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Article in Fertility and sterility · September 2015
Impact Factor: 4.59 · DOI: 10.1016/j.fertnstert.2015.08.008

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Higher pregnancy rates using testicular sperm in men with severe oligospermia

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Objective: To evaluate assisted reproductive technology (ART) outcomes using testicular sperm in oligospermic men who previously failed to achieve pregnancy using TUNEL-positive ejaculated sperm.

Design: Retrospective cohort.

Setting: Academic medical center.

Patient(s): Twenty-four oligospermic men who failed one or more ART cycles using ejaculated sperm with TUNEL-positive proportion >7%, and subsequently underwent microsurgical testicular sperm extraction (TESE).

Intervention(s): TESE followed by intracytoplasmic sperm injection (ICSI).

Main Outcome Measure(s): TUNEL-positive level in ejaculated and testicular sperm; clinical pregnancy.

Result(s): The mean TUNEL-positive level was 24.5% for ejaculated sperm, and 4.6% for testicular sperm. Clinical pregnancy was achieved in the first ART cycle with testicular sperm in 12 (50%) out of 24 couples. There was no statistically significant difference in maternal and paternal age, maternal gravity and parity, number of previous ART attempts, concentration or motility of retrieved sperm, number of oocytes retrieved, fertilization rate, or number of embryos transferred between couples who did and did not achieve pregnancy. No miscarriages occurred. All 12 pregnancies resulted in the delivery of healthy children.

Conclusion(s): The percentage of TUNEL-positive cells is lower in testicular sperm for oligospermic men who have abnormal ejaculated sperm DNA fragmentation. The use of testicular sperm for ICSI was associated with a 50% pregnancy and live-birth rate for couples who had previously failed one or more IVF–ICSI cycles with ejaculated sperm. No other clinical predictors of successful pregnancies after the use of surgically retrieved sperm could be identified. In men with elevated TUNEL-positive ejaculated sperm and failed ART, TESE may be considered. (Fertil Steril® 2015; □□□ –□□. ©2015 by American Society for Reproductive Medicine.)

Key Words: Infertility, recurrent pregnancy loss, sperm DNA fragmentation, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, TESE, testicular sperm extraction

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Excepting men with nonobstructive azoospermia, the vast majority of patients with male factor infertility have sufficient sperm in the ejaculate for use with intracytoplasmic injection (ICSI). Ejaculated spermatozoa that have completed maturation during their transit through the male reproductive tract generally have better fertilization potential than testicular sperm (1). Nevertheless, with advances in sperm preparation and selection methods, as well as advances in the techniques of assisted reproductive technology (ART), the pregnancy rates after the use of testicular versus ejaculated sperm for ICSI have been shown to be comparable among men with similar etiologies of male factor infertility (2).

Fewer studies have directly compared fertility outcomes after the use of ejaculated or testicular sperm within the same cohort of patients. Weissman et al. (3) reported improved embryo implantation rates and pregnancy rates using testicular sperm compared with ejaculated sperm in four couples with severe oligozoospermia. Similarly, Hauser et al. (4) and Ben-Ami et al. (5) reported improved implantation and pregnancy

Received June 8, 2015; revised and accepted August 6, 2015.
A.M. has nothing to disclose. A.B. has nothing to disclose. P.N.S. has nothing to disclose. D.A.P. has nothing to disclose.
Supported by the Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust.
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Fertility and Sterility® Vol. □□□, No. □□□, □□□□ 0015-0282/$36.00
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http://dx.doi.org/10.1016/j.fertnstert.2015.08.008

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rates with the use of fresh testicular versus fresh ejaculated sperm in men with either severe oligospermia or cryptozoospermia. Taken together, the results of these studies appear to favor the use of testicular sperm over ejaculated sperm in men with severe male factor infertility.

A growing body of literature suggests that sperm from infertile men contain more DNA damage than sperm from fertile men [6, 7], and that the degree of sperm DNA damage can negatively impact the fertility potential of affected men [8, 9].

Even when sperm are selected to avoid those spermatozoa with gross DNA damage, the outcome of assisted reproduction is affected when the neat (unprocessed) semen sample has increased sperm DNA damage. When used for assisted reproduction, sperm from a sample with increased DNA damage may be able to fertilize an oocyte [10], but the resulting embryo may fail to develop or implant, or may be naturally aborted at a later stage [11].

