Outcome of anticipated ICSI cycles using intentionally frozen-thawed testicular spermatozoa according to the spouse's response to ovarian stimulation

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Summary

Purpose: To investigate the outcome of ICSI cycles, using frozen-thawed testicular spermatozoa from patients suffering from non-obstructive azoospermia, in relation to the spouse's response to ovarian stimulation.

Methods: A retrospective study with two groups of couples where males suffered from non-obstructive azoospermia. In group 1 (n = 14), the female partners responded poorly to ovarian stimulation (≤ 4 oocytes retrieved). In group 2 (n=14), the female partners responded well (≥10 oocytes retrieved). Both groups underwent 14 cycles of ICSI using frozen-thawed spermatozoa obtained by TESE.

Results: The total amount of gonadotropin, the duration of stimulation, the peak estradiol concentrations and the number of oocytes were significantly different between the two groups. Despite the satisfactory fertilisation rates, the outcome in poor responders was disappointing due to a low number of oocytes. There was only one pregnancy in the poor responder group whereas there were four in the group that responded well. The pregnancy rates per oocyte collection were 7.14% in group 1 versus 28.57% in group 2. The implantation rates were 60.60% versus 55.33%, respectively.

Conclusion: It is possible to achieve satisfactory fertilisation rates using frozen-thawed, surgically-retrieved testicular spermatozoa, but the poor ovarian response to stimulation induction is the limiting factor in reaching implantation and pregnancy. It is preferable that poorly stimulated cycles be canceled, in the hope of a better subsequent response.

Key words: Frozen-thawed testicular spermatozoa; ICSI; Male factor infertility; Poor ovarian response.

Introduction

Intracytoplasmic sperm injection (ICSI) using fresh spermatozoa extracted from testicular tissue is a common practice in patients suffering from non-obstructive azoospermia [1, 2]. However, about 30% of men who have non-obstructive azoospermia do not reveal any spermatozoa in their harvested testicular tissue, even after exhaustive searching [3, 4, 5]. Therefore, using fresh testicular tissue, spermatozoa might not be available for ICSI on the day of oocyte harvest. Cryopreservation of testicular tissue obtained during a diagnostic testicular biopsy or a planned testicular sperm extraction (TESE) before an anticipated ICSI cycle is a major advance, and the use of frozen-thawed testicular spermatozoa for ICSI in non-obstructive azoospermic patients has already shown to yield satisfactory fertilisation and pregnancy rates [5-10]. In our centre we have adopted the cryopreservation of surgically obtained testicular spermatozoa and no freshly harvested testicular spermatozoa are used for ICSI cycles. The dissociation of sperm retrieval from oocyte harvest and ICSI simplifies the overall TESE/ICSI scheme without compromising the overall chances of pregnancy [5, 9, 11, 12].

Today, the only factor that seems to affect the success of couples treated with TESE, which provides available spermatozoa for ICSI, is an adequate number of oocytes from the female partner [13]. Ovarian responsiveness to exogenous stimulation with gonadotropins plays a crucial role in assisted reproductive technologies (ART). 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 [14, 15, 16]. However, ovarian stimulation with GnRH-a is often associated with a large increase in gonadotropin consumption and prolonged length of stimulation because of suppression of endogenous FSH [17] or possible direct action of GnRH-a on the ovaries [18, 19].

The aim of the present study was to investigate the outcome of ICSI cycles, using frozen-thawed testicular spermatozoa from patients suffering from non-obstructive azoospermia, in relation to the spouse's response to ovarian stimulation.

Materials and Methods

Patient selection

In this retrospective study 28 couples who underwent ICSI cycles during the period from June 1996 to November 1998 were included. All male partners suffered from non-obstructive azoospermia. They underwent TESE and the harvested testicular tissues were cryopreserved and used later in anticipated ICSI cycles.

The couples were divided into two groups according to the number of follicles produced by the female partners: group 1

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(n = 14) with four follicles or less produced after ovulation induction and group 2 (n = 14) with ten or more follicles produced. Female patients in both groups were matched for chronological age, having a mean age of 35 (range 29-42). The mean male age in group 1 was 38.2 (range 30-47) and in group 2 was 36.8 (range 29-45).

As female age is the only factor which affects the success in couples undergoing ICSI with surgically obtained spermatozoa and as the origin of spermatozoa (from epididymis or testes, frozen or fresh) makes no meaningful difference [13, 20], in our cohort of patients, we did not take into account the histopathological results of the testicular biopsies.

Technique of testicular tissue extraction

The surgical technique was as follows: under general anaesthesia, the testes were delivered out through a median raphe incision. The tunica albuginea was incised and one cm^3 piece of testicular parenchyma was harvested from the cranial and caudal sides of each testis with scissors. The pieces of testicular tissue thus obtained were placed in a Petri dish containing Ham's F10 medium. In the embryological laboratory, a piece from each harvested testicular tissue was minced in the Petri dish using a sterile surgical blade. The minced tissue was then checked for the presence of motile spermatozoa under a phase contrast microscope at x 200-400 magnification..

