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Effect on Clinical Outcome of the Interval Between Collection of Epididymal and Testicular Spermatozoa and Intracytoplasmic Sperm Injection in Obstructive Azoospermia

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ABSTRACT: We wished to determine whether the interval between surgical retrieval of epididymal and testicular spermatozoa in obstructive azoospermia and their subsequent use in intracytoplasmic sperm injection (ICSI) has an effect on their fertilizing capacity and pregnancy rates in patients undergoing ICSI. This was a retrospective review of 164 consecutive cycles of ICSI in partners of men undergoing surgical sperm retrieval for obstructive azoospermia. Seventy-three cycles used fresh testicular spermatozoa; in 35 cycles ICSI was performed within 4 hours of sperm retrieval, and in 38 cycles spermatozoa were incubated overnight before ICSI. Epididymal spermatozoa were used in 29 cycles; 22 cases within 4 hours of retrieval and 7 cases following overnight culture. Cyropreserved testicular and epididymal spermatozoa were used in 42 and 20 ICSI cycles, respectively. Fertilization and clinical pregnancy rates were calculated for each treatment group. Fertilization rates for epididymal spermatozoa were 67% at 4 hours, 56% at 24 hours, and 63% for cryopreserved spermatozoa (P = .52). Fertilization rates for testicular spermatozoa were 63% at 4 hours, 71% at 24 hours, and 60% for cryopreserved spermatozoa (P = .16). Unlike testicular spermatozoa, cryopreserved epididymal spermatozoa showed a significant increase in clinical pregnancy rates with cryopreservation, with rates of 4 of 22, 1 of 7, and 10 of 20 at 4 hours, 24 hours, and cryopreservation, respectively (P = .049). This study confirms that fertilization and pregnancy rates following ICSI with motile spermatozoa are unaffected by the duration between surgical retrieval of spermatozoa and their injection into oocytes. It also demonstrates that of all treatment modalities, the use of frozen epididymal spermatozoa was associated with the greatest pregnancy rates.

Key words: ICSI, fertilization, pregnancy, surgical retrieval.

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Prior to the development of ICSI in 1993 (Van Steirteghem et al, 1993), reported pregnancies for couples undergoing in vitro fertilization (IVF) treated with surgically retrieved epididymal spermatozoa were isolated and infrequent, with the first reported pregnancy using epididymal spermatozoa reported in 1985 (Temple-Smith et al, 1995). Although fertilization was reported with testicular spermatozoa using standard IVF, no pregnancies were reported (Hirsh et al, 1993). Most couples in which the man was azoospermic due to obstruction could be offered treatment only with donated spermatozoa, or they underwent IVF with poor yields of embryos for transfer. Fertility treatment options expanded rapidly following the widespread introduction of intracytoplasmic sperm injection (ICSI) with the first pregnancy using testicular spermatozoa reported more than 7 years ago (Craft et al, 1995). ICSI using epididymal and testicular spermatozoa has now become a common procedure for patients with azoospermia in many assisted conception units (Schoysman et al, 1993; Devroey et al, 1995; Silber et al, 1995).

Many centers have also begun to use cryopreserved, surgically retrieved spermatozoa in addition to freshly retrieved sperm. The clinical benefits to both partners are clearly evident from spermatozoal cryopreservation, in terms of a reduction in the number of surgical sperm retrievals (Oates et al, 1996; Nudell et al, 1998) as well as reducing the logistical complications in arranging concurrent oocyte and spermatozoal retrievals (Oates et al, 1996).

There is little evidence to suggest that a difference exists in fertilization, cleavage, and implantation rates or pregnancy outcome, regardless of the use of cryopreserved rather than fresh spermatozoa retrieved from the epididymis (Nagy et al, 1995; Oates et al, 1996; Cha et al, 1997; Friedler et al, 1998; Janzen et al, 2000; Cayan et al, 2001). In fact, some clinicians advocate the use of only cryopreserved epididymal spermatozoa (Oates et al, 1996). The efficacy of using cryopreserved spermatozoa extracted from the testis is slightly more questionable.