Although delays in sperm transport, varicoceles, and gonadotoxic exposures are associated with increased sperm DNA damage, the exact mechanisms by which sperm DNA damage occurs remain unclear [12]. Breaks in sperm DNA may be result of compaction errors or defects in repair of DNA breaks from free radicals as well as abnormal nuclear proteins structure. Why some men are more susceptible to developing such damage than others is also unknown. Suganuma et al. [13] have postulated that sperm are susceptible to damage during passage through the male reproductive tract. Indeed, testicular sperm have been suggested to have significantly lower levels of DNA damage compared with ejaculated spermatozoa from the same individuals [14, 15]. Conversely, however, testicular sperm have been shown to have higher rates of aneuploidy compared with ejaculated sperm from the same individual [16], a finding that could offset the advantage of using testicular sperm for ICSI if performed indiscriminately.

Thus, the optimal treatment options for patients who have previously failed ART cycles using ejaculated sperm with a high proportion of chromatin damage remains controversial. Our study evaluated reproductive outcomes using testicular sperm in oligospermic men who had previously failed to achieve pregnancy and who had TUNEL-positive ejaculated sperm.

### MATERIALS AND METHODS

#### Study Population

Oligospermic men (sperm concentration < 5 million/mL), who, in the absence of identifiable female factor infertility, had previously failed one or more IVF or ICSI cycles performed using ejaculated sperm, and who had decided to undergo microsurgical testicular sperm extraction (TESE) at our academic specialty center between 2008 and 2013, were identified for inclusion in this cohort study. A failed ART cycle was defined as a cycle that did not result in a clinical pregnancy (no heartbeat). Men with a history of obstructive azoospermia, uncorrected varicoceles, testicular trauma, chemotherapy, or pelvic radiation were excluded.

Per our standard practice, all men undergoing TESE provided an ejaculated semen sample on the day of surgery before their procedure. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed, as described herein, on ejaculated and testicular sperm samples from all men meeting the eligibility criteria for this study. Only patients with > 7% TUNEL-positive sperm in ejaculated semen samples were included in the final data analysis.

Surgically retrieved sperm was used for injection of all oocytes in fresh ICSI cycles for testicular sperm retrieval cycles. The medical records of the male patients and their female partners were reviewed to abstract data on demographics, reproductive history, semen parameters, sperm DNA fragmentation, and reproductive outcomes after the use of testicular sperm.

#### TUNEL Assay

Sperm DNA fragmentation was assessed using the in situ Cell Death Detection Kit with fluorescein isothiocyanate (FITC) (Roche Diagnostics). All assays were performed in duplicate by the same researcher (A.B.), and included one positive and one negative control. Briefly, 10-μL aliquots of each patient sample were smeared on to glass slides and air dried. Each slide was fixed with 1 mL of 4% paraformaldehyde in phosphate-buffered saline (PBS) solution and incubated at room temperature for 1 hour. Slides were washed with ice-cold PBS three times, then permeabilized with TritonX in 0.1% sodium citrate for 5 minutes. Slides were then rewashed with PBS three times then incubated with a mixture of the TUNEL enzyme solution (Roche Diagnostics) containing terminal deoxynucleotidyl transferase, and TUNEL Label Mix (Roche Diagnostics) containing deoxyuridine triphosphate. The slides were individually covered with Paraflam (Ted Pella) and were incubated in a dark, moist chamber at 37°C for 1 hour to allow time for labeling.

After removal, the slides were washed with PBS, and counterstained with Prolong Gold antifade reagent with 4',6-diamino-2-phenylindole, dihydrochloride (DAPI; Invitrogen) overnight. The slides were then analyzed using an epifluorescence microscope (Fig. 1) fitted with Nomarksi optics (Fig. 3) at ×1,000 magnification. The number of DAPI-positive and FITC-positive cells per high power field were counted. At least four separate fields of view were analyzed for each slide, and at least 200 DAPI-positive cells were counted. The number of FITC-positive cells detected were divided by the number of DAPI-positive cells to calculate the percentage of TUNEL-positive cells containing fragmented DNA.

#### Data Analysis

The primary outcome measures were [1] the TUNEL-positive proportion of sperm in ejaculated and testicular samples and [2] an intrauterine pregnancy. A paired t test was used to compare the differences in TUNEL percentages between ejaculated and testicular sperm both overall and with respect to pregnancy outcomes. Unpaired t tests for continuous variables and the chi-square and Fischer’s exact tests for categorical variables were used to analyze differences in maternal and paternal age, maternal gravity and parity, number of...
previous ART attempts, concentration and motility of retrieved sperm, TUNEL assay results for ejaculated and TESE sperm, number of oocytes retrieved, fertilization rate (2 pronuclei), and number of embryos transferred, with respect to pregnancy outcomes. All data analysis was performed using JMP 9.0 statistical software (SAS). P < .05 was considered statistically significant. This study was approved by the institutional review board of Weill Medical College.

