Outcome of ICSI using fresh and cryopreserved–thawed testicular spermatozoa in patients with non-mosaic Klinefelter’s syndrome

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BACKGROUND: Recently, intracytoplasmic sperm injection (ICSI) of testicular spermatozoa retrieved surgically from patients with non-mosaic Klinefelter’s syndrome resulted in several deliveries. The aim of this study was to evaluate the outcome of ICSI using fresh and cryopreserved–thawed testicular spermatozoa in these patients.

METHODS AND RESULTS: Following informed consent regarding the genetic risks of their potential offspring, mature testicular spermatozoa were found in five out of 12 (42%) patients who underwent testicular sperm extraction, and ICSI was performed while excess tissue was cryopreserved. The mean age of the patients was 28.7 ± 3.6 (range 23–36 years). Their baseline FSH was elevated (mean 38.3 ± 11.4; range 22–58 mIU/ml). All patients had small testicles of 2–4 ml in volume. The outcome of ICSI using fresh or cryopreserved–thawed testicular spermatozoa during five cycles in each group, was compared. No statistical significant difference was found in the two pronuclear fertilization rate (66 versus 58%), embryo cleavage rate (98 versus 90%) and embryo implantation rate (33.3 versus 21.4%) for fresh or cryopreserved sperm accordingly. The clinical outcome after using fresh testicular sperm included two singleton pregnancies (one delivered and one ongoing) and a triplet pregnancy resulting in a twin delivery (after reduction of an 47,XXY embryo). After using cryopreserved–thawed testicular spermatozoa, two pregnancies were obtained resulting in one delivery of twins and one early spontaneous abortion. CONCLUSIONS: Outcome of ICSI using cryopreserved–thawed testicular spermatozoa of patients with non-mosaic Klinefelter’s syndrome is comparable with that following the use of fresh spermatozoa. The genetic implications for the future offspring should be explained to the patients.

Key words: azoospermia/intracytoplasmic sperm injection/Klinefelter’s syndrome/TESE/47XXY

Introduction

Recently, clinical treatment of azoospermic males suffering from testicular failure has changed radically due to the introduction of testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI). In 11% of these azoospermic patients, testicular failure was caused by Klinefelter’s syndrome (KS) (Yoshida et al., 1996), due to a numeric sex chromosome aberration (47,XXY) explained by meiotic non-dysjunction (Klinefelter et al., 1942). About 15% are mosaic cases, usually with two cell lines: 47,XXY/46,XY. The others are considered as non-mosaic, upon cytogenetic examination of somatic cell lines. In these individuals, the testicular tubules become fibrotic and hyalinized, the tubule lumen gradually obliterates and germ cells disappear with time. In the adult XXY testes virtually all germ cells disappear (Ferguson-Smith et al., 1957; Foss and Lewis, 1971; Gordon et al., 1972). Occasionally, single foci of spermatogenesis do exist in the testes of KS (Steinberger et al., 1965; Skakkebaek et al., 1969; Tournaye et al., 1996), explaining the cases of sperm production and presence in the ejaculate (Ferguson-Smith et al., 1957; Foss and Lewis 1971; Tournaye et al., 1996; Palermo et al., 1998). Indeed, in non-mosaic KS, pregnancies have been reported using ICSI with ejaculated spermatozoa (Bourne et al., 1997; Hinney et al., 1997). In other cases, ICSI using testicular spermatozoa retrieved surgically is the sole mode of treatment to be offered, except sperm donation, as no mature, viable spermatozoa may be found in the ejaculate. At present, fewer than 20 deliveries have been reported following ICSI of testicular spermatozoa retrieved surgically from patients with non-mosaic KS (Tournaye et al., 1997; Palermo et al., 1998; Reubinoff et al., 1998; Nodar et al., 1999; Ron El et al., 1999, 2000a,b). The aim of this study was to evaluate the outcome of ICSI using fresh and cryopreserved–thawed testicular spermatozoa in our series of patients with non-mosaic KS.