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Although fertilization rates for cryopreserved spermatozoa extracted from the testis remain high, some studies have reported a small but significant reduction in both fertilization and implantation rates, with a corresponding reduced live birth rate (Gil-Salom et al, 1996; De Croo et al, 1998). Other studies have shown no difference (Friedler et al, 1997).

There is, however, a considerable lack of evidence for the optimum time between retrieval of spermatozoa and ICSI, when freshly retrieved epididymal or testicular spermatozoa are used in a concurrent ICSI cycle.

One large study that examined motility characteristics of fresh and cryopreserved spermatozoa suggested that epididymal spermatozoa demonstrated a gradual decrease in progressive motility for up to 5 days before all spermatozoa were immotile, whereas testicular spermatozoa could be cultured for up to 1 week before all motility was lost (Edirisinge et al, 1996). Cryopreserved spermatozoa gained little motility over 2–3 days after thawing before motility declined. A small study by Liu et al (1997) noted a small improvement in both morphology and motility in spermatozoa from patients with obstructive azoospermia after culture, reaching a peak at 72 hours. However nonmotile spermatozoa did not gain motility in this study. In a further study by Angelopoulos et al (1999), spermatozoa motility peaked 48 hours after culture, whereas vitality decreased linearly from 56% at collection to 18% at 96 hours.

In a large prospective study of 143 patients with obstructive azoospermia undergoing testicular sperm retrieval 24 hours prior to ICSI, if the spermatozoa were cultured in the presence of recombinant FSH, a significantly greater number of oocytes were injected with motile spermatozoa, and hence fertilization and pregnancy rates were greater (Balaban et al, 1999). In a small study of 20 patients by Hu et al (1999), evidence suggested that spermatozoal motility improved after culture. Finally, in a study of 87 patients undergoing ICSI after successful percutaneous epididymal sperm aspiration (PESA), or testicular sperm aspiration (TESA) or extraction (TESE) performed approximately 40 hours before the administration of human chorionic gonadotropin (hCG), then satisfactory fertilization and implantation rates per embryo of 53% and 15.7%, respectively were achieved (Urman et al, 1998).

In a further retrospective study of 47 cycles of treatment with testicular sperm from patients with nonobstructive azoospermia, no differences were noted in fertilization or clinical pregnancy rates between spermatozoa retrieved on the day of ovum pickup or 24 hours previous (Levran et al, 2001).

In this study we wished to assess the effect on fertilization and pregnancy rates of using epididymal and testicular spermatozoa within 4 hours of retrieval, following overnight culture or following cryopreservation.

Materials and Methods

Patient Population

During a 2-year period, 162 surgical sperm retrievals were performed either using PESA or, if unsuccessful, TESE. Of these, 41 were performed electively and any harvested spermatozoa were cryopreserved. One-hundred twenty retrievals were performed for obstructive azoospermia and the retrieval rate for motile spermatozoa was 100%; 33 of 120 (27%) were retrieved from the epididymis, and the remainder were from the testis. Failed epididymal retrieval was usually associated with either nonpalpable epididymii or the presence of cystic changes. Forty-two TESE procedures were performed for nonobstructive azoospermia, as diagnosed by previous biopsy, raised FSH level (>18 IU/L), and testicular size (<10 cm^3). Of these, 18 were successful in retrieving motile spermatozoa (43%).

From the spermatozoa retrieved from men with obstructive azoospermia, 164 cycles of ICSI were performed, of which 73 involved the use of fresh testicular spermatozoa and 29 used fresh epididymal spermatozoa. Sixty-two cycles used cryopreserved spermatozoa (20 cycles used epididymal spermatozoa, 42 cycles used testicular spermatozoa.)

