Birth of 16 healthy children after ICSI in cases of nonmosaic Klinefelter syndrome

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Submitted on December 11, 2012; resubmitted on January 15, 2013; accepted on February 4, 2013

STUDY QUESTION: Does the health status of infants fathered by nonmosaic Klinefelter syndrome (KS) patients whose partners underwent ICSI with sperm obtained from testicular dissection reveal any genetic risk for the offspring?

SUMMARY ANSWER: KS patients undergoing testicular sperm extraction (TESE) are capable of conceiving healthy children.

WHAT IS KNOWN ALREADY: Paternity has been successfully achieved in nonmosaic KS patients (47,XXY karyotype) by ICSI using either ejaculated or testicular spermatozoa. A crucial concern is the potential transmission of genetic abnormalities to the offspring. Some studies reported that 47,XXY spermatogonia are capable of completing spermatogenesis leading to the production of mature spermatozoa with increased aneuploidies. Other authors showed that where focal spermatogenesis is present in nonmosaic KS males, it originates from euploid germ cells and, therefore, produces normal mature gametes. In support of this finding, at present, the great majority of children born from nonmosaic KS patients are chromosomally normal.

STUDY DESIGN, SIZE, DURATION: From April 2004 to June 2010, 38 azoospermic patients with nonmosaic KS were examined for the presence of testicular spermatozoa. Spermatozoa were retrieved from 15 patients and 26 ICSI cycles were done (16 with cryopreserved sperm). There were 15 pregnancies leading to the birth of 16 babies who were karyotyped at amniocentesis and after birth.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants were recruited from couples attending the European Hospital, Rome, and Clinica MAR&Gen, Granada, for infertility treatment. Both the European Hospital and Clinica MAR&Gen are private clinics. Testicular tissue was extracted with TESE or micro-TESE. After retrieval, fresh sperm was used for ICSI or it was cryopreserved for future use.

MAIN RESULTS AND THE ROLE OF CHANCE: Spermatozoa were retrieved from 15 patients (14 TESE and 1 micro-TESE) out of 38 (39.5%). A total of 26 ICSI cycles were performed: 10 with fresh and 16 with cryopreserved-thawed sperm. Mean ages (y) of patients with positive and negative sperm retrieval were, respectively, 34.8 ± 1.72 and 35.6 ± 4.08 (NS, nonsignificant). Comparing ICSI cycles performed with fresh sperm (n = 10) to those performed with frozen—thawed sperm (n = 16): Fertilization rates per injected oocyte were 53.0% (44 of 83) and 47.8% (32 of 67), respectively (NS). The cleavage rate per injected oocyte was 90.6% (29 of 32) versus 68.2% (30 of 44); P = 0.026. Clinical outcomes were not significantly different between the fresh and the frozen—thawed sperm group: clinical pregnancy rates were 7 of 10 (70.0%) and 8 of 16 (50.0%); implanted embryos (per transferred embryo) were 8 of 23 (34.8%) and 8 of 29 (27.6%); delivery rates were 6 of 10 (60.0%) and 5 of 16 (31.3%). Sixteen babies were born, all of them are healthy with a normal karyotype, eight from the fresh sperm group and eight from the frozen—thawed sperm group.

LIMITATIONS, REASONS FOR CAUTIONS: The small numbers available for study mean that only common problems can be excluded.

WIDER IMPLICATIONS OF THE FINDINGS: This study provides further reassurance that KS men can father healthy children and that pre-implantation genetic diagnosis on embryos conceived with their sperm is not strongly indicated. However, until conclusive information is available, such couples should be offered extensive genetic counseling.

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Introduction

Klinefelter’s syndrome (KS) is a genetic male disorder associated with a numeric sex chromosome aberration. About 80% of KS men show a nonmosaic form of 47,XXY karyotype (Foresta et al., 1999). Nonmosaic KS is one of the main causes of nonobstructive azoospermia (NOA) being encountered in 10% of azoospermic men (Foresta et al., 1998, 1999). The extra X chromosome is thought to cause azoospermia via germ cell degeneration (Akslaede et al., 2006), although residual spermatogenesis may be present in some seminiferous tubules (Tournaye et al., 1996) so that spermatozoa can be found in the ejaculate in some of nonmosaic KS men. Most commonly, sperm recovery in these patients is only possible by surgical testicular sperm extraction (TESE) and micro-dissection TESE. The literature shows that sperm can be retrieved from between 16 and 69% of nonmosaic KS men (Okada et al., 2005), similar to men with NOA caused by other etiological factors (Bakircioğlu et al., 2011). Paternity has been successfully achieved in nonmosaic KS patients with the use of ejaculated or testicular spermatozoa and ICSI (review in Fullerton et al., 2010).

