

DNA Fragmentation, Mitochondrial Dysfunction and Chromosomal Aneuploidy in the Spermatozoa of Oligoasthenoteratozoospermic Males

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Purpose: This study determined the incidence of sperm nuclear DNA fragmentation, mitochondrial dysfunction, and chromosomal aneuploidy. The results were correlated with the semen analysis parameters and fertilization rates.

Methods: Semen samples from 10 men showing oligoasthenoteratozoospermia (OAT) and undergoing ICSI treatment were analyzed. Another semen samples from 10 men showing normozoospermia and undergoing IVF treatment were analyzed for comparison. The samples were prepared using a two-step discontinuous Percoll gradient (80%–50%) and analyzed using a Hamilton-Thorne Integrated Visual Optical System (IVOS) Sperm Analyzer. DNA fragmentation was detected with a terminal deoxynucleotidyl transferase-mediated dUTP nick end label (TUNEL) assay. Functional integrity of mitochondria was detected using an Apoalert™ Mitochondrial Membrane Sensor Kit. Chromosomal aneuploidy was assayed by fluorescence in situ hybridization.

Results: Higher sperm DNA fragmentation rate (18.8% vs. 2.8%), mitochondrial dysfunction rate (24.9% vs. 5.7%), and chromosomal aneuploidy rate (0.12% vs. 0.06%) were found in the oligoasthenoteratozoospermic patients in comparison with the normozoospermic patients.

Conclusions: The result indicates that spermatozoa from oligoasthenoteratozoospermic patients contain greater DNA fragmentation, mitochondrial dysfunction, and chromosomal aneuploidy. Because extremely poor semen samples are the indication for ICSI treatment, the result indicates the importance of selecting good quality sperm for oocyte injection.

KEY WORDS: Chromosomal aneuploidy; DNA fragmentation; ICSI; mitochondrial dysfunction.

INTRODUCTION

Great success has been achieved using intracytoplasmic sperm injection (ICSI) for the treatment of male

factor infertility. However, the safety of ICSI procedures remain of concern, and some studies pointed out certain pathological conditions leading to lower ICSI success rates.

It is known that men with oligoasthenoteratozoospermic (OAT) undergoing ICSI treatments exhibit low fertilization and implantation rates. The reasons for the lower ICSI outcomes in these couples are unknown, although most likely due to the absence of mature spermatozoa in the ejaculate or epididymal preparations (1,2). Oxidative stress may be a causal factor, but spermatozoa with DNA damage caused by oxidative stress have been shown extremely

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motile and capable of achieving normal fertilization rates with ICSI treatments (3,4). Apoptosis may be another causal factor for the low fertilization and implantation rates. Apoptosis is a type of physiological cellular death based on a series of cellular, morphological, and biochemical alterations, leading the cell to suicide (5,6). It is known that germ cell apoptosis exists during spermatogenesis and induction of germ cell apoptosis at specific stages of the spermatogenic cycle has been documented (7–10). Mitochondria have been demonstrated as the coordinators of apoptosis in various cellular systems, for they are involved in many apoptotic processes, including caspase activations, decreased mitochondrial membrane potential, and alterations of the intracellular reduction–oxidation potential (11–16). It is not known so far, however, whether mitochondrial dysfunction is responsible for the lower fertilization and implantation rates observed in the OAT patient undergoing ICSI treatments.

Chromosomal abnormalities have been extensively found in the spermatozoa of many infertile men (17–21). The incidence of chromosomal structural aberrations increases among patients undergoing ICSI programs than those on IVF programs (20,21). It is shown that men with low sperm count exhibit a higher incidence of reciprocally balanced translocations (22) and that sperm aneuploidy might be associated with implantation failure and/or early fetal loss (17). Thus, sperm aneuploidy analyses are of highly clinical significance, because male infertility can be treated using ICSI that has an explicit risk of transmitting chromosomal aberrations from the paternal side (17). It remains largely unknown, however, regarding whether and how sperm aneuploidy might influence the ICSI outcome in infertile patients.

This study aimed to characterize, between OAT and normozoospermic conditions, the DNA fragmentation, mitochondrial dysfunction, and chromosomal aneuploidy in semen samples, and to correlate these characteristics with conventional semen analysis, in an effort to gain new insights of the low fertilization and implantation rates observed in the patients undergoing ICSI treatments.

