Published-Ahead-of-Print September 20, 2006, DOI:10.2164/jandrol.106.000158
Journal of Andrology, Vol. 28, No. 1, January/February 2007

DOI: 10.2164/jandrol.106.000158

Case Report

Pregnancies in Cryptozoospermia With Sperm Ejaculated One Day Before ICSI: Four Case Reports

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Received for publication March 21, 2006; accepted for publication September 18, 2006.

Case Report

In cryptozoospermia, fluctuations in sperm count can lead to difficulties in finding sperm in the ejaculate on the day of oocyte retrieval. In fact, cryptozoospermia is often considered to be virtual azoospermia and often requires testicular sperm extraction (TESE) for intracytoplasmic sperm injection (ICSI). In addition to the potential andrological risks linked to testicular biopsy (vascular injuries and further androgenic defect or testicular atrophy; Harrington et al, 1996; Friedler et al, 1997; Schlegel and Su, 1997; Manning et al, 1998; Schill et al, 2003), the use of testicular gametes, which are less mature than ejaculated spermatozoa (Ariel et al, 1994; Kerjean et al, 2000) can expose the conceptus to potential risks. Even though reassuring, the follow-up of children conceived with testicular gametes is too limited to eliminate the risks linked to their use (Barri et al, 2005). Thus, the use of ejaculated sperm rather than testicular gametes should be promoted in patients with cryptozoospermia whenever possible. For this, the extended sperm preparation (ESP) protocol should be used systematically to confirm a diagnostic of azoospermia, especially in the absence of obstructive aetiology (Ron-El et al, 1997). Indeed, the retrieval of some spermatozoa, if they can constitute a safety pool of sperm, precludes the need for a testis

biopsy in the absence of sperm at the time of the ICSI. The simplest method to create this safety pool of sperm is cryopreservation. Nevertheless, spermatozoa from poor quality semen can be very fragile and unable to resist freeze/thaw procedures. Between March 2002 and April 2005, 49 cryptozoospermia (<1000 spermatozoa/ejaculate) patients have been so diagnosed by ESP at Lille assisted reproductive technique (ART) Center (France). To ensure spermatozoa on the day of the ICSI, we proposed a strategy to all cryptozoospermic patients that included a safety pool of frozen sperm and a 1-day survival test for spermatozoa in culture (Figure). After approval by the local ethics committee (January 2002), all patient couples gave written informed consent before participation in the study.

A safety pool of frozen sperm could be constituted for only 45 of the patients. For the last 4 (P1, P2, P3, P4), all spermatozoa were dead after cryopreservation. To avoid TESE in the event of an absence of spermatozoa on the day of the ICSI, we proposed the use of sperm ejaculated the day before the ICSI. We report here the results of the ICSI for these 4 cases that used spermatozoa ejaculated on the previous day.

Materials and Methods

Semen examination, cryopreservation procedure, and survival test— After 3–4 days of sexual abstinence, all cryptozoospermic patients were asked to produce semen twice in an interval of 2 hours. Freshly ejaculated semen was mixed with 5 volumes of IVF culture medium (IVF 30; Vitrolife, Gothenburg, Sweden) and centrifuged at 300 x *g* for 10 minutes. The pellet was gently resuspended in 50–300 µL of culture medium, depending on pellet size. Twenty percent of the suspension was then spread in 5-µL droplets on a culture dish, covered with mineral oil, incubated 10 minutes at 37 °C (5% CO_2), and examined under an inverted phase contrast microscope. Motile or living sperm were identified by the single-sperm curling test (SSCT; Ahmadi and Ng, 1997), were subsequently counted for each droplet, and were placed, with the use of an injection micropipette, into a second drop of culture media—the "survival droplet"—for the survival test. When at least 1 motile or live sperm could be detected, the remaining sperm preparation was frozen. This process was repeated until sperm could be observed or the suspension was finished.

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Management strategy proposed to cryptozoospermic patients.