A crucial concern in the treatment of KS patients is the potential transmission of genetic abnormalities to offspring by the retrieved spermatozoa. Some studies have demonstrated that 47,XXY spermatagonia are capable of completing the spermatogenic process leading to the formation of mature spermatozoa with an increased frequency of aneuploidy (Foresta et al., 1999). By means of preimplantation genetic diagnosis (PGD), some authors detected a significant fall in the rate of normal embryos (54%) from KS patients when compared with the controls (72%) with an increased risk of abnormalities for sex chromosomes and for autosomes 18 and 21. These authors concluded that PGD should be carefully considered for these patients in order to assess the genetic status of the embryos (Staessen et al., 2003). On the other hand, other authors pointed out that, in nonmosaic KS males, if focal spermatogenesis is present, it originates from euploid germ cells and, therefore, produces normal mature gametes (Sciurano et al., 2009). In support of this finding, at present, the great majority of children born from ICSI in nonmosaic KS patients are chromosomally normal, despite most of them never having had a genetic screening such as PGD or prenatal diagnosis (Fullerton et al., 2010). However, although several reports of successful assisted reproductive technologies (ART) in KS patients have been published over the last years, most of them are case reports and only a few are larger case series, therefore the real genetic risk cannot be evaluated and the true necessity of performing PGD for these patients remains an open question.

The aim of our study was to investigate the health status of 16 infants, fathered by a group of nonmosaic KS patients who have undergone ICSI–TESE procedures, in order to evaluate the existence of a potential genetic risk for the offspring. In addition, we evaluated the equivalent reproductive capacity of frozen and thawed testicular sperm retrieved from our nonmosaic KS men.

Materials and Methods

Study population

From April 2004 to June 2010, 38 azoospermic patients with nonmosaic KS were examined for the presence of testicular spermatozoa. For each patient, azoospermia in the ejaculate was confirmed by the absence of sperm in at least two semen samples after 600g centrifugation and screening of the pellet at ×400 magnification using an inverted microscope. Sperm recovery was possible from testicular tissues in 15 out of 38 patients (39.5%). A total of 26 ICSI cycles were performed.

The study was approved by the Ethics Committee of European Hospital. The karyotype was assessed in all patients by cytological analysis including evaluation of >30 peripheral blood lymphocyte metaphases. Before the operation, patients underwent a physical examination and a complete medical history was obtained. Hormonal profiles including testicular volume, FSH, LH and serum testosterone (T) were measured (Table I); all patients underwent testicular sonography to determine the testicular volume and to point out any pathological finding. No preoperative hormonal treatment was planned and none of the patients received testosterone replacement treatment prior to surgery. All patients have signed an informed consent relative to the hypothetical genetic risks for their offspring.

Open testicular biopsy procedure

Testicular spermatozoa were obtained either by open testicular biopsy (TESE) or by testicular micro-dissection (micro-TESE) with examination of seminiferous tubules, using an operating microscope Carl Zeiss (OPMI Pico Surgical Microscope), depending on the patient’s testicular volume. Our procedure is described in Ubaldi et al. (1999). Micro-TESE was conducted under general anesthesia; the scrotum was incised along the scrotal

| Table I Characteristics of the men with nonmosaic KS included in the study (mean ± SD). |
|---------------------------------|--------|--------|
| **Testicular sperm recovery**   | **Positive** | **Negative** |
| Number of men                   | 15     | 23     |
| Age (years)                     | 34.8 ± 1.72 | 35.6 ± 4.08 | NS |
| Testicular volume (cm³) right   | 4.6 ± 1.35 | 3.6 ± 1.33 | NS |
| Testicular volume (cm³) left     | 4.5 ± 0.86 | 3.5 ± 1.43 | NS |
| FSH (mIU/ml)                    | 33.0 ± 13.93 | 28.2 ± 10.28 | NS |
| LH (mIU/ml)                     | 14.0 ± 2.88 | 15.8 ± 5.42 | NS |
| T (ng/ml)                       | 3.5 ± 1.01 | 3.1 ± 0.63 | NS |

Means were compared using Student’s t-test (unpaired).

