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Frozen Embryos Generated from Surgically Retrieved Sperm from Azoospermic Men: Are They Clinically Viable?

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***Purpose*:** To assess the viability of frozen-thawed embryos derived from intracytoplasmic sperm injection (ICSI) in azoospermic men.
***Methods*:** Retrospective analysis of 154 consecutive ICSI cycles using surgically retrieved sperm from azoospermic men and case-control comparison of subsequent frozen transfer cycles with those using embryos generated from ejaculated sperm.
***Results*:** Patient and fresh cycle characteristics were similar in both groups. There were no differences between the two groups in the proportion of pronucleate (54% and 62%), and cleavage-stage embryos thawed (46% and 38%), post-thaw survival rates (retrievals: 69%; ejaculated: 73%) or quality of frozen embryos subsequently transferred. Implantation was significantly lower in frozen cycles where embryos were generated from surgically retrieved sperm (0% versus 11.5%; $p = 0.03$). Both clinical pregnancy rate (5% versus 21%) and live-birth rate (0% versus 21%) were lower in this group, but only the difference in LBR reached borderline statistical difference ($p = 0.10$).
***Conclusion*:** This small series demonstrates a significant impairment in implantation in FET cycles using embryos generated from surgically retrieved sperm and a trend towards a poorer pregnancy outcome.

KEY WORDS: Azoospermia; cryopreservation; frozen embryo cycles; ICSI; surgical sperm retrieval.

INTRODUCTION

Ten years ago, the use of donor sperm was the only option offering a realistic chance of parenting for severely oligoasthenoteratospermic or azoospermic men. The introduction of intracytoplasmic sperm injection (ICSI) by the work of Van Steirteghem's group in Brussels in 1992 revolutionised the management of male factor fertility, with Palermo *et al.* (1) reporting the first pregnancies and livebirths after ICSI in four women who had not benefited from IVF or subzonal sperm injection (SUZI). The ability of ICSI to achieve high fertilisation and pregnancy rates regardless of semen parameters (2) led to its application in azoospermic patients.

Studies have reported significantly lower fertilisation with the use of surgically retrieved sperm in comparison to ejaculated samples (ejaculated: 70%; epididymal: 56%; testicular: 48%) (3–5). Although implantation (ejaculated: 26%; testicular:13%) (6) and pregnancy outcome (ejacu1ated: 21%; epididymal: 22%: testicular: 10%) (7) have been reported to be significantly lower, this was found only in cases where sperm was obtained from non-obstructive

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azoospermic men. In studies of only men with non-obstructive azoospermia, impaired fertilisation has been confirmed (ejaculated: 56.9%; non-obstructive: 37.7%) (8).

Overall, the majority of large series of ICSI cycles from azoospermic men, using both fresh and cryopreserved sperm and from either obstructive or non-obstructive cases of azoospermia have reported excellent fertilisation (56–81%) and clinical pregnancy outcome (24–37% per embryo transfer) (9–15).

Embryo cryopreservation is now a well-established technique that allows the storage of supernumerary embryos created during assisted reproductive techniques (ART). Although an early report raised concerns that pregnancies from cryopreserved-thawed embryos derived from ICSI may carry an increased risk of pregnancy loss (16), later studies showed similar pregnancy (16–32% and 16–52%) and delivery rates (15–27% and 20–52%) in IVF and ICSI derived embryos respectively (17–21). However, there is little in the literature assessing the outcome of FET cycles from embryos generated following ICSI using surgically retrieved sperm from azoospermic men. In the only study that reported fresh and FET cycles from a cohort of patients with non-obstructive azoospermia (22), they reported clinical pregnancy and implantation rates of 36% and 13% respectively and post-thaw survival, clinical pregnancy and implantation rates of 68%, 24% and 8% respectively in the 17 FET cycles.

The aims of this retrospective analysis was (i) to present the results of a series FET cycles from embryos derived from ICSI cycles in azoospermic men and (ii) to compare these results with those from a matched control group.