Cryopreservation and thawing of testicular extraction

Cryopreservation and thawing of testicular tissue extraction was according to the procedure published by Al-Hasani *et al.* [21]. If spermatozoa were found on the wet preparation of harvested testicular tissue, then testicular pieces of 3-4 mm were placed separately in cryovials (Nunc, Wiesbaden, Germany) 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 tissues 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.

A vial of frozen testicular tissue was thawed the morning of oocyte retrieval in a 37°C water bath for 2-3 minutes. After washing the sample a couple of times in Ham's F10 medium it was minced in a Petri dish containing Ham's F10 medium into fine pieces by two surgical blades. An aliquot of medium was checked for the presence of spermatozoa under the microscope. If no spermatozoa were visible, a second vial was thawed for sperm retrieval. Following an incubation of 5 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). Immobilisation of single spermatozoon was performed in 10% PVP droplet.

After thawing, most spermatozoa were initially immotile, but eventually resumed motility, mostly in the form of tail twitching, indicating viability, after 5 hours of incubation. In every 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 the administration of human menopausal gonadotropins (hMG) (Menogon, Ferring, Kiel, Germany) or recombinant FSH (Gonal-F, Serono, Freiburg, Germany) after pituitary suppression with an 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 at least reached a diameter of 17 mm and serum estradiol values had been continuously rising. Transvaginal oocyte aspiration, under general anaesthesia when required, was performed 36 hours after ovulation induction. 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 six weeks of gestation.

Preparation of oocytes and ICSI

For the ICSI procedure the cumulus and corona radiata were removed mechanically under dissecting microscope, with simultaneous exposure to 0.5% hyaluronidase (Sigma Company, Deisenhofen, Germany) for 30 seconds. ICSI was performed as previously described [22, 23]. 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 fertilisation. A maximum of three cleaving embryos were replaced into the uterine cavity 48 hours after oocyte pick-up.

Statistical analysis

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

Results

The men with non-obstructive azoospermia in our study represent those in which spermatozoa had been seen in their testicular tissues and the cryopreserved sperm had been used in subsequent ICSI cycles. The mean ± SD female age was 35 ± 4.368 years (range 29-42). The 14 couples in group 1, where the female partners responded poorly to ovarian stimulation, underwent 14 cycles of ICSI using frozen-thawed testicular spermatozoa obtained during a TESE procedure. The 14 couples in group 2, where the female partners responded well to ovarian stimulation, also underwent 14 cycles of ICSI using frozen-thawed testicular spermatozoa obtained during a TESE procedure. The mean ± SEM number of collected oocytes were significantly different between the two groups (3.071 ± 0.3050 for poor responders versus 14.86 ± 0.8311 for good responders) ($p < 0.0001$). The mean ± SEM number of metaphase II oocytes collected were also significantly different between the two groups (2.357 ± 0.2695 for poor re-sponders versus 10.71 ± 1.092 for good responders) ($p < 0.0001$). Three couples failed to fertilise the oocytes and one couple had abnormal fertilisation (3PN) in poor responders (group 1) without embryo transfer. There were no differences in 2 PN fertilisation rates between poor and good responders (Fisher's exact test, p = 6990). The total dosage of gonadotropins to achieve ovarian stimulation was significant different between poor and good responders (81.64 ± 17.46 ampou-

les versus 36.20 ± 2.299 ampoules, respectively) ($p = 0.0238$). The duration of stimulation was significant different between poor and good responders (16.08 ± 0.6566 days versus 12.64 ± 0.3878 days) ($p = 0.0002$). The peak plasma estradiol concentration on the day of hCG administration was significantly different between the two groups (864.0 ± 156.3 pg/ml versus 2568 ± 141.5 pg/ml, respectively) ($p < 0.0001$). The mean ± SEM number of embryos transferred was also significantly different between the two groups (1.286 ± 0.3043 in poor responders versus 2.786 ± 0.1138 in good responders) ($p < 0.0001$). There was one pregnancy for the poor responders, while four patients of the good responders conceived; however one of them aborted (all the pregnancies were singleton). Seven out of 14 patients who responded well to ovarian stimulation had 34 supernumerary 2 PN oocytes which were cryopreserved and available for transfer, thus allowing additional pregnancies to occur.

The results are summarised in Table 1.

