TITLE
ASSISTED REPRODUCTIVE TECHNOLOGY OUTCOMES IN AZOOSPERMIC MEN:
TEN YEARS OF EXPERIENCE WITH SURGICAL SPERM RETRIEVAL
SHORT TITLE
REPRODUCTIVE MEDICINE AND INFERTILE MEN

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ABSTRACT

An azoospermic man suffers from an absence of sperm in the ejaculate and this condition is present in about 10% of infertile men. Obstructive azoospermia (OA) is characterized by an occlusion or partial absence of the reproductive tract with the presence of normal spermatogenesis. On the other hand, non-obstructive azoospermia (NOA) is characterized by impaired spermatogenesis. In these cases, spermatozoa can be obtained by percutaneous epididymal or testicular sperm aspiration (PESA and TESA, respectively) and used for intracytoplasmic injection (ICSI). To compare ICSI outcomes using spermatozoa that were surgically retrieved by PESA and TESA, azoospermic patients were divided into the following categories: (i) TESA-NOA (n=102), (ii) TESA-OA (n=103) and (iii) PESA-OA (n=171). Fertilization, pregnancy, and implantation rates were compared between the groups. We noted a lower normal fertilization rate (P=0.0017) and a higher abortion rate (P=0.0387) among men in the TESA group who had OA as compared to men in the PESA group who had OA. On the other hand, a lower normal fertilization rate (P=0.05) and a lower rate of non-cleaved embryos (P=0.034) was found in the TESA group of NOA patients as compared to the TESA group of OA patients. No statistically significant differences were detected between the TESA and PESA groups and the OA and NOA groups, respectively. The clinical outcomes of embryos arising from ICSI cycles using spermatozoa harvested via PESA and TESA were similar, regardless of whether the patient had obstructive or non-obstructive azoospermia.
INTRODUCTION

Since its introduction in 1993 (1,2) intracytoplasmic injection (ICSI) of oocytes using testicular and epididymal sperm has become a routine treatment procedure for patients with azoospermia as male infertility factor. Azoospermic men suffer from an absence of sperm in the ejaculate, and this condition is present in about 10% of infertile men (3,4). Obstructive azoospermia (OA) is characterized by an occlusion or partial absence of the reproductive tract with the presence of normal spermatogenesis, such as previous vasectomy, congenital absence of vas deferens, or testicular trauma. Non-obstructive azoospermia (NOA) is characterized by impaired spermatogenesis (5), and its manifestation ranges from varying degrees of maturation arrest to Sertoli-cell-only syndrome.

Sperm from the testis and epididymis can be used for ICSI (1,2) with successful fertilization rates and satisfactory pregnancy rates (6,7). In these cases, spermatozoa can be obtained by percutaneous epididymal sperm aspiration (PESA) or testicular sperm aspiration (TESA). However, the effects of the source of spermatozoa and the etiology of the azoospermia on ICSI outcomes has not been well established and may be influenced by various factors related to sperm quality, such as genetic alterations and/or male imprinting (8,9). The quality of spermatozoa derived from the epididymis may differ from that of spermatozoa collected from the testis, and embryo quality has been found to be related to the origin of the injected sperm (testicle or epididymis) in a previous study (10). O’Connell (11) observed that testicular sperm have fewer mitochondrial and nuclear DNA mutations and less DNA fragmentation than epididymal sperm. It has also been observed that embryos from ICSI cycles using ejaculate or epididymal spermatozoa are of a much higher quality than embryos derived from testicular sperm (10). Previous studies have shown that TESA results in lower fertilization and pregnancy rates and higher abortion rates than PESA (12,13,14), illustrating that the use of mature male gametes may result in better embryos.

The type of azoospermia can also affect ICSI outcomes. Some studies have found no differences in the outcomes of patients with OA vs. NOA (15,16), but most studies show that sperm harvested from NOA
patients results in lower fertilization and pregnancy rates as compared to patients with normal spermatogenesis (12,17,18). The fertilization rate after ICSI using testicular spermatozoa from men with impaired spermatogenesis may be reduced because the spermatozoa are less mature than those from men who have normal spermatogenesis (17). Impaired spermatogenesis may also have an underlying genetic cause, leading to poor ICSI outcomes (19).