RESULTS
Twenty-four couples met the study criteria and were included in the final analysis. The mean age of the male and female partners was 42 ± 9.8 and 36.5 ± 4.9 years, respectively. Twelve of the 24 couples had achieved one or more clinical pregnancies in the past, resulting in a total of two singleton and one twin births. Before considering TESE at our institution, these 24 couples had undergone, on average, 3.2 failed cycles (range: 1–10) of IVF or ICSI, at either our institution or elsewhere.

Overall, the mean TUNEL-positive score was 24% for ejaculated sperm (95% CI, 14%–34%), and 5% for testicular sperm (95% CI, 3%–7%) \( (P = .0013) \). These data are summarized in the box plot in Figure 3. Testicular sperm were retrieved in all 24 male partners. All couples underwent a programmed ICSI cycle using the freshly retrieved sperm (either on the day of or the day before oocyte retrieval). Clinical pregnancy was achieved in 12 (50%) of 24 couples in the first cycle.

Table 1 compares various characteristics of couples who did and did not achieve pregnancy. No statistically significant differences were noted in maternal and paternal age, number of previous IVF attempts, concentration of retrieved sperm, number of oocytes retrieved, oocyte fertilization rate, or number of embryos transferred. Additionally, maternal gravity...
and parity were comparable between the two groups. Six couples in each group had previously achieved a clinical pregnancy together, resulting in a singleton birth in the nonpregnant group and one twin and one singleton birth in the pregnant group.

Couples who achieved pregnancy using testicular sperm were observed over the course of their pregnancy. No miscarriages occurred. All 12 pregnancies resulted in the delivery of healthy children. A subanalysis of the couples who achieved a pregnancy using testicular sperm was performed. Ten of these 12 couples (83%) had undergone a previous IVF attempt using ejaculated sperm at our center.

**DISCUSSION**

There is growing evidence that among couples with isolated male factor infertility, the source of sperm used for IVF—ejaculated versus testicular—may have a differential impact on fertility outcomes. We postulated that differences in sperm DNA fragmentation between ejaculated and testicular sperm could be a reason for the differences seen in fertility outcomes. The results of our study demonstrate that the proportion of TUNEL-positive sperm is lower in testicular samples compared with ejaculated samples from the same cohort of men. The use of testicular sperm for ICSI was associated with a 50% pregnancy and live-birth rate for couples who had previously failed one or more IVF/ICSI cycles with ejaculated sperm. There were no other statistically significant differences in patient characteristics between the couples who did and did not achieve pregnancy with testicular sperm in this study.

Our results are in agreement with the findings of other investigators. Greco et al. (14) measured sperm DNA fragmentation using the TUNEL assay in ejaculated and testicular specimens from 18 patients, and found a lower proportion of TUNEL-positive sperm in testicular samples compared with ejaculated samples (4.8% vs. 23.6%, P < .001). The couples in their study first underwent an ICSI cycle using ejaculated sperm; in case of failure, they underwent a second ICSI cycle using testicular sperm. Although no statistically significant differences in fertilization and cleavage rates or in embryo morphology were seen in ICSI attempts performed using ejaculated or testicular sperm, the ICSI cycles with ejaculated sperm led to only one pregnancy with spontaneous miscarriage whereas the ICSI cycles with testicular sperm led to eight clinical pregnancies with healthy deliveries (14). To our knowledge, the study by Greco et al. (14) is the only other published study that examines differences in fertility outcomes using testicular versus ejaculated sperm in the context of sperm DNA integrity results.

Other studies in the published literature have either compared sperm DNA integrity in ejaculated and testicular samples without assessing fertility outcomes (15), or compared fertility outcomes after the use of ejaculated or testicular sperm without measuring DNA integrity (3, 5, 17–19). Nevertheless, collectively, these studies suggest that implantation, pregnancy, and live-birth rates after the use of testicular sperm are better than the corresponding rates for ejaculated sperm in the setting of severe male factor infertility, including cryptozoospermia, oligoasthenoteratozoospermia, necrozoospermia, and virtual azoospermia. Some advantage of testicular sperm over ejaculated sperm may be presumed in these settings.

Moskovtsev et al. (15) reported a threefold higher prevalence of DNA damage in ejaculated sperm versus testicular sperm in men with a history of abnormal sperm DNA integrity who failed antioxidant therapy. Although interesting in and of themselves, these results serve as a reminder of our imperfect understanding of the pathophysiology and treatment of sperm DNA damage. The testis has substantial antioxidant activity (20). After their release from Sertoli cells, sperm are susceptible to damage from oxidative stress, created by reactive oxygen species that are produced by the immature spermatids. Direct visualization of sperm DNA damage. The testis has substantial antioxidant activity (20). After their release from Sertoli cells, sperm are susceptible to damage from oxidative stress, created by reactive oxygen species that are produced by the immature spermatids. Direct visualization of sperm DNA damage, resulting in sperm DNA fragmentation. In such men, testicular sperm may represent a healthier albeit more immature sperm population for assisted reproduction.