MATERIALS AND METHODS

This retrospective analysis involved 154 consecutive ICSI cycles performed between November 1996 and July 2002 (from 91 couples) using surgically retrieved sperm in patients with either obstructive or non-obstructive azoospermia. From these cycles, good-quality supernumerary embryos were cryopreserved at the pronucleate or cleavage stage. FET cycles were subsequently performed at the request of the couples (Group 1).

An age-matched and embryo-quality (at embryo transfer) matched group was selected from all frozen embryo transfer cycles performed that followed fresh ICSI cycles using ejaculated sperm in our unit over a similar time period (Group 2).

Sperm Retrieval and Assisted Reproduction

In all cases, surgical sperm retrieval was performed by the same surgeon. Epididymal sperm was retrieved by microsurgical sperm aspiration (MESA) under general anaesthesia. The samples were prepared in the IVF laboratory by washing in 1–2 mL of culture medium. The suspension was centrifuged at 1000 rpm for 10 min and the pellet redispersed in 50–100 μL of culture medium. Presence of motile sperm was assessed under an inverted microscope ($\times$400 magnification, Hoffman modulation). The prepared samples were left in the incubator, set at 37° in an atmosphere of 5% CO_2 in air, until use. In cases where no motile sperm were found in the MESA sample, a testicular biopsy was performed (TESE). The tubules were minced using two insulin-needles and sterile glass slides, and sperm suspension prepared as described above. Epididymal or testicular sperm suspension was pipetted into microdrops of culture medium under paraffin oil. Under the inverted microscope, a motile sperm was immobilised using a microinjecting pipette and placed into polyvinylpyrolidone drop (PVP; Medicult, U.K.) prior to ICSI.

For fresh ejaculated semen, samples were allowed to liquefy at room temperature for approximately 20 min. The samples were either washed with culture media as described earlier or a two-layer density gradient (45% and 90%; Puresperm, Nicadon, Sweden) was used. Following centrifugation at 1000 rpm for 20 min, the pellet was washed twice in culture medium and the final pellet resuspended in approximately 100 μL of medium.

For retrieved sperm, surplus spermatozoa were cryopreserved for future ICSI attempts, thus avoiding the need for repeated microsurgery. The sperm suspension was diluted with an equal volume of sperm cryopreservation medium (Medicult, U.K.), aliquoted into cryovials and suspended in liquid nitrogen vapour for 30 min before being submerged in liquid nitrogen for storage. On the morning of oocyte retrieval, a vial of frozen sample was thawed at room temperature for 20 min. Samples were subsequently prepared as described earlier.

Oocyte retrieval was performed after pituitary desensitisation with gonadotrophin-releasing hormone agonists (long protocol) and ovulation stimulation with gonadotrophins. The oocytes were stripped

of surrounding cumulus cells using hyaluronidase (Medicult, U.K.) and ICSI performed between 38–42 h post human chorionic gonadotrophin (hCG) injection. Fertilisation was assessed 16–18 h post ICSI, and top quality embryos were subsequently transferred on either Day 2 (cleaved to the 2- to 4-cell stage by 42–49 h post injection) or Day 3 (cleaved to the 5- to 8-cell stage by 64–72 h post injection) post oocyte retrieval. Luteal support was administered in the form of progesterone pessaries (800 mg/day; Cyclogest®; Shire Pharmaceuticals, Basingstoke, U.K.) from the day of transfer and continued until the twelfth week of pregnancy where appropriate.

Embryo Cryopreservation

Embryos were cryopreserved at the pronucleate or cleavage stage (2- to 8-cell), in 1,2-propanediol (PROH) and sucrose as the cryoprotectants using the slow cooling method. The medium for preparation of the cryoprotectant and the sucrose solutions is phosphate-buffered saline (PBS) containing 20% human serum albumin (HSA). Embryos were incubated at room temperature in a mixture of 1.5 M PROH and HSA for 10 min before being transferred to a solution containing 1.5 M PROH, 0.1 M sucrose and HSA, and loaded into straws. Cooling was performed using a biological programmable cell freezer (Kryo 10 Series II, Planer Products Ltd) with liquid nitrogen vapour. Embryos were cooled initially to −7°C at a rate of −2°C/min, where seeding was performed using forceps, after which they were cooled to −30°C at a rate of −0.3°C/min, then to −150°C at a rate of −10°C/min. The straws were finally plunged into liquid nitrogen at −196°C for storage.