STUDY FUNDING/COMPETING INTEREST(S): No external funding was obtained for the present study. None of the authors has any conflict of interest to declare.

TRIAL REGISTRATION NUMBER: Not applicable.

Key words: Klinefelter syndrome / azoospermia / testicular sperm extraction / sperm cryopreservation / healthy offspring
Sperm extraction was always performed using multiple testicular samples. Tissue samples were placed into small Petri dishes with 6–8 ml of Medium 199 (Sigma-Aldrich, Missouri, USA) and 2.5% Human Serum Albumin (Albutein, Alpha Therapeutic, Milan, Italy). In order to open the seminiferous tubules, samples were mechanically micro-dissected immediately after the collection, with the use of sterile glass microscope cover slides or fine needles, on the heated stage of a stereomicroscope.

The presence of spermatozoa in the Petri dishes was assessed under an inverted microscope (Nikon DIAPHONT 300) at ×400 magnification. When no spermatozoa were observed, the dissected tissue was transferred into a sterile tube and centrifuged at 600g for 10 min; 0.5 ml of a buffered medium (Quinn’s Advantages Medium with Hepes, 5% Human Serum Albumin, SAGE, CooperSurgical, Pasadena, USA), pre-warmed at 37°C, was added to the pellet. A little amount (~20–50 μl) of the resuspended pellet was observed in the inverted microscope to assess the presence of spermatozoa, their motility and morphology. When the sperm search was positive, the sample was cryopreserved for future use. An equal volume of cryoprotectant (Sperm-Freeze, FertiPro, Belgium) was added very slowly to the suspension and gently mixed. The specimen was partitioned into 0.3 ml portions and loaded into sterile high-security straws (CBS, Cryo Bio System’s, Washington, USA). After a 10 min incubation with the cryoprotectant, the straws were kept for 15 min in liquid nitrogen vapors (~80°C) and then plunged into liquid nitrogen (~196°C) for long-term storage.

When frozen sperm was used for treatment, one or more straws were removed from liquid nitrogen 4 h before ICSI and the content was washed in 5 ml of pre-warmed (37°C) buffered medium and centrifuged at 600g for 10 min. The pellet was resuspended in 0.3 ml of the same fresh medium and gently mixed. Finally, all the cells suspensions were smeared on the lid of a Petri dish, covered with mineral oil and observed fresh medium and gently mixed. Finally, all the cells suspensions were mechanically micro-dissected immediately after the collection, with the use of sterile glass microscope cover slides or fine needles, on the heated stage of a stereomicroscope. When no spermatozoa were observed, the dissected tissue was transferred into a sterile tube and centrifuged at 600g for 10 min. The pellet was resuspended in 0.3 ml of the same fresh medium and gently mixed. The specimen was partitioned into 0.3 ml portions and loaded into sterile high-security straws (CBS, Cryo Bio System’s, Washington, USA). After a 10 min incubation with the cryoprotectant, the straws were kept for 15 min in liquid nitrogen vapors (~80°C) and then plunged into liquid nitrogen (~196°C) for long-term storage.

Ovarian stimulation and ICSI procedure
All female partners underwent ovulation induction using a gonadotrophin-releasing hormone (GnRHa) analog suppression protocol (short or long) or a GnRHa antagonist protocol. Ovarian stimulation was performed as described in Ubaldi et al. (1999). Metaphase II (MII) oocytes were injected with, preferably, morphologically normal motile sperm. Fresh as well as frozen–thawed testicular sperm were used. All the ICSI procedures were performed as described elsewhere (Rienzi et al., 1998). Since 10 March 2004, the Italian law regulating ART (Benagiano and Gianaroli, 2004) restricted to three number of oocytes to be treated by ICSI and forbad embryo cryopreservation. Under such conditions, a maximum of three morphologically ideal oocytes were selected for ICSI, according to previously described criteria (Rienzi et al., 2003). From April 2009, after the Constitutional Court sentence no.151, more than three MII oocytes could be subjected to ICSI, as to achieve the optimal treatment for each individual couple, and freezing of the eventual extranumerary embryos was readmitted and imperative. Normal fertilization was achieved when two clearly distinct pronuclei were present on Day 1. Further embryonic development was assessed 24 h later. The embryos were classified according to previously described morphological criteria (Rienzi et al., 1998). Based on the number of morphologically good-quality embryos as well as on the age of the female partner, up to three (in most instances two) embryos were replaced into the uterine cavity. 70 h post-insemination (Day 3).