MATERIALS AND METHODS

Patients

Ten OAT patients, who were not medically or surgically treated in the last 3 months prior to this study, were chosen for semen analyses. The OAT patients were selected on the basis of the following criteria:

less than 20×10^6 cells/mL, forward motility <25%, and abnormal morphology >14%. Another 10 normozoospermic patients, who were undergoing IVF treatment and were not medically or surgically treated in the last 3 months prior to this study, were chosen for comparison with the OAT subjects. The OAT patients were aged between 23 and 39 years, and the normozoospermic patients were aged between 22 and 41 years. All patients were informed with written consents, and the study was reviewed and approved by the institutional review board.

Sample Preparation and Routine Semen Analyses

Samples were prepared using a two-layered (80%–50%) discontinuous Percoll gradient (Pharmacia Biotech AB, Uppsala, Sweden). Each aliquot of liquefied semen was layered on top of the Percoll and centrifuged at $450 \times g$ for 12 min. The final sperm preparation was suspended in a suitable volume of Biggers, Whitten, and Whittingham medium supplemented with 600 mg of albutein (Alpha Therapeutic UK Ltd, Thetford, Norfolk, UK). Routine semen analyses were performed according to the procedures described by the World Health Organization (23).

Sperm Morphology Evaluation

Sperm morphology was assessed in semen using the “strict criteria” as described by Kruger *et al.* (24). Each sample (5 μ L) was evenly spread along the length of a microscope slide that had been thoroughly cleaned with 95% (v/v) methyl alcohol (Adams Healthcare, Leeds, UK). Sperm morphology was assessed at $\times 1000$ magnification by using oil-immersion. At least 100 spermatozoa were counted on each slide preparation. Results were expressed as the percentage of normal spermatozoa observed on each slide. Spermatozoa with borderline morphologies were counted as abnormal.

TUNEL Assay

DNA fragmentation was evaluated using the TUNEL assay (Boehringer Mannheim, Cat. No 1684795, Mannheim, Germany). Briefly, the semen samples were washed twice in phosphate-buffered saline (PBS) for 5 min, followed by centrifugation for collecting spermatozoa at 200 g . The spermatozoa were then treated with a solution containing 0.1% Triton X-100 (Sigma, St Louis, MO, USA; T-8787) and 0.1% sodium citrate (Sigma, C-8532) for 2 min on

ice. A 30- μ L TUNEL mixture, composed of terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP, was added to the same volume of each sample. After thorough mixing, the preparations were spread on air-dried clean slides and were covered with 22 \times 22-mm coverslips. The samples were incubated for 60 min at 37°C in a moist chamber in darkness, washed three times with PBS, and then analyzed using a fluorescence microscope (Leitz Dialux 22, Leica, Wetzlar, Germany). At least 500 cells were counted on each slide, with the presence of green fluorescent signals regarded as positive. For positive controls, spermatozoa were processed in the same way, except for a prior incubation with DNase I (1 μ g/mL, Sigma, D-4263) for 10 min at room temperature to induce DNA fragmentation before the addition of TUNEL mixture. For negative controls, the spermatozoa were similarly processed, but the TUNEL mixtures were added without presence of TdT.

Determination of Sperm Mitochondria Functional Integrity

Sperm mitochondria functional integrity was determined using an Apoalert™ Mitochondrial Membrane Sensor Kit (MitoSensor; Clontech Laboratories Inc., Palo Alto, CA, USA) that allowed detection of the changes in mitochondrial transmembrane potential during early initiation of apoptosis. Briefly, aliquots of spermatozoa samples were centrifuged at 200 g for 5 min to pellet the cells. The cells were then resuspended in 1 mL of MitoSensor incubation buffer, gently mixed, and incubated under 5% CO₂ at 37°C for 40 min in darkness. MitoSensor (1 μ L) was then added and the preparation was incubated at 37°C for an additional 10 min before observation using a Nikon E600 epifluorescence microscope equipped with a 450–490-nm excitation filter and a 520-nm barrier filter. In healthy cells, MitoSensor is taken up by the mitochondria where it forms aggregates exhibiting intense red/orange fluorescence. In dysfunctional (possibly apoptotic) cells, the MitoSensor cannot aggregate in the mitochondria due to alterations in the membrane potential. The stain remains as monomers in the cytoplasm and exhibits green fluorescence. For each sample, at least 500 spermatozoa were counted.