This study was designed to compare ICSI outcomes from patients with either obstructive or non-obstructive azoospermia who underwent PESA and TESA procedures between 1997 and 2007. We sought to examine whether or not the source of sperm used for ICSI or the type of azoospermia (AO or NOA) that patients had influenced ICSI outcomes.
MATERIALS AND METHODS

Patients

Patients who underwent PESA or TESA between January 1997 and March 2007 were included in the analysis. During this time, 376 patients who underwent PESA or TESA were treated at the Fertility - Assisted Fertilization Center, Brazil. Patients were divided into experimental groups according to the origin of the sperm used for ICSI and the type of azoospermia the patients had as follows: (i) TESA-NOA (n=102), when the injected sperm were retrieved from the testicle of patients with non-obstructive azoospermia (ii) TESA-OA group (n=103), when the injected sperm were retrieved from the testicle of patients with obstructive azoospermia, and (iii) PESA-OA (n=171), when the injected sperm were retrieved from the epididymis of patients with obstructive azoospermia. In order to analyze whether or not the surgical approach and/or the origin of azoospermia would lead to differences in treatment success, comparisons were performed between the TESA-OA vs. PESA-OA groups and the TESA-OA vs. TESA-NOA groups. This was done because epididymal sperm aspiration (PESA) is not performed in non-obstructive azoospermic patients. Furthermore, the non-obstructive TESA and obstructive PESA groups are not comparable since there are two potential sources of variations (i.e., surgical approach and type of azoospermia). Pituitary down-regulation and ovarian stimulation, as described later, were performed in all cycles, followed by ICSI. All patients provided written informed consent for all the procedures before inclusion in this study. Institutional review board approval was not required because all procedures are routinely performed.

TESA and PESA Procedures

After spinal cord block anesthesia, TESA was performed using a 21-gauge butterfly needle that was longitudinally inserted into the superior pole of the testicle, avoiding the epididymis. Forward and backward movements of the needle as well as slight changes in needle orientation were performed to ensure
parenchymal disruption to allow needle aspiration. A standardized testis mapping procedure was performed with an average of 8 needle passes.

Epididymal sperm aspiration was performed after induction of local anesthesia using a 27-gauge needle inserted into the epididymis. Gentle, negative pressure was applied as epididymal fluid was aspirated. For both PESA and TESA, the aspirated material was collected into a conical tube and washed with a minimum volume of Modified Human Tubal Fluid medium (HEPES, Irvine Scientific™, Santa Ana, California, USA) at 37 ºC. The recovered material was checked for the presence of spermatozoa and centrifuged at 300g for 8 min. The fraction was diluted or concentrated as necessary.

For patients with non-obstructive azoospermia, TESA was the procedure of choice. Percutaneous epididymal sperm aspiration was the first approach tried in patients with obstructive azoospermia, and TESA was used when the former was not effective. If the initial aspiration was unsuccessful, the same procedure was performed on other sites of the testicle or epididymis (in TESA or PESA, respectively).

**Stimulation Protocol in Retrieval Cycle**

Patients were stimulated with a standard long GnRH (GnRH agonist, Lupron Kit™, Abbott S.A Société Française des Laboratoires, Paris, France) agonist protocol. Controlled ovarian stimulation was achieved with human recombinant FSH (Gonal-F; Serono Laboratories, Norwell, MA) in a step-down protocol. Transvaginal oocyte retrieval was performed 34–36 hours after urinary (uhCG, Profasi™, Serono, Geneve, Switzerland) or recombinant human chorionic gonadotropin (rhCG, Ovidrel™, Serono, Geneve, Switzerland) administration. Oocytes were incubated in HTF culture medium (Irvine Scientific) supplemented with 10% HSA (Human Serum Albumin, Irvine Scientific™, Santa Ana, California, USA) under oil (Ovoil, Vitrolife, Kungsbacka, Sweden) for a maturation culture interval of 3 to 4 hours. Oocyte–corona–cumulus complexes were treated with HEPES-buffered medium containing hyaluronidase (80 IU/mL, Irvine Scientific™, Santa Ana, California, USA) for 30 seconds to allow visualization of the ooplasm and polar body.
ICSI Procedure

Oocytes in metaphase II were transferred into drops of HTF–HEPES medium, covered with oil, and placed on the warmed plate of an inverted Nikon Diaphot microscope. Intracytoplasmic sperm injection was performed according to the technique described by Palermo et al. (20). Fertilization was confirmed by the observation of the presence of two pronuclei and the extrusion of the second polar body. Embryos were kept in a 50µL drop of HTF medium supplemented with 10% HSA under oil in a humidified atmosphere containing 5% CO₂ at 37°C until transfer. The following parameters were analyzed after ICSI: the normal fertilization rate, defined as the presence of two pronuclei 16 to 18 hours after ICSI; the abnormal fertilization rate, defined as the presence of only one or three or more pronuclei 16 to 18 hours after ICSI; the fertilization failure rate, defined as the lack of appearance of pronuclei after ICSI, even when there were two polar bodies; and the non-cleaved zygote rate, the rate of normal fertilized embryos that still had only one cell on day 2 of fertilization. The implantation rate was defined as the number of gestational sacs divided by the number of high quality transferred embryos (i.e., embryos containing an adequate number of cells and that had a lack of fragmentation).

