Comparison of poor responders with good responders using intentionally frozen-thawed epididymal spermatozoa in subsequent ICSI cycles

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Summary

Purpose: To compare the outcome of intracytoplasmic sperm injection (ICSI) cycles performed with frozen-thawed epididymal spermatozoa between patients who respond poorly and patients who respond well to ovarian stimulation.

Methods: 17 patients suffering from obstructive azoospermia underwent microsurgical retrieval of epididymal spermatozoa (MESA) and the spermatozoa were frozen. The frozen-thawed spermatozoa were used in subsequent ICSI cycles. In six patients, the female partners responded poorly to ovarian stimulation. They accomplished nine ICSI cycles. In 11 patients, the female partners responded well to ovarian stimulation and they accomplished 16 cycles.

Results: Poor responders were older than those who reponded well. The mean number of metaphase II oocytes collected was lower in the poor responder group. In the poor responders, two couples failed to fertilise the oocytes in two ICSI cycles. In the good responders, one couple failed to fertilise the oocytes in an ICSI cycle. There were no significant differences in fertilization rates between the two groups. The estradiol concentrations on the day of hCG administration were significantly higher in the good responders. There was no pregnancy in the poor responder group, while three patients who responded well conceived. Eight good responders had 34 supernumerary 2PN oocytes which were cryopreserved.

Conclusion: Frozen-thawed epididymal spermatozoa from men with obstructive azoospermia are potent to achieve satisfactory fertilization rates. Poor ovarian response to stimulation induction appears to be the main limiting factor in reaching the stage of embryo transfer. It is preferable in older women to cancel cycles with poor response in the hope that a better response might be obtained in a subsequent cycle. Thus, the frozen-thawed epididymal sperm can be preserved and the most stressful and expensive phase of IVF-ICSI treatment can be avoided.

Key words: Epididymal spermatozoa; ICSI; Male factor infertility; Ovarian response.

Introduction

Intracytoplasmic sperm injection (ICSI) has been successful in cases of obstructive azoospermia due to total blockage or congenital absence of the vas deferens, failed vasoepididymostomy and otherwise irreparable obstruction, by using microsurgically retrieved epididymal spermatozoa (MESA) [17, 19]. MESA combined with ICSI allows pregnancy rates and delivery rates in cases with obstructive azoospermia that are no different from those of men with normal sperm samples [17]. Recently, the cryopreservation of surgically obtained spermatozoa has been adopted by many assisted-reproductive technology (ART) programs around the world, dissociating with this way the sperm retrieval from oocyte harvest and ICSI. In our center we have also adopted this approach and no freshly harvested epididymal spermatozoa are used for ICSI cycles. This approach simplifies the overall MESA/ICSI scheme without compromising the overall chances of pregnancy [5, 14].

Success of in vitro fertilization – embryo transfer and ICSI is also affected by the ovarian responsiveness to exogenous stimulation with gonadotropins. Patients with a poor response to classical ovarian stimulation protocols have a higher cancellation rate and a lower pregnancy rate with ART than patients who demonstrate a 'good' response to ovarian stimulation [8, 9, 10]. Various regimens have been used for ovarian stimulation in poor responders. Among these, it has been suggested that pituitary down-regulation with GnRH agonists may help to overcome the problem of 'poor' response to ovarian stimulation with gonadotropins [4, 16].

The aim of this study was to compare the outcome of ICSI cycles of patients who respond poorly by producing four follicles or less, with patients who respond well by producing ten or more follicles after ovarian stimulation. In all ICSI cycles frozen-thawed surgically obtained epididymal spermatozoa from men suffering from obstructive azoospermia were used.