Luteal phase support and establishment of pregnancy
The luteal phase was supported by means of natural progesterone in oil, 50 mg/day i.m. (Prontogest® 100 mg; Amsa, Barberino del Mugello, Italy) starting on the day of the oocyte retrieval. Pregnancy was confirmed by a serum rise in β-HCG on two consecutive screenings, 12 days after embryo replacement. Clinical pregnancy was determined by ultrasound observation of cardiac activity at 7 weeks. An abortion was considered preclinical when β-HCG levels did not reach 1000 ml U/ml and no gestational sac was detected by ultrasound examination.

Statistical analysis
Data are presented as mean ± SD. Statistical evaluation was performed using unpaired two-sided Student’s t-test for age differences between groups and for the clinical parameters of the patients included in the study. Fisher’s exact test was used to analyze differences between groups in the laboratory and clinical outcomes. Differences were considered significant at P < 0.05.

Results
A total of 26 ICSI cycles were performed, 10 with spermatozoa from fresh biopsy samples and 16 with spermatozoa from frozen–thawed biopsy samples; two of the latter were carried out with both fresh and cryopreserved oocytes. There were no significant differences in age, testicular volume or reproductive hormone concentrations between men with successful and unsuccessful sperm recovery (Table I). In the group with successful sperm recovery, the average ages of the male and the female partner (mean ± SD) were 34.8 ± 1.7 and 33.6 ± 3.8 years, respectively, in the fresh biopsy group, and 33.0 ± 2.75 and 33.7 ± 1.9, respectively, in the frozen–thawed biopsy group (Table II). No signs of gynecomastia were noted in our patients.

In order to obtain testicular spermatozoa for ICSI, TESE was performed in 28 cases and micro-TESE in 10 cases. Spermatozoa were recovered in 14 out of the 28 TESE procedures (50.0%), but only in 1 out of the 10 micro-TESE procedures (10.0%).

Laboratory outcomes of the ICSI attempts performed with fresh and cryopreserved sperm are presented in Table II. Similar numbers of oocytes were collected in both groups. ICSI using fresh testicular spermatozoa resulted in a fertilization rate not significantly different from that obtained with cryopreserved–thawed testicular sperm. However, a higher cleavage rate was achieved with frozen–thawed sperm (90.6%) than with fresh sperm (68.2%; P = 0.026) (Table II). Clinical outcomes of 26 ICSI cycles performed with fresh and frozen–thawed testicular spermatozoa in our population of KS patients are presented in Table III. No difference was observed between fresh and frozen–thawed testicular sperm.

Obstetric outcomes after the ICSI attempts leading to pregnancy are shown in Table IV. A total of 11 pregnancies and 11 deliveries
Nonmosaic KS is typically a 47,XXY karyotype associated with a male genetic disorder that often leads to NOA. Our study on azoospermic nonmosaic KS patients confirms that, for this condition, fertility is possible with the use of testicular spermatozoa and ICSI insemination. Sixteen babies were born, all of them were healthy with a normal karyotype. Moreover, cryopreservation of testicular tissue did not compromise testicular sperm competence in our set of patients.

Two techniques were used to retrieve sperm from our azoospermic patients: conventional TESE (28 cases) or micro-TESE (10 cases). Interestingly, only in 1 micro-TESE procedure spermatozoa could be retrieved when compared with 14 successful retrievals from 28 conventional TESE procedures. These findings disagree with those reported by Schlegel (1999) who obtained improved sperm retrieval from micro-TESE when compared with conventional TESE. In patients who underwent ICSI cycles, testicular biopsy confirmed Leydig cell hyperplasia, tubular hyalinization, thickening of baseline membrane and Sertoli-only phenotype, compatible with nonmosaic KS. In the rest of the patients, sperm-line agenesis was observed. We noted that nonmosaic KS men have small testis and a decreased spermatogenesis, which is even more compromised in the adult age resulting in germ-cell and Leydig-cell loss (Oates, 2012).

The percentage of successful sperm recovery with TESE in KS patients ranges between 21 and 45% (Madgar et al., 2002). Sperm recovery rate was similar to those described in other studies (Tournaye et al., 1996; Levron et al., 2000; Kyono et al., 2007; Yarali et al., 2009). Studies comparing TESE–ICSI outcomes between KS and other NOA patients described similar rates of sperm retrieval (review in Fullerton et al., 2010) similar fertilization, implantation and live birth rates (Yarali et al., 2009), as well as similar pregnancy, spontaneous abortion and live birth rates (Bakircioglu et al., 2011). It can be assumed that KS patients have similar chance of conceiving as other NOA patients.