Clinical Follow-up

Clinical pregnancy was determined by the visualization of at least one gestational sac on transvaginal ultrasonography.

Statistical analysis

Results were expressed as means ± standard deviations and confidence interval for the mean for numeric variables, while proportions (%) were used for categorical variables. Mean values were compared using the parametric unpaired Student’s t-test or the non-parametric Mann-Whitney test, according to the Gaussian distribution of the data. Proportions were compared using the Chi-squared test or Fisher’s exact test. The latter was used when the expected frequency of an event was five or less. Results were considered
statistically significant when the P-value was less than 0.05. Data analysis was carried out using GraphPad Prism version 4.0.
RESULTS

ICSI outcomes according to the origin of surgically retrieved spermatozoa in OA patients.

The 171 patients in the PESA-OA group were compared to the 103 patients from the TESA-OA group. There were no differences in the mean maternal (P=0.11) or paternal (P=0.10) age of patients in the two groups. Similar results were observed regarding the mean number of follicles retrieved (P=0.55), the mean number of oocytes retrieved (P=0.63), the mean number of intact oocytes after ICSI (P=0.15), and the mean number of transferred embryos (P=0.13) (Table 1).

Sperm was found in 100% of the cases, and ICSI using motile spermatozoa was performed in 58.9% of the TESA and in 93.6% of the PESA ICSI cycles (P<0.05), while immotile spermatozoa were retrieved and injected in 14.0% of the TESA and in 6.4% of the PESA ICSI cycles (P<0.05). In the TESA cycles, both motile and immotile spermatozoa were injected in 27.1% of cycles. These data were not used in this study.

Although a higher normal fertilization rate was achieved in the PESA group than in the TESA group (P=0.0017), there were no statistically significant differences noted in the abnormal fertilization rate (P=0.94) or in the mean number of non-fertilized oocytes (P=0.11) of these two groups. No significant differences in clinical pregnancy (P=0.88) or implantation rates (P=0.93) were observed between the TESA and PESA groups. The miscarriage rate was significantly higher for patients with OA who underwent TESA (P=0.038) as compared to PESA (Table 2).

ICSI outcomes according to the type of azoospermia in TESA cycles

A total of 205 patients underwent the TESA procedure prior to ICSI. They were divided between patients with obstructive azoospermia (n=103) and those with non-obstructive azoospermia (n=102).

There were no differences in the mean maternal (P=0.28) or paternal (P=0.125) age of patients in the two groups. Similar results were observed from regarding the mean number of follicles retrieved (P=0.72), the
mean number of oocytes retrieved (P=0.58), and the mean number of intact oocytes present after ICSI (P=0.92) (Table 3).

Sperm was not found in 27.9% of TESA cycles from patients with non-obstructive azoospermia (n=28). ICSI using motile spermatozoa was performed in 83.2% of the obstructive cases and in 52.4% of the non-obstructive cases (P<0.0001), whereas immotile spermatozoa were retrieved and injected in 9.7% of the obstructive and in 12.6% of the non-obstructive azoospermia ICSI cycles (P=0.45). Both motile and immotile spermatozoa were injected in 7.1% of the ICSI cycles of patients with obstructive azoospermia. In the ICSI cycles of patients with non-obstructive azoospermia, spermatozoa with and without motility were injected in 17.5% of the ICSI cycles, while round cells were injected in 1.4% of the ICSI cycles, and elongated spermatids were injected in 16.1% of the cases. These data were not used in this study. The rate of normally fertilized oocytes was higher in the OA-TESA group than in the NOA-TESA group (P=0.05). The abnormal fertilization rate between groups did not differ significantly (P=0.4421), but both the non-fertilized oocyte rate (P=0.0023) and the non-cleaved zygote rate (P=0.0340) were higher in the NOA-TESA group. However, these results did not influence the clinical pregnancy rates (P=0.42), implantation rates (P=0.85), or miscarriage rates (P=0.99), which did not differ significantly between OA-TESA and NOA-TESA patients (Table 4).
DISCUSSION

The introduction of ICSI has resulted in a great enhancement of fertilization and pregnancy rates in patients with severely reduced sperm concentrations and quality. The availability of ICSI enabled the first attempts of fertilization using epididymal or testicular spermatozoa (21). The results obtained by the surgical retrieval of sperm are satisfactory, and so far, many babies have been born in cases in which it would otherwise have been impossible (22).