Surgical sperm retrievals were performed on a fixed operating day and the female partners underwent controlled ovarian hyperstimulation using the standard long regimen so that oocyte retrieval could be performed either on the same day as sperm retrieval or the following day. In patients for whom cryopreserved spermatozoa were used, either excess spermatozoa cryopreserved from a previous fresh cycle were used, or sperm were electively harvested before controlled ovarian stimulation (COH).

Sperm Retrieval Methods

Surgical sperm retrieval was performed under general anesthesia on a fixed day (day 12 of ovarian stimulation). PESA was performed using the method first described by Craft et al in 1993. The epididymis was identified and secured between the thumb and index finger, and the remaining fingers and palm were used to cup and stabilize the testicle. Epididymal fluid was aspirated blindly from the epididymis via percutaneous puncture of the epididymis with a 19-gauge needle with suction obtained using a 20-mL syringe. The procedure was performed under general anesthesia and the epididymal fluid was aspirated and immediately examined under ×400 brightfield microscopy for the presence of motile spermatozoa. If no motile spermatozoa were observed the patient underwent immediate TESE while still under general anesthesia. TESE was chosen over microsurgical epididymal sperm aspiration (MESA) because it is a simpler and quicker procedure, it is well tolerated, and has few side effects. Although MESA is associated with retrieval of larger quantities of epididymal spermatozoa than PESA, the requirement for enough spermatozoa to treat patients in up to 20 ICSI cycles (Silber, 1996) is limited. With most data suggesting that the use of mature testicular spermatozoa is not associated with any reduction in clinical outcome compared with epididymal spermatozoa, many clinicians believe that the much simpler testicular sperm extraction or aspiration techniques are more cost-effective options than MESA.

TESE was performed as an open biopsy in which a small

incision was made in the scrotal skin and the layers of tissue were opened through the spermatic fascia and the tunica vaginalis to the tunica albuginea. After opening the tunica albuginea, seminiferous tubules were removed. In order to extract usable spermatozoa from the tubules the tissue was minced and the tubes were ''milked'' with sterile needles (Tucker et al, 1995) to achieve the mechanical isolation of spermatozoa for ICSI.

Oocyte Stimulation and Retrieval, ICSI, and Embryo Transfer

Female patients underwent a standard, long-protocol pituitary desensitization with a gonadotropin hormone-releasing agonist (Suprecur, Hoechst Marion Roussel, Frankfurt, Germany). COH began on a fixed day with human menopausal gonadotropin hMG (Menogon, Ferring GmBh, Kiel, Germany). The cycle was monitored via ultrasound after 10 days of stimulation. For patients who achieved three follicles or more of >16 mm, oocyte retrieval was arranged for day 12 or 13 depending upon the size of the lead follicle. Oocyte retrieval was performed under sedation anesthesia. Retrieved oocytes were processed in the laboratory in the manner described by Hillier et al (1984) and cultured in P1 medium (Irvine Scientific, Santa Ana, Calif) supplemented with 10% v/v SSS (Irvine Scientific) in an atmosphere of 5% CO_2 in air at 37°C. After approximately 4 hours of incubation the oocytes were mechanically denuded of their cumulus and coronal cells following brief exposure to hyaluronidase (Hyase, IVF Science Scandinavia, Goteborg, Sweden).

Epididymal spermatozoa were washed by centrifugation at 600 × *g* in P1 medium for 10 minutes, followed by resuspension in 1 mL of fresh medium. Testicular spermatozoa extracted from the seminiferous tubules were washed by centrifugation at 600 × *g* in P1 medium for 10 minutes followed by resuspension in 3 mL of fresh medium. If required, the following day the sample was cultured overnight at 37°C in an atmosphere of 5% CO_2 in air and observed for motile spermatozoa the following morning. Only motile spermatozoa with normal morphology were used for ICSI, and these were transferred to PVP (ICSI-100, IVF Science Scandinavia) for immobilization before injection. Injected oocytes were cultured in P1 medium and examined for the presence of pronuclei approximately 18 hours following ICSI.