Fluorescent In Situ Hybridization Analyses

Semen samples were centrifuged in HTF medium (Medicult, Copenhagen, Denmark) and the pellets

were fixed in methanol acetic acid (3:1) on slides. Sperm nuclei were decondensed by incubation in 5 mmol/L of dithiothreitol (DTT) and 1% of Triton X-100. Hybridization was performed following the manufacturer's instruction (Vysis Inc, Downers Grove, IL, USA). An aliquot of the triple-color probe hybridization solution from Vysis (the X-, Y-, or an 18-chromosome probe was labeled with the fluorescent hapten CEP Spectrum Orange, Aqua, or Green, respectively) was added to each prepared slide and then covered with a coverslip. The nuclear and probe DNA were denatured simultaneously for 4 min at 75°C, followed by incubation for hybridization in a moist chamber at 37°C for 4 h. The slides were then washed with 60% formamide, 2 \times SSC twice at 42°C, and then with 4 \times SSC and 0.05% Tween 20 at room temperature. Each wash took 5 min. After wash, the slides were dehydrated through ethanols, counterstained with 4', 6-diamino-2-phenylindole (DAPI, Vysis), and mounted. The nuclei were examined using a fluorescent microscope (Nikon) equipped with a Tetra band-pass filter set (Vysis) for simultaneous observations of Spectrum Orange, Green, Aqua, and DAPI. At least 2000 sperm nuclei per slide (4000 per patient) were scored. The sorting criteria was according to that published by Williams *et al.* (25).

Statistical Analysis

Student's *t* test and chi test were used to assess the statistical differences between the data from analyses of semen samples from normozoospermic and OAT patients. *P* < 0.05 was considered significant.

RESULTS

Routine Semen Analysis

Results of routine semen analysis are shown in Table I. Semen specimens from 10 patients undergoing ICSI treatments and another 10 patients undergoing IVF treatment programs were included in this study. The parameters included the semen volume, days of abstinence, sperm concentration, percent motility, and the percentage of sperm with normal morphology. The normozoospermic samples showed sperm concentration at $74.2 \pm 33.0 \times 10^6$ /mL (mean \pm SD), with $72.4\% \pm 13.5\%$ of the spermatozoa with motility and $36.2\% \pm 7.6\%$ of the spermatozoa with normal morphology. The OAT semen samples showed a sperm concentration of $8.0 \pm 7.1 \times 10^6$ /mL (mean \pm

Table I. The Routine Semen Analysis for Normozoospermic and OAT Patients

	Patient no.	Sperm volume (mL)	Abstinence days	Sperm conc. ($\times 10^6$)	Sperm motility (%)	Sperm with normal Morphology ^b
Normozoo-spermia	10	3.0 \pm 2.6 ^a	5.0 \pm 4.0	74.2 \pm 33.0	72.4 \pm 13.5	36.2 \pm 7.6*
OAT	10	3.2 \pm 1.5	4.4 \pm 1.8	8.0 \pm 7.1	22.5 \pm 15.8	2.9 \pm 2.8*

^a mean \pm SD.^b (% normal).* χ^2 test, $P < 0.05$.

SD), with 22.5% \pm 15.8% with motility and 2.9% \pm 2.8% with normal morphology. The results clearly showed lower sperm concentration, sperm motility, and percentage with normal morphology in the OAT samples, as compared to the samples from the normozoospermic patients.

TUNEL Assay

Figure 1(A) shows a typical example of cells stained positive for DNA fragmentation, as represented by the intense green fluorescence in the nuclear region, in contrast to several sperms showing negative results without the FITC-labeled dUTP fluorescence signal, although with DAPI as counterstain (compare Fig. 1(A) with Fig. 1(B)). The overall DNA fragmentation rate of samples from the OAT patients were 18.8% (942/5012), in contrast to the 2.8% (141/5013) of samples from the normozoospermic patients (Table II). The negative control samples showed a signal in less than 3% of the cells. The positive con-

trol samples showed more than 85% of the cells with a signal.

Mitochondrial Dysfunction

Sperm mitochondrial damage was tested using an Apoalert™ Mitochondrial Membrane Sensor Kit, showing red/orange color for the healthy cells and green color in cells with altered mitochondrial membrane potential (see Fig. 2 and Table II). The percentage of spermatozoa with mitochondrial dysfunction in the normozoospermic group was 5.7% (292/5121), whereas it was 24.9% (1264/5077) in the OAT group.

