocyte. Paternal effects on preimplantation embryo development have been shown to be responsible for repeated failures of assisted reproduction attempts (8, 22). Previous results demonstrated that infertile male patients produce cytogenetically abnormal spermatozoa, despite a normal somatic karyotype, as a result of an altered intratesticular environment that negatively af-

fects the mechanisms controlling chromosome segregation during cell division. Azoospermic men have a significantly higher rate of meiotic chromosomes abnormalities and aneuploidy in the spermatozoa compared with ejaculated spermatozoa (7, 23, 24).

Although an increased sperm aneuploidy rate could negatively affect the clinical outcome of assisted reproduction, our data show no relationship between sperm maturity and

FIGURE 1

A comparison of post-thaw results between cryopreserved embryos generated using testicular or epididymal spermatozoa and those generated using ejaculated spermatozoa.
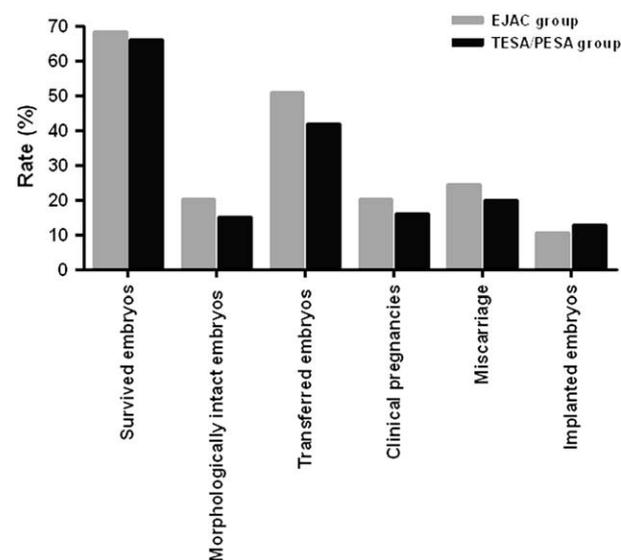
Figueira. Post-thaw results from ICSI. Fertil Steril 2009.

TABLE 2

Demographic profile of TESA and PESA frozen-thawed ET cycles.

| | Study group | | |
Variable	TESA	PESA	*P* value
No. of frozen-thawed cycles	17	19	
No. of transfer cycles/no. of frozen-thawed cycles (%)	88.2	84.2	.7274[a]
Maternal age at freezing (y)	33.3 ± 4.9	30.8 ± 3.9	.0949[b]
Maternal age at thaw (y)	34.4 ± 4.5	31.7 ± 6.4	.0949[b]
Cryopreservation period (mo)	6.8 ± 11.3	7.0 ± 12.3	.8779[b]
No. of embryos transferred	2.3 ± 1.2	1.9 ± 1.5	.4559[b]

Note: Values are mean ± SD, unless otherwise noted.
[a] Statistical χ^2 test.
[b] Student's *t*-test.

Figueira. Post-thaw results from ICSI. Fertil Steril 2009.

post-thaw outcomes. The immaturity of spermatozoa may affect embryo development (25, 26), resulting in a lower number of good-quality embryos selected for cryopreservation. However, because those embryos were selected for cryopreservation, indicating a high development rate in in-vitro conditions, such embryos may provide good results in a future freeze–thaw cycle.

Although high preclinical abortion rates after transfer of ICSI embryos generated from testicular sperm retrieval have been reported (5, 6, 27), in our study we observed only one miscarriage, in the PESA group. Further studies, including a large casuistic, are required to confirm those results.

In conclusion, this is the first report demonstrating that the outcome of frozen–thawed cycles was not dependent on the origin of sperm. These findings indicate that embryos obtained from ICSI cycles using testicular or epididymal spermatozoa from azoospermic patients survive cryopreservation in a range similar to those generated from ejaculated sperm.

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TABLE 3

Outcomes of frozen-thawed transfer cycles after ICSI using testicular or epididymal spermatozoa.

| | Study group | | |
Variable	TESA	PESA	*P* value
Embryos that survived post-thaw/no. of embryos thawed	59/86 (68.6)	64/100 (64.0)	.5082[a]
Morphologically intact embryos after thawing/embryos thawed	10/86 (11.6)	18/100 (18.0)	.2256[a]
Clinical pregnancies/cycles transferred	2/15 (13.3)	3/16 (18.8)	.5324[b]
Implanted embryos/embryos transferred	4/33 (12.1)	4/30 (13.3)	.5901[b]

Note: Values are number (percentage).
[a] Statistical χ^2 test.
[b] Fisher exact test.

Figueira. Post-thaw results from ICSI. Fertil Steril 2009.

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