A maximum of three embryos were transferred into the uterus approximately 2 days after ICSI. Embryo quality was assessed by standard morphological appearances with light microscopy (Scott et al, 1991). Successful implantation was determined by positive urinary β-hCG and a clinical pregnancy by the presence of a fetal heartbeat during vaginal scan 4–6 weeks following embryo transfer.

Data on the number of oocytes collected, injected, fertilized, and cleaved were recorded for each cycle, as were the number of high-quality embryos available for transfer and cryopreservation, and pregnancy outcomes.

Cryopreservation and Thawing

Excess epididymal and testicular spermatozoa not used for ICSI were cryopreserved (using SpermFreeze, FertiPro N.V.) in 0.5-mL straws using a slow-freeze method (Mahedevan et al, 1993). Straws were thawed rapidly at 37°C for 10 minutes. Cryoprotectant was removed with the dropwise addition of P1 medium followed by centrifugation at 600 × *g* for 10 minutes and resuspension in 0.5 mL of fresh medium.

Statistical Analysis

Statistical analyses were performed using independent *t* tests, ANOVA, and chi-square tests to assess differences between the results achieved using epididymal and testicular spermatozoa. To compare fertilization rates between groups in which spermatozoa were used within 4 hours, sperm cultured overnight or with cryopreservation, we used ANOVA with a Tukey post-hoc analysis. To compare the demographics of age, FSH levels in women, and metaphase II oocytes available for ICSI, we used independent *t* tests. Wilcoxon tests were used for nonparametric data, including assessment of embryo cleavage rates and number of grade I embryos available for transfer or freezing. Chi-squares were used to detect differences in pregnancy rates. Results were considered significant at $P < .05$.

Results

In the cycles using fresh epididymal spermatozoa, 22 patients underwent ICSI within 4 hours of PESA and 7 underwent ICSI between 24 and 28 hours later. Of the 73 patients undergoing ICSI with testicular spermatozoa, 35 procedures were performed within 4 hours of spermatozoa collection, and 38 underwent ICSI between 24 and 28 hours of TESE.

There was no significant difference between the ages of men and women, number of metaphase II oocytes available for injection or fertilization, and cleavage rates between fresh, overnight cultured, and cryopreserved epididymal or testicular spermatozoa. There were also no significant differences in the number of high-quality (grade I) embryos available for transfer or cryopreservation, number of embryos transferred, or clinical pregnancy rate, when fresh or frozen spermatozoal retrieval from the epididymis or testicle were compared (Table 1).

Most ICSI treatments with fresh epididymal spermatozoa were performed within 4 hours of collection. However, if motile spermatozoa were used, the following day (24–28 hours after collection) there was a small but nonsignificant decrease in fertilization rates from 67.4% to 55.9% ($P = .23$), but a small, nonsignificant increase in the number of grade I embryos were available from 3.4 to 4.9, and there was no significant effect on clinical pregnancy rates (Table 2). Good fertilization rates were achieved with cryopreserved epididymal spermatozoa compared with those when fresh spermatozoa were used, and this was associated with the greatest pregnancy rate for all modes of treatment. There was a significant increase in pregnancy rates of 50% compared with 17%; $P = .049$, compared with freshly retrieved epididymal spermatozoa (Table 2).

When motile retrieved testicular spermatozoa were

Table 1. *Results of ICSI using epididymal, testicular, and cryopreserved spermatozoa*

	Epididymal Sperm	Testicular Sperm	Frozen-Thawed Epididymal Sperm	Frozen-Thawed Testicular Sperm	*P* Value*
Number of ICSI cycles	29	73	20	42	
Average age of men (years)	38.4	41.9	42.9	39.4	NS
Range	29–56	27–61	26–58	28–62	.62
Average age of women (years)	32.2	32.9	33.4	33.0	NS
Range	23–45	20–42	22–43	22–42	.42
Number oocytes injected/cycle	8.2	8.9	11.0	8.9	NS
Mean ± SD	4.5	5.3	4.3	4.96	.76
Fertilization rate (%)	67.6	65.6	55.9	59.9	NS
Mean ± SD	27.5	23.2	20.5	25.6	.28
Cleavage rate (%)	92.0	96.0	92.9	97.0	NS
Mean ± SD	18.7	12.4	18.9	13.4	.26
Number of grade 1 embryos	3.6	4.0	4.9	4.11	NS
Mean ± SD	2.8	3.1	3.9	3.2	.83
Number of embryos transferred	2.1	2.2	1.95	1.97	NS
Mean ± SD	0.7	0.6	0.4	0.6	.89
Clinical pregnancy rate	5/29	17/73	10/20	6/42	
	17%	23%	50%	14%	.049