Aneuploidy

Sperm aneuploidy rates for chromosomes X, Y, and 18 were evaluated in OAT patients and normozoospermic patients using triple-color FISH techniques (see Fig. 3 and Table II). The aneuploidy rate in the OAT group was 0.12% (63/50121), in contrast to the 0.06% (31/50230) in the normozoospermic group.

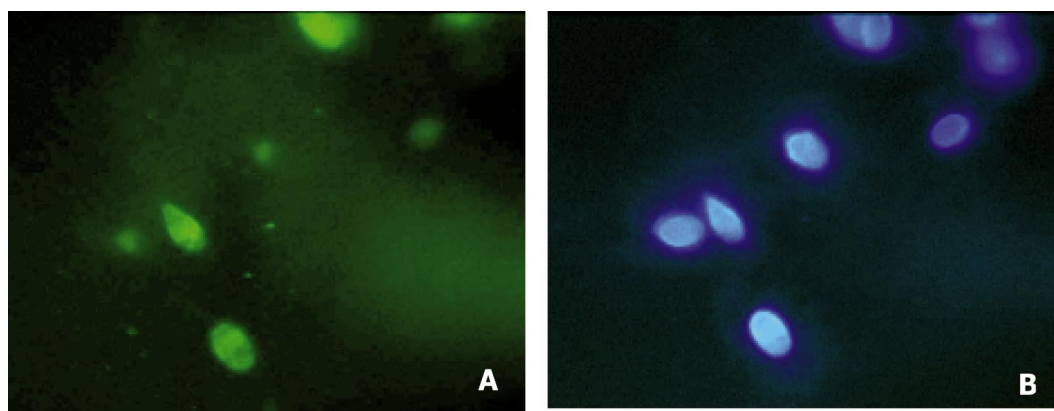


Fig. 1. Results of the TUNEL assay. (A) Spermatozoa with DNA fragmentation showing intense green fluorescence in the nuclear region, in contrast to the negative results without green fluorescence; (B) The spermatozoa (at same field as A) with combined green fluorescence and DAPI, showing the same TUNEL-positive signals (with abnormal morphology) and several TUNEL-negative spermatozoa with only DAPI as counterstain (with normal and abnormal morphological features).

Table II. Results of the DNA Fragmentation, Mitochondria Dysfunction and Sperm Chromosomal Aneuploidy

	DNA fragmentation rate ^a	Mitochondria dysfunction rate ^b	Aneuploidy rate ^c
Normozoospermia	2.8* (141/5031)	5.7 [#] (292/5121)	0.06 (31/50230)
OAT	18.8* (942/5012)	24.9 [#] (1264/5077)	0.12 (63/50121)

^a (%) Sperm DNA fragmentation no./sperm count.

^b (%) Sperm mitochondria dysfunction no./sperm count.

^c (%) Aneuploid sperm no./sperm count.

*,[#] χ^2 test, $P < 0.05$.

Comparison of IVF and ICSI Outcomes

We calculated and compared the outcomes of IVF (normozoospermic group) and ICSI (OAT group) in terms of maternal age, duration of infertility, follicle number, oocyte retrieve number, fertilization rate, embryo cleavage rate, embryo transfer number, and pregnancy rate (Table III). The fertilization rates for OAT patients in the ICSI group were significantly less than that in the normozoospermic patients undergoing IVF treatments (68.4% vs. 84.3%). We also calculated the outcome of IVF from normozoospermic patients and compared with that of ICSI from OAT patients at Lee women's hospital from Jan 1st, 2001 to Jun 30th, 2001 (see Table IV). The fertilization rates of ICSI from OAT patients (67.1%) were still significantly less than that of IVF from normozoospermic patients (85.5%).