Materials and methods

Study population

During the period of January 1996 and June 1999, 12 azoospermic patients diagnosed after cytogenetic evaluation as suffering from
non-mosaic KS were referred for treatment at the Infertility and IVF unit of Assaf Haroofeh Medical Center. Karyotype in all patients was assessed by cytological analysis including evaluation of >30 peripheral blood lymphocyte metaphases. The patients underwent physical examination of their genitalia as well as testicular and transrectal sonography to determine existence of normal anatomy of seminal vesicles, prostate, distal vasa deferentia and ejaculatory ducts and to rule out any pathological findings in their testes. Their hormonal profile was also assessed.

Following informed consent from the patients regarding the genetic risks of their potential offspring, extensive sperm preparation (ESP) (Ron-El et al., 1997) from ejaculated spermatozoa was offered as first line treatment.

**Sperm retrieval and preparation**

On the day of oocyte retrieval, the male partners produced fresh ejaculates, and ESP revealed mature, non-motile spermatozoa in two out of the 12 patients. In 10 patients ESP confirmed that no spermatozoa were found in the specimen and TESE was performed immediately. In the first patient with spermatozoa in the ejaculate, ICSI was performed using non-motile ejaculated spermatozoa; however, no fertilization was achieved. Therefore, TESE was offered in the following cycle. In the second patient with ejaculated immotile spermatozoa, TESE was offered immediately. Finally, all 12 patients underwent TESE.

**Methodology of TESE procedures**

The technique of surgical sperm retrieval by TESE, sperm preparation and ICSI has been described in detail elsewhere (Friedler et al., 1997). In all patients, tissue taken from their testicular biopsy was sent for histological evaluation.

When testicular spermatozoa were found enabling ICSI, excess tissue was cryopreserved using a simple freezing protocol (Friedler et al., 1997). This enabled performance of ICSI using testicular spermatozoa extracted from the thawed specimen, in consecutive cycles, of the same patients.

Ovulation induction and oocyte retrieval were performed using a routine long protocol of mid-luteal pituitary suppression with gonadotrophin-releasing hormone (GnRH) agonist, followed by a routine long protocol of mid-luteal pituitary suppression with a GnRH agonist, followed by human menopausal gonadotrophins for ovarian stimulation. Oocytes were retrieved by vaginal ultrasound guided follicular puncture. After assessment of fertilization ~24 h later, embryo transfer was performed on day 2 or 3, after recording embryonic cleavage and morphological quality. No more than three embryos were transferred, in the first trial and up to four, if available, in the repeated attempt. The reason for transferring one more embryo (if available) was to increase the chances of the couple to achieve pregnancy, according to their wish, fearing that the performance of cycles using cryopreserved–thawed testicular spermatozoa might be somewhat inferior compared with that with fresh spermatozoa. Routine pregestative luteal support was given, as described previously (Friedler et al., 1997). Only clinical pregnancies, including sonographic demonstration of a gestational sac, were counted. As only a few spermatozoa were needed in all ICSI cycles (up to 16 in fresh and up to 11 in cryopreserved–thawed cycles), not all the testicular biopsy specimen was extracted and survival rate was not quantified. However, after thawing in four out of five cycles, enough motile spermatozoa were found to enable ICSI of the available oocytes, giving the impression that cryopreservation was quite efficient.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years)</th>
<th>FSH (mIU/ml)</th>
<th>Testosterone (µg/ml)</th>
<th>Spermatozoa found in TESE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>28</td>
<td>11.3</td>
<td>–</td>
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<tr>
<td>2</td>
<td>25</td>
<td>22</td>
<td>3.4</td>
<td>+</td>
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<td>3</td>
<td>30</td>
<td>45</td>
<td>3.2</td>
<td>–</td>
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<tr>
<td>4</td>
<td>23</td>
<td>58</td>
<td>5.8</td>
<td>–</td>
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<tr>
<td>5</td>
<td>31</td>
<td>48</td>
<td>15.2</td>
<td>+</td>
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</tr>
<tr>
<td>12</td>
<td>28</td>
<td>43</td>
<td>4.5</td>
<td>–</td>
</tr>
</tbody>
</table>

Mean ± SD | 28.7 ± 3.6 | 38.3 ± 11.4 | 7.3 ± 4.1 | 5/12 |

**Statistical analysis**

Statistical evaluation was performed using $\chi^2$ test and Fischer’s exact test, where appropriate. Difference was considered significant at $P < 0.05$.