Embryo Thawing

Embryos were thawed using the rapid thaw method by a four-step process to remove the cryoprotectants; pronucleate and cleavage stage embryos were thawed one day prior to and same day of FET respectively. Straws were removed from liquid nitrogen and incubated at room temperature for 40 s before incubating for 5 min in thawing media containing a mixture of PROH, sucrose and HSA. Thawed embryos were subsequently washed and rehydrated in pre-equilibrated culture media and incubated for either 24 h (for pronucleate stage embryos) or 3–4 h (for cleavage stage embryos) at 37°C in an atmosphere of 5% CO_2 in air prior to FET.

Pregnancy was detected using urinary or serum beta HCG tests and clinical pregnancy determined by observation of a gestational sac at a six-week ultrasound scan. Implantation rate was defined as the number of gestation sacs observed divided by the number of embryos transferred.

Statistical analysis was performed using Fisher's exact test and Mann-Whitney U tests.

RESULTS

Of the 154 ICSI cycles commenced, 64 used fresh sperm and 90 cryopreserved sperm. 127 used sperm retrieved from the epididymis and 27 from the testicle. The aetiology of azoospermia was a combination of obstructive (127 cycles) and non-obstructive azoospermia (25 cycles) with insufficient data to correctly classify a further two cycles.

137 of 154 cycles proceeded to embryo transfer (89%). From these 25 yielded supernumerary embryos that were suitable for cryopreservation.

To date, 19 FET cycles have been performed in 10 couples where a total of 71 embryos (38 zygotes and 33 cleavage stage) were thawed. Details of the fresh cycles from which these FET cycles ensued can be seen in Table I.

Table II indicates the patient characteristics and fertilization outcome for the fresh ICSI cycles (from azoospermic men and control group) from which supernumerary embryos were later used. Mean paternal (37.9:37.4) and maternal age (33.2:33.6) were similar between the two groups. Maternal serum FSH levels and, in consequence total dose of gonadotrophins required were slightly higher in the ejaculated sperm group (7.4 IU/L and 2313 IU: 6.3 IU/L and 2118 IU). The mean number of eggs collected was similar (18.2: 17.5), although the fertilization rate (FR) was higher in the group using ejaculated sperm (72% versus 59%). Despite this a similar number of embryos were transferred per cycle in the two groups (2.53 and 2.50).

The pregnancy outcome of the fresh cycles is shown in Table III. Similar clinical pregnancy (CPR: 29% versus 25%) and livebirth rates (LBR: 14% versus 19%) per transfer were seen for the retrieved and ejaculated group respectively. The retrieval group (Group 1) had a higher implantation rate (IR) than the ejaculated group (18% versus 10%), although this did not reach clinical significance. This difference can be explained by a triplet pregnancy in this group, where selective reduction was performed, but led to miscarriage of all three fetuses. The numbers of

Table I. Details of Fresh Cycles in Azoospermic Men Yielding Frozen Embryos Later Used in FET Cycles