Materials and Methods

Patient selection

Between October 1995 and December 1998, 17 patients suffering from obstructive azoospermia underwent MESA and the

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epididymal spermatozoa were frozen once the microscopic survey revealed spermatozoa in the aspirates. The frozen-thawed spermatozoa were used in subsequent ICSI cycles. In our program, no freshly harvested epididymal spermatozoa are used for ICSI cycles. The mean male age was 40.71 years (range 27-51). In six patients, the female partners responded poorly to ovarian stimulation. The definition of low responders in this study was four or less retrieved oocytes. In this group of patients a total of nine subsequent ICSI cycles using thawed spermatozoa were accomplished. The mean ± SD age of the female patients was 38 ± 9.187 years. In 11 patients, the female partners responded well to ovarian stimulation. The patients in which ten or more oocytes were obtained were defined as good responders. In this group of patients a total of 16 subsequent ICSI cycles using thawed epididymal spermatozoa were accomplished. The mean ± SD age of the female patients was 29.91 ± 2.737 years.

Technique of microsurgical epididymal sperm aspiration

Epididymal aspirates were obtained from patients with obstructive azoospermia as a source of spermatozoa in the ICSI program. The surgical technique was as follows: under general anaesthesia, scrotal contents were extruded through a small incision, the tunica vaginalis was opened and the epididymis was exposed. A tiny incision was made in the epididymal tunic to expose the underlying epididymal tubules of the obstructed epididymis. Spermatozoa were aspirated directly from the opening in the epididymal tubule into a syringe with a micropipette.

Cryopreservation and thawing of epididymal aspirates

After the microscopical survey revealed the presence of motile spermatozoa, the epididymal sperm was diluted in HEPES-buffered medium and then was centrifugated for 5 min and resuspended with HEPES-buffered medium. Aliquots were placed separately in cryovials containing 0.5 ml of HEPES-buffered medium (SpermFreeze, Medicut, Hamburg, Germany) consisting of modified Earle's balanced salt solution with 0.4% human serum albumin and 15% glycerol as a cryoprotectant. The spermatozoa were frozen by a Planner Kryo 10 III apparatus (Messer Griesheim, Germany). The cooling procedure was performed in liquid nitrogen vapour down to -30°C within the first 5 minutes and exponentially to -150°C in the next 55 minutes. The cryovials were then transferred directly to liquid nitrogen.

Early in the morning of oocyte retrieval a vial of frozen epididymal spermatozoa was thawed in a 37°C water bath for 3 minutes. After washing the sample in Ham's F10 medium, the mini swim-up technique [1] was used because of low progressive motility and an aliquot of medium was checked for the presence of spermatozoa under the microscope. Following an incubation of five hours in Ham's F10 medium in a humidified atmosphere with 5% CO_2 at 37°C, the supernatant was put into 2 ml Eppendorf tubes and centrifuged at 500 g for one minute. The pellet was resuspended with 3 µl Ham's F10. One µl of this suspension was transferred into a Petri dish containing droplets of Ham's F10 medium and 5 µl PVP (polyvinylpyrrolidone, Medicult, Hamburg, Germany). Immobilization of single spermatozoa was performed in 10% PVP droplets. After thawing, few spermatozoa showed local motility, but eventually they resumed better motility, especially after 5 hours of incubation. In each case, it was possible to recover enough motile spermatozoa upon thawing for microinjection of all metaphase II (MII) oocytes.

Ovarian stimulation

Ovarian stimulation was achieved by administering human menopausal gonadotropins (hMG) (Menogon, Ferring, Kiel, Germany) or recombinant FSH (Gonal-F, Serono, Freiburg, Germany) after pituitary suppression with a GnRH-agonist (Decapeptyl Depot; Ferring, Kiel, Germany) according to the long protocol. All patients received different dosages of hMG or recFSH in their treatment cycle according to the serum estradiol levels and transvaginal ultrasound measurements of the follicles. Ovulation was induced by the administration of 10,000 IU human chorionic gonadotropin (hCG) when the follicles reached a diameter of at least 17 mm and serum estradiol levels had been continuously rising. Thirty-six hours after ovulation induction, a vaginal ultrasound-guided puncture of the follicles for oocyte retrieval was carried out. When required general anaesthesia was given. The luteal phase was supported with 600 mg natural progesterone administered vaginally (Utrogestan, Besins-Iscovesco, Paris, France). Clinical pregnancy was defined as the presence of a gestational sac by ultrasonography at approximately seven weeks of gestation.