DISCUSSION

Infertility caused by male factor may originate from different pathological conditions. On the DNA level,

the presence of poor quality sperm DNA might be a major factor in male infertility (26). Semen from infertile men has been found to contain a greater percentage of spermatozoa with fragmented DNA than semen from fertile men (27,28). On the chromosome level, aneuploidy has been documented as a causal factor of male infertility (17,18,20–23). On the cellular level, mitochondrial function has been reported to associate with reproductive performance, both in males and in females. As ATP production takes place in mitochondria, when the mitochondria are functionally defective, adverse effects will ensue. Rotter *et al.* found increased germ cell apoptosis in transgenic animals, in which p53 genes for the proteins involved in apoptosis control were deleted or over-exposed, leading to oligozoospermia and infertility (29). Knudson *et al.* also found that bax-deficient mice displayed lymphoid hyperplasia and male germ cell death (30). Thus, the process of apoptosis, a physiological cell death, is not only important in spermatozoa quality control, but may also be an important factor of male sterility when it has gone away. It is so far largely unknown, however, regarding the mechanisms

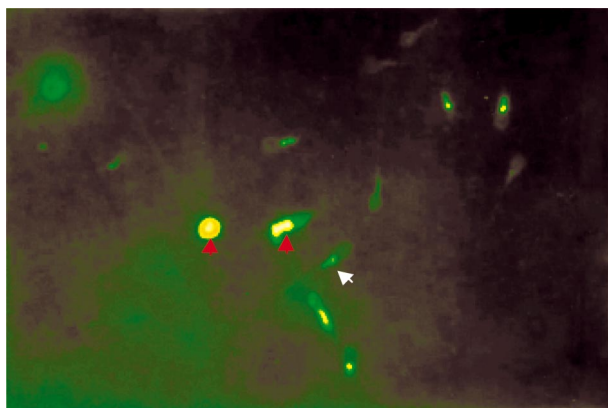


Fig. 2. Results of mitochondrial dysfunction as detected by an Apoalert™ Mitochondrial Membrane Sensor Kit. The white arrow indicates mitochondria damaged in the neck of the spermatozoa (signals in green color). Normal spermatozoa mitochondria are indicated by red arrows (signals in red color).

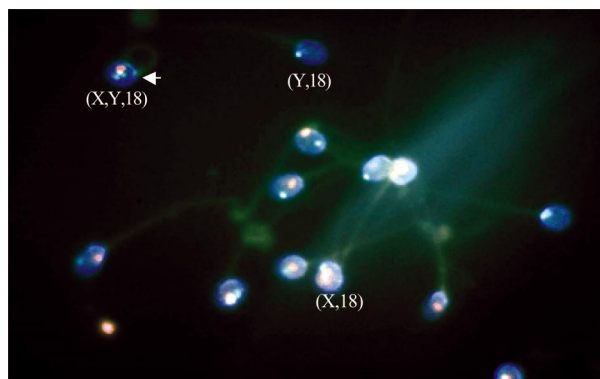


Fig. 3. An example of the result of fluorescence in situ hybridization (FISH). Sperm aneuploidy was detected on chromosomes X, Y, and 18 using triple-color FISH techniques. The probes for X-, Y-, and chromosome 18 were labeled with the fluorescent hapten CEP Spectrum Orange, Aqua, or Green, respectively. The aneuploidy spermatozoa in this patient is indicated by an arrow.

Table III. Comparison of the Outcome of IVF from the Normozoospermic Patients with That of ICSI from the OAT Patients

	Normozoospermia (IVF)	OAT(ICSI)
Patient no.	10	10
Maternal age	31.5 ± 4.7	28.2 ± 2.0
Duration of infertility	3.2 ± 2.6	4.0 ± 1.8
Follicle no.	17.4 ± 10.3	25.6 ± 11.4
Oocyte retrieve no.	16.1 ± 9.2	25.0 ± 11.3
Fertilization rate (%)	84.3*	68.4*
Embryo cleavage rate (%)	95.2	77.8
Embryo transfer no.	4.8 ± 2.4	5.4 ± 1.9
Pregnant rate (%)	60.2	80.0

* χ^2 test, $P < 0.05$.

of increased DNA fragmentation and/or aneuploidy as causal factor(s) for the damage of mitochondrial functions in the male germ cells.

In the present study, we investigated sperm quality by three criteria, namely DNA fragmentation, mitochondrial dysfunction, and chromosomal aneuploidy, in both OAT and normozoospermic patients. The results showed increased rates of the three criteria in the OAT patients, as compared with those in the normozoospermic group. The results here do not necessarily reflect DNA fragmentation and chromosomal aneuploidy as causal factors of mitochondrial dysfunction. However, they are consistent with the data from the routine semen analysis, in that the OAT patients exhibited worse sperm concentration, motility, and morphology as compared to the normozoospermic patients. It will be interesting to unveil whether and how DNA fragmentation, chromosomal aneuploidy, and mitochondrial dysfunction will affect sperm motility and morphology, and even sperm concentration. Interestingly, it is known that a number of testicular germ cells will undergo apoptotic process under hormonal control during normal spermatogenesis.