	Fresh/Frozen sperm	Source of spermatozoa	Type of azoospermia	Aetiology
1	Frozen	Epididymal	Obstructive	Epididymo-orchitis
2	Frozen	Epididymal	Obstructive	Epididymo-orchitis
3	Frozen	Epididymal	Obstructive	Primary Anorgasmia
4	Frozen	Epididymal	Obstructive	Vasal Aplasia
5	Frozen	Epididymal	Obstructive	Vasal Aplasia
6	Frozen	Epididymal	Obstructive	Vasal Aplasia
7	Frozen	Epididymal	Obstructive	Vasectomy (1981)
8	Frozen	Epididymal	Obstructive	Vasectomy (1983)
9	Frozen	Epididymal	Obstructive	Vasectomy (1983)
10	Fresh	Epididymal	Obstructive	Vasal Aplasia
11	Fresh	Epididymal	Obstructive	Young's Syndrome
12	Fresh	Epididymal	Obstructive	Young's Syndrome
13	Fresh	Epididymal	Obstructive	Ejaculatory failure
14	Fresh	Testicular	Obstructive	Young's Syndrome
15	Fresh	Testicular	Non-Obstructive	Bilateral Undescended Testes

embryos frozen per treatment cycle were similar at 4.9 and 6.1 for the retrieved and ejaculated group respectively.

The pregnancy outcome of the 19 FET cycles from embryos cryopreserved from the fresh cycles outlined above can be seen in Table IV. There were no differences between the two groups in the proportion of pronucleate (54% and 62%), and cleavage-stage embryos thawed (46% and 38%). Furthermore, post-thaw survival rates (retrievals: 69%; ejaculated: 73%), and quality of embryos subsequently transferred at FET were comparable. The mean number of embryos transferred was higher in Group 2 (2.74 versus 2.32) but this was of borderline statistical significance ($p = 0.08$).

Implantation rate was significantly lower in FET cycles using embryos generated from surgically retrieved sperm compared to those from ejaculated sperm (0% versus 11.5%: $p = 0.03$). Both CPR (5% versus 21%) and LBR (0% versus 21%) were also lower but only the difference in LBR reached borderline statistical difference ($p = 0.10$).

Table II. Patient Characteristics and Fertilization Outcome of Cycles Yielding Frozen Embryos Later Used

	Group 1	Group 2
	Fresh cycles from which FET later performed (MESA/TESE)	Fresh cycles from which FET later performed (ejaculated)
No. of patients	10	15
No. of fresh cycles	15	16
Paternal age at retrieval[a]	37.9 ± 7.6	37.4 ± 5.8
Maternal age at egg collection[a]	33.2 ± 3.3	33.6 ± 4.6
Maternal serum FSH (u/L)[a]	6.3 ± 1.5	7.4 ± 1.6
Total dose of FSH used (IU)[a]	2118 ± 669	2313 ± 948
No. of eggs collected	273	280
No. of eggs collected[a]	18.2 ± 5.5	17.5 ± 5.5
No. of eggs ICSI'd	241 (88.3%)	227 (81.1%)
No. of 2PN embryos	143 (59.3%)	163 (71.8%)
No. of embryo transfers	14	16
No. of embryos transferred	38	40
No. transferred[a]	2.53 ± 0.83	2.50 ± 0.50

[a] All values are mean ± standard deviation.

Table III. Pregnancy Outcome of Fresh Cycles from Which Frozen Embryos Later Used

	Group 1	Group 2
	Fresh cycles from which FET later performed (MESA/TESE)	Fresh cycles from which FET later performed (ejaculated)
Clinical pregnancy	4	4
% CPR/cycle	26.7%	25.0%
% CPR/transfer	28.6%	25.0%
Miscarriages	2	1
Ongoing pregnancy	2	3
Singleton delivery	1	3
Twin delivery	1	0
% LBR/cycle	13.3%	18.8%
% LBR/transfer	14.3%	18.8%
No. of fetal hearts	7	4
Implantation rate %	18.4%	10.0%
Total No. of embryos frozen	74	97
No. of embryos frozen[a]	4.9 ± 1.9	6.1 ± 3.0

[a] All values are mean ± standard deviation.

This clinical pregnancy rate of 21.1% for Group 2 is similar to the overall rate from all FET cycles in our unit over the time period of this study of 18.7%.

DISCUSSION

To our knowledge, this is the first report that compares the viability of frozen embryos generated from ICSI cycles using either ejaculated or surgically retrieved sperm. The data demonstrate impaired implantation and a trend towards a poorer pregnancy outcome for cycles using frozen/thawed embryos generated from surgically retrieved sperm.