Preparation of oocytes and ICSI

For the ICSI procedure the cumulus cells and corona radiata were removed under dissecting microscope by two fine needles to shorten the time in hyaluronidase enzyme. Then the oocytes were exposed to 0.5% hyaluronidase (Sigma Company, Deisenhofen, Germany) for 30 seconds. ICSI was performed as previously described [1, 21]. After 18 hours of incubation at 37°C in a humidified atmosphere with 5% CO_2 oocytes were examined for the presence of two or more pronuclei as a sign of fertilization. Up to three cleaving embryos were replaced into the uterine cavity 48 hours after the ICSI procedure, according to the German Embryo Protection Law.

Statistical analysis

The Fisher's exact test and unpaired t-test were used for the comparisons of the rates and means between the groups; $p < 0.05$ was considered as statistically significant.

Results

The men with obstructive azoospermia in our study represent those in which spermatozoa had been seen in their epididymal aspirates for a diagnostic as well as therapeutic procedure. The cryopreserved sperm was used in subsequent ICSI cycles. The mean male age was 40.71 years (range 27-51). A total of six patients in which the female partners responded poorly to ovarian stimulation, underwent nine cycles of ICSI, using frozen-thawed epididymal spermatozoa obtained during a MESA procedure. A total of 11 patients, in which the female partners responded well to ovarian stimulation underwent 16 cycles of ICSI using frozen-thawed epididymal spermatozoa also obtained during a MESA procedure. The mean ± SD female age in poor responders was 38 ± 9.187 years and in good responders 29.91 ± 2.737 years. The mean female age was statistically significant different between the two groups ($p = 0.0143$). The mean ± SD numbers of metaphase II oocytes collected were also statistically significant different between the two groups (2.111 ± 1.269 for poor responders versus 10.38 ± 5.87 for good ones) ($p = 0.0004$). Two couples failed to fertilise the oocytes in two ICSI cycles in the poor responder group and one couple failed to fertilise the oocytes in one ICSI cycle in the good responder group. There were no significant differences in 2PN fertilization rates between poor and good responders (Fisher's exact test, $p = 0.1507$). There were no differences in the percent of regular cleaved embryos

transferred between poor and good responders (Fisher's exact test, p = 0.3285). The total dosage of gonadotropins to achieve ovarian stimulation was also not significantly different between poor and good responders (53.88 ± 37.30 ampoules versus 48.13 ± 14.69 ampoules, respectively) (p = 0.6002). The duration of stimulation was not different between poor and good responders (13.38 ± 4.534 days versus 14.20 ± 2.007 days) (p = 0.5483). However the peak plasma estradiol concentration on the day of hCG administration was significantly different between the two groups (571.5 ± 411 pg/ml versus 2339 ± 1071 pg/ml, respectively) (p = 0.0002). There was no pregnancy in the poor responders, while three patients in the good responders conceived. All the pregnancies were singleton. Eight out of 11 patients who responded well to ovarian stimulation had 34 supernumerary 2PN oocytes, which were cryopreserved and are available for transfer, allowing additional pregnancies to occur. The results are summarised in Table 1.

Table 1. — *Outcome of ICSI cycles using frozen-thawed epididymal spermatozoa obtained by MESA in poor and good responders.*

	'Poor' responders	'Good' responders
No. of patients	6	11
Female age*	38 ± 9.187	29.91 ± 2.737
No. of ICSI cycles	9	16
Mean number of metaphase II oocytes*	2.11 ± 1.269	10.38 ± 5.87
No. of cycles with failed fertilization	2	1
No. of cycles with embryo transfer	7	15
No. embryos per transfer	1.857	2.733
E_2 on hCG day (pg/ml)*	571.5 ± 411	2339 ± 1.071
No. of gonadotropin ampoules*	53.88 ± 37.30	48.13 ± 14.69
No. of days of stimulation period*	13.38 ± 4.534	14.20 ± 2.007
Fertilization rate (%)	68	50
Clinical pregnancies	-	3
Supernumerary cryopreserved 2 PN oocytes	-	34