* NS indicates not significant.

used, an even distribution of patients underwent ICSI with spermatozoa retrieved within 4 hours, with spermatozoa retrieved between 24–28 hours, and with cryopreserved spermatozoa. There was a small but nonsignificant increase in fertilization rates with spermatozoa used after culture for 24–28 hours, and no significant differences in terms of cleavage rates, grade I embryos, or clinical pregnancy rates whether the spermatozoa were used within 4 hours, 24–28 hours, or after cryopreservation(Table 3). The reduction in pregnancy rates between fresh and frozen testicular spermatozoa was 24% (17 of 72) to 14% (6 of 42), respectively (P = .334) and was not statistically significant.

The data collected on obstetric outcome of these pregnancies showed no evidence of abnormalities, gestation, or birth weights.

Discussion

As long as motile spermatozoa are used in ICSI, there appears to be no optimum time for oocyte injection with surgically retrieved spermatozoa. It has been established that testicular spermatozoa may retain their motility for up to 6 days and epididymal spermatozoa remain motile and viable for up to 5 days (Edirisinghe et al, 1996; Angelopolous et al, 1999). This would lead us to suggest that good results in fertilization can be expected at any time within these time periods. The improvements demonstrated in the percentage of motile spermatozoa in a specimen of testicular spermatozoa following in vitro culture are clear (Edirisinghe et al, 1996; Liu et al, 1997; Angelopolous et al, 1999; Hu et al, 1999). Indeed, it has

Table 2. *Results of ICSI with epididymal sperm within 4 or 24–48 hours of collection and cryopreservation*

	Epididymal Spermatozoa <4 Hours	Epididymal Sperm 24–48 Hours	Frozen-Thawed Epididymal Spermatozoa	*P* Value
ICSI cycles	22	7	20	
Age of women	33.2	33.0	33.4	NS
Mean ± SD	4.5	5.2	3.3	.98
Oocytes injected	8.1	11	8.2	NS
Mean ± SD	5.6	4.3	3.9	.36
Fertilization rate (%)	67.4	55.9	62.9	NS
Mean ± SD	23.6	20.5	24.6	.518
Cleavage rate (%)	96.9	92.9	94.0	NS
Mean ± SD	10.1	18.9	16.9	.74
Grade 1 embryos	3.4	4.9	3.9	NS
Mean ± SD	3.2	3.9	3.3	.53
Embryos transferred per cycle	2.1	2.3	1.9	NS
	0.57	0.77	0.42	.32
Clinical pregnancy rate	4/22	1/7	10/20	.049
	18%	14%	50%	

Table 3. *Results of ICSI with testicular sperm within 4 or 24–28 hours of collection and cryopreservation*

	Fresh Testicular Spermatozoa <4 Hours	Testicular Sperm 24–48 Hours	Frozen-Thawed Testicular Spermatozoa	*P* Value
ICSI cycles	35	38	42	
Age of women	31.6	32.4	33.0	NS
Mean ± SD	4.3	4.2	5.3	.46
Oocytes injected	8.9	7.5	8.9	NS
Mean ± SD	4.0	4.9	4.9	.29
Fertilization rate (%)	62.3	71.1	59.9	NS
Mean ± SD	26.2	26.7	26.5	.159
Cleavage rate (%)	94	92	97	NS
Mean ± SD	13.2	21.5	11.2	.25
Grade 1 embryos range	3.8	3.6	4.11	NS
Mean ± SD	2.5	2.2	2.6	.26
Embryos transferred per cycle	1.94	1.7	1.97	NS
Mean ± SD	0.4	0.5	0.6	.26
Clinical pregnancy rate	8/35	9/38	6/42	NS
	23%	24%	14%	.49