The TUNEL assay provides direct quantification of sperm DNA fragmentation, which can be measured using either fluorescent microscopy or flow cytometry (22). Sperm are classified as TUNEL positive or negative and expressed as a percentage of the total sperm in the population. Using flow cytometric analysis, Sharma et al. (23) have specified a TUNEL-percentage cutoff of approximately 20% to differentiate the infertile men with DNA damage from the fertile men. This guideline has been used by several other investigators.

However, flow cytometry can overestimate the number of TUNEL-positive sperm; immature sperm and spermatids undergoing DNA condensation always appear TUNEL positive because this physiologic process requires DNA breaks directed by helicases. In our laboratory, TUNEL assays are performed using epifluorescent microscopy, which allows for direct visualization and the opportunity to distinguish between mature sperm and spermatids. Direct visualization of sperm provides better assessment of sperm DNA damage, which often manifests as clumping (Fig. 1). The addition of
Nomarski optics to epifluorescent microscopy allows for the TUNEL assay to be performed on sperm meeting the morphologic criteria for selection for ICSI (Fig. 2). In our laboratory, we have established a strict TUNEL-positive cutoff of 7%, based on the screening of 150 healthy male volunteers and 150 infertile men, with internal and external validation studies (data not presented).

Several different assays besides TUNEL are available for the assessment of sperm DNA integrity. Their results should be interpreted in the context of the methodology they use. We recommend that investigators use the assay they have the greatest familiarity with, and which has been validated in their patient population.

The American Society of Reproductive Medicine (ASRM) does not recommend the routine use of sperm DNA fragmentation assays in the evaluation and treatment of infertile couples, as existing data do not indicate that the predictive value of abnormal sperm DNA integrity on reproductive outcomes is strong enough to change the management of patients who are considering ART (24). However, this recommendation may not apply for couples who have multiple failed IVF cycles or recurrent spontaneous miscarriages. Our results suggest that the assessment of sperm DNA integrity may have an important role in the evaluation of infertile couples who have experienced repeated failures of IVF–ICSI cycles. In these couples, elevated rates of sperm DNA fragmentation in ejaculated semen samples may prompt use of testicular spermatozoa for ICSI.

This study was subject to the limitations of any uncontrolled cohort study. As an academic specialty center, many of our patients are referred from other centers. Therefore, there was some heterogeneity in terms of the IVF–ICSI technique used for previous cycles that the couples had undergone and failed. However, 83% of couples who went on to achieve a pregnancy using testicular sperm had failed at least one IVF cycle at our center, suggesting that differences in IVF techniques from outside centers are not likely to have played a large role in pregnancy outcomes with the use of testicular versus ejaculated sperm. We did not specifically evaluate what proportion of the previously failed cycles were IVF cycles and what proportion additionally involved ICSI, as this information was not consistently available. Accordingly, we were also unable to evaluate the quality of the embryos resulting from the previous cycles. Our results suggest that elevated sperm DNA fragmentation in ejaculated samples was a contributing factor to repeated failures of previous ART cycles. Other contributing factors—other important differences between ejaculated and testicular sperm—may also have been present but were not specifically evaluated in this cohort study.

Repeated failures of ART cycles represent a considerable challenge for both patients and physicians. In the vast majority of cases, the exact cause of the repeated failures is unknown. Related research has focused primarily on maternal factors contributing to failure. With the exception of paternal karyotypic abnormalities, sperm DNA fragmentation, and sperm aneuploidy, no other paternal factors have been identified (25). Various interventions have been advocated for couples who have repeatedly failed IVF or ICSI cycles (26), but none appear to be consistently efficacious in achieving implantation and live births.

Currently, azoospermia is the sole accepted indication for surgical retrieval of testicular spermatozoa, and the presence of even a few sperm in the ejaculate can render this procedure unnecessary. Special techniques to increase the yield of spermatozoa from the ejaculate have also been developed (27), with the goal of avoiding testicular biopsies. The elective use of surgically retrieved spermatozoa instead of ejaculated spermatozoa in patients with severe male factor infertility represents a novel therapeutic alternative for this specific patient population that merits consideration.

CONCLUSION

The percentage of TUNEL-positive cells is lower in testicular sperm samples from infertile, oligospermic men who have abnormal ejaculated sperm DNA fragmentation. The use of testicular sperm for ICSI was associated with a 50% pregnancy and live-birth rate for couples who had previously failed one or more IVF–ICSI cycles with ejaculated sperm. Therefore, TESE can be considered in appropriately selected men with elevated TUNEL-positive ejaculated sperm and otherwise unexplained infertility.

REFERENCES