Table 1. — *Outcome of ICSI cycles using intentionally frozen-thawed testicular spermatozoa in poor and good responders to ovarian stimulation.*

	Poor responders	Good responders
No. of patients	14	14
Female age (mean ± SD)	35 ± 4.368	35 ± 4.368
Mean number* of retrieved oocxytes	3.071 ± 0.3050	14.86 ± 0.8311
Mean number * of MII oocytes	2.357 ± 0.2695	10.71 ± 1.092
Mean number* of transferred embryos	1.286 ± 0.3043	2.786 ± 0.1138
No. of cycles with failed fertilization	3	-
No. of cycles with abnormal fertilization	1	-
No. of cycles with embryo transfer	10	14
E_2 on hCG day (pg/ml)*	864.0 ± 156.3	2568 ± 141.5
No. of gonadotropin ampoules*	81.64 ± 17.46	36.20 ± 2.299
No. days of stimulation period*	16.08 ± 0.6566	12.64 ± 0.3878
Fertilization rate (%)	60.60	55.33
Clinical pregnancies	1	4
Pregnancy rate (%)	7.14	28.57

* Values are mean ± SEM

Discussion

Intracytoplasmic sperm injection (ICSI) with testicular spermatozoa has become a valuable method in the treatment of patients suffering from non-obstructive azoospermia [1, 2, 13, 24, 25]. However, testicular sperm retrieval may not always be successful in all patients [5, 26]. Therefore, there is always a possibility of not having any spermatozoa for ICSI on the day of oocyte retrieval. There are no clear-cut clinical or biological parameters to predict the success of sperm retrieval in patients with azoospermia. Tournaye *et al.* [4] found that no strong predictors for successful testicular sperm retrieval are available except for testicular histopathology. On the other hand, histopathological examination was found to be an accurate predictor in patients with germ-cell aplasia but not in patients with maturation arrest [4].

With the cryopreseving of the testicular tissue, the sperm retrieval procedure does not have to coincide with the female partner's cycle. Multiple aliquots of testicular tissue can be cryopreserved and used in several subsequent ICSI cycles. Numerous recent reports document the successful use of frozen-thawed spermatozoa obtained from testicular biopsies in patients suffering from obstructive or non-obstructive azoospermia and ICSI with frozen-thawed testicular spermatozoa gives rise to fertilisation and cleavage rates comparable to that obtained with fresh testicular spermatozoa [5, 8, 9, 10, 11, 12, 27, 28]. In our centre we have adopted the policy of cryopreservation of all harvested testicular spermatozoa before an anticipated ICSI cycle. With this way, 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 testicular biopsy procedures for successive ICSI cycles in this group of patients. Vials to be frozen should simply be held over liquid nitrogen vapour for 30 minutes before being plunged into the liquid nitrogen or they may be frozen with a computer controlled freezing unit utilising a slow controlled freezing program (29).

Eliminating the male factor problem by using frozen-thawed testicular spermatozoa with ICSI, the ovarian responsiveness seems to be the only predictor for ICSI treatment outcome. Traditionally, most ART cycles are carried out under the combination of GnRH agonist with gonadotropins, but in some patients the results are disappointing. In the literature, a uniform definition of what is a poor responder is lacking [15, 16, 17, 30]. In our study, we defined as poor responders those patients in which no more than four oocytes had been obtained and good responders those patients in which ten or more oocytes had harvested. Poor responders represent a heterogeneous group of patients consisting of older patients with a low ovarian reserve, younger patients with borderline ovarian reserve and young patients with normal ovarian reserve but no good response to gonadotropin stimulation [31, 32, 33]. Pituitary down-regulation with GnRHa can block the premature LH peak, which often occurs in poor responders, and can show lower cancellation rates. However, there is a significant increase in the total amount of gonadotropins to achieve stimulation and a need for longer stimulation [17]. The use of GnRHa in poor responders can be accompanied sometimes by a lack of ovarian responsiveness, despite a high dose of gonadotropins [34]. On the contrary, poor responders do not benefit from a stimulation protocol with higher doses of gonadotropins [35] and high dose of hMG may negatively influence fertilisation, conception and pregnancy outcome [36].

To our knowledge, this is the first study investigating the outcome of anticipated ICSI cycles, using intentionally frozen-thawed testicular spermatozoa according to the spouse's response to ovarian stimulation. Due to our results, the total amount of gonadotropin to achieve ovarian stimulation, the duration of stimulation, the peak estradiol concentrations and the number of oocytes obtained were all significantly different when poor responders were compared with good responders. Despite the satisfactory fertilisation rates, which are comparable to those obtained by other investigators [6, 7, 9], the outcome in poor responders was disappointing as a result of the very low number of oocytes retrieved. The group of poor responders in comparison to that of good ones, had much lower pregnancy rates per oocyte collection, 7.14% versus 28.57% respectively, although the implantation rates were 60.60% versus 55.33%, respectively.

Conclusion

The results presented here indicate that it is possible to achieve satisfactory fertilisation rates using, as a source of sperm, frozen-thawed surgically-retrieved testicular spermatozoa. However, the poor ovarian response to stimulation induction is the limiting factor in reaching implantation and pregnancy. In view of our findings, we believe that it is preferable to cancel the poor stimulated cycles, in the hope that a better response might be obtained in a subsequent cycle. In this way, the enormous physical, emotional and financial efforts, as well as the expenses that couples endure to reach the point of oocyte harvest are avoided.

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