Hormonal levels and testicular volumes had no predictive value on sperm recovery. Other authors have reported contradictory results in relation to the predictability of recovering spermatozoa on the basis of clinical parameters and, overall, to date, there is no clinical parameter accepted as predictive of sperm retrieval (Tournaye et al., 1996; Seo et al., 2004; Vernaeve et al., 2004). Interestingly, although the difference was not statistically significant, in our observations, there was a tendency for younger male age in patients with successful sperm retrieval: the mean age of patients with successful sperm recovery was 34.8 ± 1.72, whereas the mean age of patients with unsuccessful sperm recovery was 35.6 ± 4.08, although this did not reach statistical significance. Our data are supported by other studies, indicating that aging reduces successful sperm recovery for men with KS (Ulug et al., 2003; Okada et al., 2005; Emre Bakircioglu et al., 2006; Ferhi et al., 2009; Yarali et al., 2009).

Cryopreservation of testicular spermatozoa has extensively been reported to be a successful approach for patients with NOA. In our experience, cryopreservation of testicular sperm allows a better planning of ICSI cycles so as to make the cycle timing most favorable for patients. Based on the time spent on sperm retrieval on the day of testicular biopsy, the thawing of testicular tissue can be planned well in advance before oocyte pick-up. In this manner, the best-quality spermatozoa needed for ICSI can be searched and retrieved in advance so that ICSI can be performed at the right time after hCG administration, avoiding oocyte in vitro aging. Given the possibility of failing sperm recovery from the testicles, azoospermic nonmosaic KS patients are ideal candidates for performing a diagnostic testicular biopsy prior to oocyte pick-up. In cases of successful sperm retrieval,
Healthy children from Klinefelter patients

Table IV Obstetric outcomes of the ICSI cycles included in this study.

<table>
<thead>
<tr>
<th>KS patient age (years)</th>
<th>Spouse age (years)</th>
<th>Sperm source</th>
<th>Babies delivered</th>
<th>Mode of delivery and gestational week</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>31</td>
<td>Frozen</td>
<td>2F 1990 g, 1880 g</td>
<td>CS (37 weeks)</td>
</tr>
<tr>
<td>34</td>
<td>38</td>
<td>Frozen</td>
<td>2F 1950 g, 1960 g</td>
<td>CS (29 weeks)</td>
</tr>
<tr>
<td>38</td>
<td>32</td>
<td>Frozen</td>
<td>1M 3400 g</td>
<td>CS (38 weeks)</td>
</tr>
<tr>
<td>36</td>
<td>35</td>
<td>Fresh</td>
<td>1M 3300 g</td>
<td>CS (40 weeks)</td>
</tr>
<tr>
<td>35</td>
<td>40</td>
<td>Fresh</td>
<td>1M 3500 g</td>
<td>VD (38 weeks)</td>
</tr>
<tr>
<td>34</td>
<td>34</td>
<td>Fresh</td>
<td>1F 3230 g</td>
<td>CS (39 weeks)</td>
</tr>
<tr>
<td>33</td>
<td>32</td>
<td>Fresh</td>
<td>1M 3100 g</td>
<td>CS (39 weeks)</td>
</tr>
<tr>
<td>34</td>
<td>28</td>
<td>Frozen</td>
<td>2M 1980 g, 1990 g</td>
<td>CS (30 weeks)</td>
</tr>
<tr>
<td>35</td>
<td>27</td>
<td>Fresh</td>
<td>2F 1990 g, 2000 g</td>
<td>CS (30 weeks)</td>
</tr>
<tr>
<td>37</td>
<td>29</td>
<td>Fresh</td>
<td>1F 3350 g</td>
<td>CS (38 weeks)</td>
</tr>
<tr>
<td>35</td>
<td>30</td>
<td>Fresh</td>
<td>1M 3500 g</td>
<td>CS (39 weeks)</td>
</tr>
</tbody>
</table>

All infants had normal karyotype at birth.
CS, Cesarean section; VD, vaginal delivery; M, male; and F, female.

the tissue can be cryopreserved and used for a future ICSI cycle. Overall, our data support previous findings from Friedler et al. (2001) who compared ICSI with fresh and frozen–thawed spermatozoa. Despite the small size of the two groups, the authors showed that testicular biopsies can be successfully cryopreserved in patients with nonmosaic KS without compromising ICSI outcomes.