been suggested that the immobility of testicular spermatozoa may be a physiological rather than a pathological process resulting from immaturity or attachment to Sertoli cells, and that extended culture may allow spermatozoa to mature and detach from Sertoli cells (Jow et al, 1993). This is, however, unlikely to affect the overall clinical outcome as long as ICSI is performed only with motile spermatozoa. Thus, although culture of epididymal and especially testicular sperm may increase the number of motile sperm for injection, it is likely that if motile spermatozoa are seen immediately, there will be sufficient numbers for ICSI. There is also evidence to suggest that motility is not gained in samples that are completely immotile when they are initially collected (Liu et al, 1997), and therefore, culture is unlikely to effect this outcome.

Fertilization success achieved with cryopreserved spermatozoa is also significant in offering couples greater ease in planning their treatment by electively collecting spermatozoa before a woman undergoes ovarian stimulation and oocyte collection. Indeed, some proponents suggest that this is the optimum form of treatment (Oates et al, 1996) and should be used exclusively.

Of interest is a significant improvement in clinical pregnancy rates with the use of cryopreserved epididymal spermatozoa. This improvement in pregnancy rates does not occur when cryopreserved testicular spermatozoa are used. The reason for this is not known, but we do know that epididymal spermatozoa show higher rates of DNA damage (Steele et al, 1999) presumably due to the heterologous population of spermatozoa that accumulate and are stored within the epididymis. It could therefore be postulated that these damaged spermatozoa may lose motility or even viability in the cryopreservation process, and thus motile spermatozoa after thawing may exhibit less damage or even lower incidences of aneuploidy. Further studies are needed to assess whether motile spermatozoa after cryopreservation differ from those in the precryopreservation population.

Motile testicular spermatozoa have less DNA damage (Steele et al, 1999) and this may be why there is no significant difference in pregnancy rates when either fresh or frozen thawed testicular spermatozoa are used.

Previous papers have already stated the benefit to couples undergoing treatment and clinicians who care for them in advancing the sperm retrieval process up to and even before women receive hCG (Levran et al, 2001). These benefits include increasing the time available for embryological staff to search for motile spermatozoa, better planning of operating room timetables, and allowing the couple more time to decide whether they wish to proceed with donor treatment if no spermatozoa are found.

Even though the benefits of establishing an optimal timing for using freshly retrieved spermatozoa are clear, the case for a large prospective trial would be difficult to justify given the obvious benefits to both patients and clinicians in using cryopreserved spermatozoa. This study, like others (Nagy et al, 1995; Oates et al, 1996; Cha et al, 1997; Friedler et al, 1998, Janzen et al, 2000; Cayan et al, 20001) has shown no decrease in fertilization rates, embryo cleavage rates, grade 1 embryos, or clinical pregnancy rates when using frozen thawed spermatozoa. Indeed, this study is the first to have shown an improvement in clinical pregnancy rates when using cryopreserved epididymal spermatozoa.

From this study we would suggest that the major benefit from culturing retrieved spermatozoa, particularly testicular spermatozoa, is to not alter fertilization or pregnancy rates, but to optimize the number of motile spermatozoa from the sample for cryopreservation for use in future cycles. Indeed, a recent paper has suggested that testicular spermatozoa recovered for obstructive azoospermia and cultured for 72 hours showed both a signif-

icant increase in motility (20% vs 53%) and a significant increase in spermatozoa with double-stranded DNA (46% vs 80%). This significantly increased the number of spermatozoa available for cryopreservation, and more significantly, it increased the subsequent postthaw survival rate (Emiliani et al, 2001). This pattern was not seen in patients with nonobstructive azoospermia.

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