**Results**

Characteristics of the patient group with non-mosaic KS are presented in Table I. The mean age of the male patients was 28.7 ± 3.6 (range 23–36 years). The mean age of the female partners was 26.4 ± 3.9 (range 20–33 years). The male patients baseline FSH was elevated ranging from 22 to 58 mIU/ml (mean 38.3 ± 11.4). Their mean serum testosterone concentrations were 7.3 ± 4.1 mg/l (mean ± SD). All patients had small testicles of 2–4 ml in volume. TESE was performed in all 12 patients, resulting in retrieval of motile testicular spermatozoa in five cases (42%) and cryopreservation of excessive testicular tissue. In all patients, testicular biopsy confirmed tubular hyalinization, thickening of baseline membrane and Sertoli cell-only with no evidence of spermatozoa in the specimens evaluated.

Outcome of ICSI using fresh or cryopreserved–thawed testicular spermatozoa in these patients was compared.

Outcome of ICSI using fresh or cryopreserved–thawed testicular spermatozoa in these patients is presented in Table II. ICSI using fresh testicular spermatozoa resulted in a total fertilization of rate of 66%. In all cases sufficient numbers of motile spermatozoa were found to be injected into the mature oocytes retrieved. Transfer of three embryos in each patient resulted in three pregnancies. All 15 embryos had good morphology grade. The first woman delivered a healthy neonate, the second delivered two healthy twins, following first trimester embryo reduction of a third fetus diagnosed prenatally as 47,XXY and the third also delivered a healthy neonate. ICSI using cryopreserved–thawed testicular spermatozoa resulted in a total fertilization rate of 58%. In one cycle all six mature oocytes were injected with immotile spermatozoa; two oocytes were fertilized, cleaved
azoospermia, as described by Klinefelter syndrome. Gynaecomastia, hypergonadotrophic hypogonadism and KS. These infertile males characterized by small testicles, evaluation of treatment options for patients diagnosed as reproductive techniques has led to renewed interest in the introduction of TESE and ICSI into the arena of assisted reproduction.

Discussion

The introduction of TESE and ICSI into the arena of assisted reproductive techniques has led to renewed interest in the evaluation of treatment options for patients diagnosed as KS. These infertile males characterized by small testicles, gynaecomastia, hypergonadotrophic hypogonadism and azoospermia, as described by Klinefelter et al. (Klinefelter et al., 1942), were considered sterile. 47,XXY (KS) is the most prevalent chromosome anomaly, diagnosed in 3% of infertile male patients (Guichaoua et al., 1993), and up to 11% of patients with azoospermia (De Braekeleer and Dao 1991; Yoshida et al., 1996; Chiang et al., 2000). Cytogenetic analysis reveals that some are mosaic cases, usually with two cell lines: 47,XXY/46,XY and severity increases in parallel with proportion of aberrant cell population. Frequency of azoospermia in non-mosaic Klinefelter has not been estimated precisely. Compiling patients with non-mosaic KS in the reports found in the literature may not express the true incidence in this population, because they include patients who are virile enough to be married and prefer to achieve conception using their own spermatozoa. Surgical retrieval of spermatozoa is not always indicated, because when using recent methodology for sperm search, occasionally sperm cells may be identified in the ejaculate. In the current study, two out of 12 (17%) of patients with non-mosaic KS had spermatozoa in the ejaculate. Successful use of motile, cryopreserved–thawed spermatozoa from the ejaculate was reported in one case, leading to the birth of healthy normal twins (Bourne et al., 1997). However, the spermatozoa found by ESP are not always motile. In the report by Bourne et al., five other patients had immotile spermatozoa (Bourne et al., 1997). In our patient, ICSI of immotile spermatozoa failed to fertilize any of the 10 oocytes injected. When no viable spermatozoa are available for injection in the ejaculate, surgical retrieval of testicular spermatozoa is indicated to allow ICSI. In our series TESE resulted in retrieval of testicular spermatozoa in five out of 12 patients (42%). According to our literature survey, the success rate of TESE in non-mosaic KS is 56% (18/32), based on series taken from the literature (Tournaye et al., 1997; Reubinoff et al., 1998; Palermo et al., 1999) and from our own. This rate is similar to the chance of finding spermatozoa after TESE in the general population of patients with non-obstructive azoospermia (Silber et al., 1997; Palermo et al., 1999), but no clinical parameters are available at the present time to predict the success in a particular patient.