* Values are mean ± SD

Discussion

This study confirms that ICSI using frozen-thawed epididymal spermatozoa obtained by MESA is an effective approach in the therapy of patients with obstructive azoospermia. In fact, successive reports have shown that the use of frozen-thawed epididymal spermatozoa gives fertilisation rates as high as with fresh epididymal sperm, high cleavage rates and satisfactory pregnancy rates in patients suffering from obstructive azoospermia [5, 13, 14]. Cryopreservation of the epididymal spermatozoa at the time of sperm aspiration helps to avoid both unnecessary ovarian stimulation of the female partner, in case of sperm nonavailability and the need for repetitive sperm retrieval for successive ICSI cycles. In our centre we have adopted the policy of cryopreservation of all harvested epididymal spermatozoa before an anticipated ICSI cycle and this was why we did not compare the outcome of ICSI cycles using frozen-thawed epididymal spermatozoa with the ICSI outcome using fresh collected epididymal spermatozoa. Using cryopreserved epididymal spermatozoa we know if any spermatozoa will be available at the time of ICSI and having multiple vials of frozen spermatozoa in hand, we also avoid repetitive microsurgical epididymal aspirates for successive ICSI cycles in this group of patients.

In the present study, we defined the female patients in which no more than four oocytes had been obtained as poor responders and good responders the females in which ten or more oocytes were retrieved. Poor responders represent a heterogeneous group of patients who can be differentiated: older patients with a low ovarian reserve, younger patients with borderline ovarian reserve, and young patients with normal ovarian reserve but inadequate response to gonadotropin stimulation [2, 6, 11]. Traditionally most ART cycles are carried out under the combination of GnRHa with gonadotropins. Several authors have investigated the role of GnRHa in the treatment of poor responders [6, 7, 12, 16]. Pituitary downregulation with GnRHa can block the premature LH peak that often occurs in poor responders, and can present lower cancellation rates. However, there is a significant increase in the total amount of gonadotropins to achieve stimulation and a need for longer stimulation [4]. The use of GnRHa in poor responders can sometimes be accompanied by a lack of ovarian responsiveness, despite a high dose of gonadotropins [3]. On the other hand, poor responders do not benefit from a stimulation protocol with higher doses of gonadotropins [20] and a high dose of hMG may negatively influence fertilisation, conception and pregnancy outcome [15].

In our study, the comparable fertilisation rates and the good quality of the produced pronuclear stage oocytes in both groups allow us to suggest that the potential of frozen-thawed epididymal spermatozoa to fertilise is independent of female age, however the outcome of ICSI cycles is directly dependent on the number of oocytes collected. Our present results are in accordance with the findings of Silber *et al.* [18], who have pointed out that there are very few cases of obstructive azoospermia that cannot be successfully treated with sperm retrieval methods and ICSI, as long as the female has an adequate number of oocytes.

The concept of cryopreserving epididymal spermatozoa obtained by MESA for later use, during actual sterility treatment by ART, has resulted in high expectations of a successful outcome for couples suffering from obstructive azoospermia. In our group of patients frozen-thawed epididymal spermatozoa were successfully used as a source of spermatozoa in subsequent ICSI cycles. Despite the satisfactory fertilization rates, which are

comparable to those obtained by other investigators [14, 18], the outcome was disappointing in the poor responder group, as a result of the very low number of oocytes retrieved, compared with patients who responded well to ovarian stimulation. In contradiction to the three pregnancies achieved in the good responder group and the supernumerary cryopreserved 2 PN oocytes which are available for transfer in the future, no pregnancy was achieved in the poor responder group.

Conclusion

It is obvious from our data that it is possible to achieve satisfactory fertilisation rates using frozen-thawed epididymal spermatozoa obtained from men with obstructive azoospermia in older women, but the limiting factor in reaching the stage of embryo transfer is the poor ovarian response to stimulation induction. On the basis of our data, it appears clear that in older women the outcome of cycles with ICSI using frozen-thawed epididymal spermatozoa is related to the number of oocytes collected and that it is preferable in cases of poor response to cancel these cycles. In this way, the most stressful and expensive phase of IVF treatment can be avoided. At the same time, the frozen spermatozoa are preserved, with the hope of being used in a subsequent, better cycle.

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