Table IV. Outcome of IVF and ICSI at Lee Women's Hospital from Jan 1st, 2001 to Jun 30th, 2001

	Normospermia (IVF)	OAT (ICSI)
Patient no.	310	55
Maternal age	32.5 ± 4.8	32.0 ± 4.6
Duration of infertility	3.4 ± 2.8	5.2 ± 4.0
Follicle no.	16.3 ± 10.3	15.0 ± 9.7
Oocyte retrieve no.	15.6 ± 10.2	14.5 ± 10.0
Fertilization rate (%)	85.5*	67.1*
Embryo cleavage rate (%)	95.3	90.0
Embryo transfer no.	5.2 ± 2.5	4.3 ± 2.2
Pregnant rate (%)	57.2	50.0

* χ^2 test, $P < 0.05$.

Oxidative and mitochondrial changes have been proposed to play a role in the apoptosis of many cell types (13,15,16). Our data correlated well the higher number of TUNEL-positive cells with lower sperm concentration in OAT patients, implying that the lower sperm concentration might be caused by higher rate of apoptosis.

Numerous conventional tests are used to examine the quality of human sperm. The World Health Organization recently described criteria by which semen could be deemed sufficient for potential fertility (23). While conventional routine semen assays provide considerable information, there are limitations in predicting the ability to achieve pregnancy. In particular, the routine semen assays provide no help in specifying a single sperm without DNA fragmentation and chromosomal aneuploidy, which is particularly in need with the advent of ICSI treatments where only one single sperm will be chosen. Such limitation is shown by extensive observations. For example, it is shown that no difference could be concluded among parameters of spermatozoa concentration and motility in relation to the outcome of pregnancy, if the parameters of sperm were above certain minimum levels (31,32). In the present study, we observed a better result of routine semen analyses, as well as higher rates of fertilization and embryo cleavage in the 10 cases of the normozoospermic group, but the pregnancy rate was found lower than that of OAT patients (Table III). To exclude the potential bias of limited sample number in the present study, we extended the comparison by pooling the outcomes of 310 cases of normozoospermia undergoing IVF treatments and 55 cases of OAT on ICSI programs (Table IV). To our surprise, despite that the fertilization rate and the embryo cleavage rate were higher in the normozoospermic group, the overall pregnancy rate did not show much difference in the OAT patients (57.2% of normozoospermia vs. 50.0% of OAT). Thus, the limitation of routine semen analyses was also reflected by our results.

We do not know exactly why higher rates of fertilization and embryo cleavage rates did not achieve higher pregnancy rates, as shown by the present study and by our calculations of previous outcomes. Nevertheless, the results presented here seemed to imply that the male factors are most detrimental to the processes of fertilization and embryo cleavage, but not as harmful after the two processes are completed. This does not mean that a better semen analysis is not needed, as fertilization and embryo cleavage are critical to the overall success rate of ICSI treatments.

Obviously, further studies are needed to confirm the potential benefits of using the three criteria investigated here in a clinical setting.

In recent years, severe oligoasthenoteratozoospermic (OAT) couples have produced offspring due to ICSI techniques, although some reports suggested that these patients exhibited increased frequency of sperm aneuploidy (33,34). These patients are prone to mitotic and meiotic error risks during cell division and proliferation (35), raising concern on the safety of ICSI procedures. If spermatozoa with chromosomal aneuploid were allowed for fertilization with subsequent embryonic implantation, higher rates of recurrent abortion, fetal abnormality, and stillbirth would result (36,37). Whereas, in clinical surveys, although couples undergoing ICSI with OAT have a slightly reduced fertilization rate, their chances of delivery or pregnancy loss are similar to those of other patients undergoing IVF treatments with nonmale infertility (38); an observation similar to our finding in the present study.

We concluded that spermatozoa from OAT patients contain greater DNA fragmentation, mitochondrial dysfunction, and chromosomal aneuploidy. Because extremely poor semen samples are the indication for ICSI treatments, selection of sperm with good quality for oocyte injection is important.

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