For freezing, the sperm preparation was mixed at room temperature with an equal volume of Freezing Medium (TEST Yolk Buffer; Irvine Scientific, Santa Ana, Calif), and 30–50 µL of this suspension was loaded into straws and subjected to a standard cooling program (–10 °C/min from room temperature to –7 °C, then –20 °C/min to –70 °C; immersion into liquid nitrogen) in a controlled temperature freezer (Nicool LM10; AirLiquide, Marne la Vallée, France). As a test, 1 straw was thawed for 2 minutes at 37 °C. Its contents were diluted 5 times very slowly with IVF 30, incubated at 37 °C (5% CO_2) for 10 minutes, and centrifuged at 300 x *g* for 10 minutes. The pellet, after resuspension in

30–50 µL of IVF medium, was spread in 5-µL droplets and examined as previously described to detect motile sperm cells. When fewer than 10 motile spermatozoa were observed, a SSCT was performed with the motionless spermatozoa. At least 10 motile or living thawed spermatozoa were required for a successful "safety pool." When this number was not reached, the procedure was repeated a maximum of 6 times in the next months, or until a minimum of 10 live spermatozoa was obtained. If this minimum was obtained, the safety pool was considered an alternative to freshly ejaculated sperm in the event of transient azoospermia on the day of the ICSI. When this minimal pool of 10 frozen spermatozoa could not be identified, the treatment depended on the survival test as described below.

Survival test— For all cryptozoospermic patients, the survival test consisted of examining the cultured survival droplet after 1 day for sperm motility and vitality (SSCT). The survival test of 1-day-old sperm was performed without motility enhancer at the same time as sperm examination for the cryopreservation assay (up to 7 times, but an average of 3 times before the ICSI attempt and with a last assay 2 weeks before the ICSI attempt). If at each examination at least 10 motile/alive spermatozoa could be found in the survey droplet, the survival test was considered positive, and 1-day-old sperm was considered an alternative to freshly ejaculated sperm. In the absence of any safety pool of ejaculated sperm (either frozen or incubated), a testicular biopsy was used to make a safety pool of frozen testicular spermatozoa. Thus, the ICSI would be performed with ejaculated sperm (produced twice on the morning of the attempt), which if necessary would be supplemented, in order of preference, with cryopreserved sperm, sperm cultured for 24 hours, and, finally, frozen testicular spermatozoa (Figure).

For 45 of 49 patients, a safety pool of frozen sperm was successfully obtained; for these patients, the survival test was also positive. For practical reason, and to optimize sperm production the day of the ICSI with several days of sexual abstinence, we planned to use freshly ejaculated sperm from these patients for the ICSI, although frozen spermatozoa could be used in case of insufficient sperm count (or absence). For the 4 others, despite up to 5 attempts at cryopreservation, creation of safety pool of frozen sperm was not possible: spermatozoa were observed and cryopreservation performed, but after thawing/rehydration all spermatozoa were dead (SSCT). In contrast, the survival test was regularly positive. For these 4 patients, the use of spermatozoa cultured for 1 day was proposed as an alternative to freshly ejaculated spermatozoa in the event of azoospermia the day of the ICSI.

We report here the ICSI results for these 4 patients, using spermatozoa cultured for 1 day. Duration of infertility, male and female age and pathology, testis size, male seric FSH and testosterone levels, as well as karyotype and results of a search for Y microdeletions are summarized in Table 1. Even if testis size was low in all cases, FSH was abnormally elevated in only 1 patient. All 4 patients regularly had sperm counts under 50 spermatozoa per ejaculate, with at least 1 episode of transient azoospermia.

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Table 1. *Characteristics of the patients**

The day before, as well as the day of, oocyte retrieval, patients were asked to produce semen twice in 2 hours; this semen was then treated as described above. Because no spermatozoa were in the ejaculates on the morning of the ICSI, spermatozoa from the previous day were used for ICSI.

Ovarian stimulation, oocyte retrieval, and ICSI procedure were performed according to standard practices (Van Steirteghem et al, 1993; Rienzi et al, 1998).