However, various factors affect ICSI results, including the method used for sperm retrieval, the maturity and motility status of retrieved gametes, the timing of sperm retrieval in relation to oocyte collection, and the possibility of freezing the retrieved male gametes for repeated use. Several studies have reported differences in ICSI outcomes from spermatozoa derived from PESA vs. TESA, including higher fertilization and pregnancy rates (23) and lower miscarriage rates (13) observed after PESA.

When the two different surgical procedures were compared, we found a higher rate of zygotes with only two pronuclei on the day after ICSI in the group in which PESA was performed. The lower fertilization rates observed with testicular spermatozoa may be related to physiological differences in the characteristics of the sperm membrane, possibly involving differences in cholesterol, glycolipids, and membrane lipids (24). Abnormal cytoskeletal events that can occur during fertilization may also explain fertilization failures and abnormal embryonic development (24). Therefore, because the sperm from the testis has been through a longer process of maturation than the sperm from the epididymis, there is a chance that the genetically normal sperm have not yet been selected, thus increasing the chances of choosing a spermatozoon with only one nucleus in the PESA procedure.

An important finding of this study is that, in the majority cases in which spermatozoa were present in epididymal sperm retrieval, it was possible to find motile spermatozoa. It is known that the motility of the spermatozoon used for ICSI is an important predictive factor for successful fertilization. Even though fertilization and pregnancy have been reported after the injection of immotile spermatozoa, the rates are lower when compared to those observed with the injection of motile spermatozoa (25, 26). Sperm acquire the capacity for vigorous forward motility during transit through the epididymis, which plays an important
role in sperm maturation (23). Therefore, it is important to perform aggressive sperm immobilization in immature spermatozoa, since it seem to increase sperm membrane permeability (20). The immature sperm from the testis may have been responsible for the lower fertilization rate found in this study when the results from azoospermic patients with some kind of obstructive pathology who underwent PESA vs. TESA were compared. Several studies (19,20,27), including one from our own unit, have compared the outcomes of ICSI cycles in patients with OA using epididymal and testicular sperm that were identified as suitable, and a total number of 734 ICSI cycles were analyzed. Fertilization rates varied from 45%–72% for epididymal and 34%–81% for testicular sperm, but only one article (28) found a significant difference in the fertilization rate between epididymal and testicular sperm. No differences in cleavage, pregnancy, or implantation rates were reported in any of the individual articles, a finding that is in accordance with the results of the current study.

Another interesting result that we observed when comparing PESA and TESA in patients suffering from obstructive azoospermia was the higher miscarriage rates observed in the TESA group, which is in accordance with the findings of other reports. One explanation for that may be that the use of immature testicular spermatozoa from patients with normal spermatogenesis can impair the results of ICSI, through mechanisms that may involve genomic imprinting (21). Moreover, genetically defective sperm can lead to fetal loss and genetic disease in the offspring (18).

Because we did not only compare the type of surgical procedure used for spermatozoa collection, but also the reason why the patient was azoospermic (i.e., OA vs. NOA), we were able to demonstrate that NOA patients have the lowest normal fertilization rate and the highest rate of non-cleaved zygotes. Even though obstructive azoospermia leads to low quality spermatozoa due to their immaturity or to the fact that this condition is often associated with infection or inflammation (29, 30), spermatozoa retrieved from the testicle of a patient that has NOA have another potential set of problems. The common causes for NOA include Klinefelter’s syndrome, orchitis, and cryptorchidism, and the genetic components of these conditions can certainly become a barrier to reproduction. The genetic components described above are even more prominent in cases of non-obstructive azoospermia (31). Therefore, the possible genetic
deficiencies in the sperm of NOA patients combined with poorer spermatozoa motility and maturity, conditions observed with the use of testicular sperm, may have had a crucial influence on the higher rates of non-fertilized oocytes and non-cleaved zygotes observed in NOA patients in the present study. However, at some point there must have been another factor that corrected for the presence of these conditions, leading to pregnancy and implantation rates that did not differ between patients with obstructive vs. non-obstructive azoospermia.

PESA and TESA are established surgical procedures commonly used in assisted reproductive technology and thanks to them, patients with various pathologies can be offered *in vitro* fertilization. Our study shows that even though the quality of surgically collected spermatozoa may differ according to the origin of sperm, the ICSI outcomes were similar when the TESA and PESA groups were compared. Although our larger data set showed that the fertilization rate is higher in the PESA group, embryos arising from ICSI cycles using surgically retrieved testicular or epididymal spermatozoa derived from azoospermic patients, had similar clinical pregnancy and implantation rates, regardless of whether the azoospermia was obstructive or non-obstructive.
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REFERENCES


DECLARATION OF CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.