Several factors have been shown to affect the outcome of FET cycles, including maternal age, number of embryos transferred, and possible differences in freeze-thaw protocols and culture conditions (23, 24). In addition, the factors associated with the fresh cycle, namely the total number of oocytes collected, quality of embryos available for cryopreservation (25, 26) and pregnancy outcome (27), have all been shown to impact upon the corresponding FET cycle.

Table IV. Frozen Embryo Transfer Cycle Details and Outcome

	Group 1	Group 2	
	(FET/retrieved sperm)	(FET/ejaculated sperm)	
Number of FET cycles	19	19	
No. of embryos thawed	71	71	
% 2PN	53.5% (38/71)	62.0% (44/71)	
% Cleaved	46.5% (33/71)	38.0% (27/71)	
Embryo survival rate (%)	69.0% (49/71)	73.2% (52/71)	
No. of embryos transferred	44	52	
No. of embryos transferred[a]	2.32 ± 0.83	2.74 ± 0.57	(NS: $p = 0.08$)
	Grade of embryos transferred		
% Grade 1	29.5% (13/44)	34.6% (18/52)	
% Grade 2	61.4% (27/44)	57.7% (30/52)	
% Grade 3	9.1% (4/44)	7.7% (4/52)	
Clinical pregnancy rate	5.3% (1/19)	21.1% (4/19)	(NS: $p = 0.34$)
Miscarriage	0	0	
Ectopic	1	0	
Livebirth rate	0% (0/19)	21.1% (4/19)	(NS: $p = 0.10$)
Singleton	0	2	
Twin	0	1	
Implantation rate	0% (0/44)	11.5% (6/52)	(NS: $p = 0.03$)

Note. Group 1: FET cycles from embryos generated from surgically retrieved sperm.
Group 2: FET cycles from embryos generated from ejaculated sperm.
[a] All values are mean ± standard deviation.

In our report, the maternal age was matched between groups 1 and 2, and the fresh cycles from which the embryos were cryproserved had a comparable number of oocytes collected, comparable pregnancy outcome and a similar proportion of embryos available for cryopreservation (Tables II and III). The only factor discrepant between the groups was the mean number of embryos transferred at FET (2.32 and 2.74 for retrieved and ejaculated sperm respectively), which was of borderline significance ($p = 0.08$).

The first report of FET cycles from ICSI-generated embryos using surgically retrieved sperm for injection (frozen testicular sperm from non-obstructive azoospermic patients) was published in 1999 (22). From a total of 17 FET cycles analysed, the authors reported a CPR, ongoing pregnancy rate and IR of 23%, 17% and 8% respectively. Unlike our report, the fresh cycles from which these embryos were cryopreserved were not separately assessed (FR, IR and CPR of 59%, 18% and 29% respectively in our patient group).

The data presented in this study of FET cycles by source of sperm suggests significantly impaired clinical outcome with the use of frozen-thawed ICSI-generated embryos where surgically retrieved sperm from azoospermic men was used. The origin of the sperm appears to be a major contributory factor since the two groups were matched for maternal age and FSH, mean number of oocytes collected and embryos transferred at fresh cycle, outcome of fresh cycle and post-thaw survival. One possible explanation for the poorer clinical outcome is that such embryos, originating from suboptimal sperm, are potentially associated with higher levels of faulty DNA (28). This in turn, may render them more susceptible to cryodamage thus compromising their viability.

CONCLUSIONS

In view of the small numbers of FET cycles performed following ICSI using surgically retrieved sperm, these findings need to be confirmed in larger multi-center studies. Further studies are also needed to assess the impact of source of sperm (i.e. epididymal or testicular), aetiology of azoospermia (i.e. obstructive or non-obstructive) and use of fresh versus frozen sperm on FET outcome. If these findings are confirmed, counseling of couples embarking on ICSI cycles using surgically retrieved sperm from azoospermic men will have to include the poorer outcome of such FET cycles.

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