Spermatozoa injected were selected according to their motility and morphology. All had either progressive or nonprogressive motility, without any use of motility enhancers. Morphology was assessed under an inverted microscope (400x). Most spermatozoa met our criteria: no angulation, normal head size, and a maximum of only 1 vacuole. Spermatozoa with cytoplasmic rest on the first day were very often motionless by the second day. The assessment of fertilization, embryonic cleavage, and morphologic quality were performed according to standard practices (Ron-El et al, 1997). Embryo transfers occurred 70 hours after ICSI and a good quality embryo was defined as having at least 3 blastomeres on the second day or 6 on the third day, with less than 20% of anucleated fragments. No more than 3 embryos were transferred, and routine progestational luteal support was given. Clinical pregnancies were defined as a sonographic visualization of at least 1 gestational sac.

Results

ICSI results are summarized in Table 2. For all 4 patients, neither ejaculate produced on the day of the oocyte retrieval contained spermatozoa. Thus, spermatozoa of the previous day were employed for ICSI; the count of spermatozoa available ranged from 10 to 32 (mean ± SD, 18 ± 10). The fertilization rates ranged from 27% to 75% (mean 41%). Table 3 summarizes the characteristics (morphology and motility) of spermatozoa used for successful fertilizations: in most cases, zygotes resulted from spermatozoa with normal morphology and progressive motility. In the 4 attempts, the cleavage rates were good (mean 92%), and 4 out of 5 embryos were of "good quality" for patient P1, and 2 out of 2 for P3 and P4. For P2 however, all 3 embryos were of poor quality. Nevertheless, in keeping with the patients' wishes, an embryo transfer took place for each of the couples. For the 2 patients in which fertilization rates were highest (50% for P1 and 75% for P4), the transfer of good quality embryos resulted in evolutionary pregnancies. In the 2 other cases (P2 and P3), in which the fertilization rate was around 25%, the proportion of good quality embryos was low, and no pregnancy occurred. It is important to note that the poor quality embryos for P2 could have been due to the woman's pathology (polycystic ovarian syndrome). For P1 and P4, 2 healthy children were born. For P1, the transfer of a second frozen embryo led to a second healthy child.

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Table 2. *Description of intracytoplasmic sperm injection (ICSI) attempts**

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Table 3. *Morphological characteristics of injected spermatozoa and subsequent zygote*

Discussion

In the case of cryptozoospermia, fluctuations in sperm number can lead to the absence of sperm in the ejaculate on the day of oocyte retrieval. In this case, testicular spermatozoa are often used as

an alternative to ejaculated ones. But, in patients with severe primary testicular defect and likely only very focalized spermatogenesis, testicular biopsy can fail to provide spermatozoa (Ron-El et al, 1997), and some authors suggest the TESE be repeated when negative to enhance sperm retrieval (Vernaeve et al, 2006). Moreover, in addition to potential andrological defects, testicular biopsies can involve the risk of nonmature gametes. To have the greatest possible chance of using ejaculated sperm for ICSI attempts, to have the greatest possible chance of using ejaculated sperm from patients with cryptozoospermia, our center proposes a strategy including the creation of 2 safety pools of spermatozoa: 1 frozen and 1 collected the day before ICSI and cultured overnight. In the event that the cryopreserved sperm are nonviable, we propose as an alternative the use of sperm ejaculated 1 day earlier instead freshly ejaculated sperm.

In the cases presented here, patients' ejaculates regularly contained fewer than 50 sperm. They agreed to come to the center at least 3 times in the months before the ICSI attempt to check the survival of their sperm during a 24-hour period of culture. Survival was regularly more than 50%, and in all cases, at least 10 spermatozoa were motile after the 24-hour incubation. They declared, retrospectively, that this procedure permitted them to approach the ICSI day without fear of the absence of spermatozoa on the day of oocyte retrieval.

Interestingly, bacterial contamination was rarely a problem. In more than 200 survival tests for 49 patients, we recorded only 3 instances of contamination. This was likely because spermatozoa were, in effect, washed twice: they were centrifuged, resuspended in sterile medium, then isolated and transferred to fresh, sterile medium with an injection micropipette.