Although several studies have already reported successful ICSI and high pregnancy rates in men with nonmosaic KS using testicular spermatozoa, there are still many concerns that offspring will be at risk of inheriting genetic problems. In the literature, from 1996 to date, 101 children were born to nonmosaic KS fathers with ART and, in most cases, healthy offspring was born without the use of PGD and selection of normal embryos (review in Fullerton et al., 2010). We report here 16 more healthy infants born to nonmosaic KS men. Overall, in the literature, most of the children born to KS patients are reported to be healthy. In few occasions, however, fetuses or embryos were diagnosed as 47,XXY karyotype (Reubinoff et al., 1998; Ron-El et al., 2000; Friedler et al., 2001).

Given the conflicting observation reported, it remains an open question whether embryo biopsy (PGD) should be offered to couples with KS male partner. One article from Staessen et al. (2001, 2008) is extremely convincing on the necessity of PGD on KS patients. The authors reports data on PGD offered to 20 nonmosaic KS patients and show that 54% of the embryos were normal, versus 77.2% in a control group, and show that embryos from KS patients have a higher incidence of sex chromosome abnormalities (13.2 versus 3.1%: KS versus control), as well as autosomal abnormalities (15.6 versus 5.2%: KS versus control), with an increased risk for chromosomes 18 and 21 (Staessen et al., 2003). Magli suggested that sex chromosome mosaicism may be linked to autosomal mosaicism (Magli et al., 2002). Based on these observations, KS patients are not only at risk of transmitting KS to offspring but also at higher risk of generating embryos with autosomal aneuploidies, such as trisomy 21 (Hennebicq et al., 2001; Staessen et al., 2003). A high incidence of sperm chromosome abnormalities, especially sex chromosomes aneuploidies, was found in NOA patients. It has been suggested that the increased anomalies in embryos from KS patients could be due to the use of testicular sperm more than to the KS karyotype of their father (Staessen et al., 2003). If this is the situation, PGD should not be offered to KS males because of the chromosomal anomaly but it should be more extensively offered to all NOA patients who undergo TESE. However, in azoospermic KS patients, embryos are originated with rare sperms found in surgically excised testicle tissue; this should be taken in consideration as PGD manipulation could affect embryo viability and implantation potential in particular when performed on Day 3. However, a Day 5 blastocyst biopsy might be recommended as it is not documented to have a negative impact on embryo viability.

In conclusion, in our experience with azoospermic nonmosaic KS men, healthy children are delivered. Sixteen healthy babies with normal karyotype were born from KS men using either fresh or cryopreserved–thawed testicular sperm. Two more deliveries of healthy babies from nonmosaic KS patients are reported in a previously published article from our group (Greco et al., 2001, 2008). Newborns from KS patients are reported to be healthy by many authors; however, the scientific opinion is still divergent. We suggest that an extensive genetic counseling should be provided to couples with nonmosaic KS male partner. The risk of transmission of genetic disorders as well as the pros and cons of PGD and/or prenatal genetic diagnosis should be carefully explained so that patients are provided with sufficient tools to finally choose the treatment most suitable for them. However, PGD and/or prenatal diagnosis should be (*) suggested to those couples with KS male partners (**).

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements
The authors would like to thank Dr Mario Terribile for his help in the acquisition of data and Dr Alessandro Colasante and Dr Anna Maria Lobascio for their precious participation to laboratory procedures.
**Authors’ roles**

E.G. was involved in study conception and design, data interpretation, drafting manuscript and final approval. F.S. played a central role in all laboratory procedures, was involved in the acquisition of data, critical revision of the article and final approval. M.G.M played a role in laboratory procedures, data analyses and final approval of the manuscript. V.C. was involved in drafting the manuscript and took part in the laboratory procedures, critical discussion and final approval. D.Z. was involved in surgical procedures of testicular biopsies and final approval. D.D took part in surgical procedures and final approval. J.T. provided a critical discussion and extensive improvement of the manuscript. G.F. was involved in surgical procedures of testicular biopsies, in critical discussion and revision and in the final approval of the manuscript.

**Funding**

No external funding was either sought or obtained for this study.

**Conflict of interest**

None declared.

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