Testicular spermatozoa in KS patients may have a different genetic composition, with an increased rate of hyperhaploidy (Forest et al., 1999) than those suffering from azoospermia from a different cause and their freezability might differ. It appears that the outcome of ICSI using fresh or cryopreserved–thawed testicular spermatozoa in patients with KS has not yet been compared.

The results in the current study show that testicular tissue may be successfully cryopreserved in patients with non-mosaic KS.
mosaic KS without compromising significantly fertilization and implantation rates. Two pregnancies and a single delivery were previously reported using cryopreserved–thawed testicular spermatozoa among patients with non-mosaic KS (Ron-El et al., 2000a).

The ability to use successfully cryopreserved–thawed testicular spermatozoa for ICSI in patients with non-obstructive azoospermia (NOA) has been evaluated in several reports. Following the use of fresh or cryopreserved–thawed testicular spermatozoa, in patients with NOA, a similar fertilization rate of 47 versus 44%, embryo cleavage rate of 94 versus 89% and implantation rates of 9 versus 11% were observed (Friedler et al., 1997). Interestingly, ICSI using both fresh or cryopreserved testicular spermatozoa from patients with non-mosaic KS patients resulted in somewhat better fertilization rates and implantation rates compared with those from patients with NOA; however to reach significant conclusions the size of the groups should be enlarged.

The risk of transmission of gonosomal aneuploidy by using spermatozoa from non-mosaic KS patient is probably not high. This finding should in fact not be surprising, as it has been demonstrated recently that 47,XXY spermatogonia are probably capable of undergoing meiosis, completing the spermatogenic process culminating in formation of cytogenetically normal spermatozoa (Foresta et al., 1999; Bielanska et al., 2000). However, in one of the conceptions in our series, prenatal diagnosis of a 47,XXY fetus was performed, demonstrating the risk of injecting an abnormal spermatozoon during ICSI in these patients. This is in concordance with the higher prevalence of sex chromosome disomy found in the spermatozoa of non-mosaic KS patients (Guttenbach et al., 1977; Estop et al., 1998; Foresta et al., 1998; Okada et al., 1999). Of course, non-dysjunction in the oocyte injected may not be ruled out completely. Nevertheless, there is no doubt that genetic counselling is warranted in these cases. Preimplantation genetic diagnosis (PGD) by embryo biopsy may serve as a potent tool for embryo selection. As PGD cannot be considered yet as an absolutely reliable technique (Munné et al., 1995) nor is it available in each unit, prenatal genetic diagnosis should be recommended in all pregnancies achieved. So far, all 15 newborns after ICSI of testicular spermatozoa in non-mosaic KS patients are healthy with normal karyotype (Tournaye et al., 1997, two healthy newborns) (Palermo et al., 1998, 1999, five healthy newborns) (Reubinoff et al., 1998, one healthy newborn) (Ron-El et al., 1999, 2000a,b, five healthy newborns) (Nodar et al., 1999, two healthy newborns). One conception was prenatally diagnosed as 47,XXY, and terminated (Ron-El et al., 2000b).

In conclusion, patients with KS, even the non-mosaic type, may be offered treatment by ICSI. Preferably motile spermatozoa should be identified by ESP in their ejaculates. If only non-motile spermatozoa are found, there is still a possibility of finding motile testicular spermatozoa following TESE that may lead to pregnancy and delivery. Outcome of ICSI using cryopreserved–thawed testicular spermatozoa seems comparable with that following use of fresh spermatozoa. The genetic implications for the future offspring should be explained to the patients, warranting pretransfer or prenatal genetic evaluation of their potential offspring.

References


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