On the day before oocyte retrieval, the number of motile (or living) sperm was very low, especially for 2 patients, P1 and P4, who obtained a child. Not surprisingly, we observed that zygotes came most often from spermatozoa with apparently normal shape (assessed with a standard inverted microscope at 400x magnification; Table 3) and progressive motility and that pregnancies occurred for the patients with the 2 best fertilization rates. Fertilization rates (FR, 41%), cleavage rates (CR, 92%), and the proportion of good quality embryos (GE, 67%) were similar to those observed in our center with freshly ejaculated sperm of cryptozoospermic patients (n = 40: FR 49%, CR 95%, GE 43%, pregnancy/delivery rate 12%, 5 deliveries) or with safety pools of frozen spermatozoa (n = 5: FR 54%, CR 92%, GE 33%, 1 child). Over the same period, our center found that these ICSI results were not significantly different of those obtained using TESE-testicular spermatozoa for patients with nonobstructive azoospermia (n = 42; FR 30%, CR 95%, GE 50%, pregnancy/delivery rate 19%, 8 deliveries). For Ron-El et al (1997), too, the results of ICSI with ESP fresh-ejaculated sperm (FR 41%, CR 88%, GE 56%) are very similar to those with testicular sperm (FR 49%, CR 93%, GE 58%) in cryptozoospermic men, and both are somewhat lower than with ejaculated spermatozoa in the regular ICSI program.

In the 4 cases reported here, the use of ejaculated sperm cultured for 24 hours rather than TESE spermatozoa resulted in ICSI parameters similar to those of other cryptozoospermic patients using fresh or frozen sperm or with nonobstructive azoospermic patients using frozen testicular spermatozoa. The incubation of spermatozoa for 24 hours in culture medium does not seem to have impaired their fertility. This was not entirely expected, considering that others have observed a decrease in the quality of ejaculated sperm cultured for 24 hours (but after selection by swim-up technique; Calamera et al, 2001) or testicular sperm (Dalzell et al, 2004). In ejaculated and cultured sperm, the major alteration observed during 24 hours of in vitro incubation was a reduction in the number of motile spermatozoa, together with an impairment in the quality of sperm movement. Importantly, however, no substantial differences in sperm viability or chromatin condensation were observed (Calamera et al, 2001); this is in contrast to immature testicular spermatozoa, in which

increased DNA fragmentation was observed and probably linked to a greater vulnerability to environment (Dalzell et al, 2003). Muratori et al (2000, 2003) suggested the involvement of endogenously produced reactive oxygen species as the possible cause of in vitro sperm DNA fragmentation; they found that DNA fragmentation rate was increased in teratozoospermic samples, particularly in the case of persistent cytoplasmic residues, which are indicative of immature spermatozoa. Still, it is not possible to demonstrate that the risk of DNA damage is not increased in these patients. Indeed, with a severe defect in spermatogenesis, a significant proportion of spermatozoa injected into oocytes can contain damaged DNA. Injection of oocytes with spermatozoa containing abnormal chromatin would probably result in a failure of fertilization; this could explain the poor fertilization rates in cryptozoospermic patients, although Twigg et al (1998) demonstrated that chromatin-damaged spermatozoa can result in a zygote, and Gandini et al (2004) reported full-term pregnancies achieved by ICSI despite high levels of sperm chromatin damage. Today, it appears difficult to tell whether the use of cultured cryptozoospermic spermatozoa is more or less safe than the use of frozen/thawed testicular spermatozoa. Large-scale studies are necessary to determine the effects of sperm DNA damage on the outcome of ART, especially on children's health.

Conclusion

The use of ejaculated sperm cultured for 24 hours can help avoid the need both for an invasive procedure like TESE and for the use of immature testicular spermatozoa. Considering the ICSI results of the 4 cases reported here, this method could be an alternative to freshly ejaculated sperm for cryptozoospermia patients with azoospermia on the day of ICSI. Finally, it improves the psychological state of cryptozoospermic patients who feel much stress and anguish in the absence of a safety pool of frozen sperm. Today, whereas reproductive medicine tries to treat each patient couple on a case-by-case basis in hopes of maximizing the chance of conception while minimizing the risks and discomfort, the 4 cases described here provide an interesting avenue of treatment.

Acknowledgments

The authors thank L. Acitokahn and MacLean Sellars for language help.

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