Journal of Assisted Reproduction and Genetics, Vol. 21, No. 4, April 2004 (© 2004)

Male Infertility

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

Chung-Hsien Liu,[1] Hui-Mei Tsao,[2] Tzu-Chun Cheng,[2,3] Hui-Mei Wu,[2] Chun-Chia Huang,[2,3] Chung-I Chen,[3] David Pei-Cheng Lin,[3] and Maw-Sheng Lee[1,2,4,5]

Submitted March 28, 2003; accepted February 27, 2004

***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

[1] Institute of Medicine, Chung-Shan Medical University, Taichung, Taiwan.
[2] Division of Infertility Clinic, Lee Women's Hospital, Taichung, Taiwan.
[3] Institute of Biochemistry, Chung-Shan Medical University, Taichung, Taiwan.
[4] China Medical College School of Medicine, Taichung, Taiwan.
[5] To whom correspondence should be addressed at The Infertility Clinic Division, Lee Women's Hospital. No. 263, Pei-Tun Road, Taichung 406, Taiwan; e-mail: msleephd@giga.net.tw.

1058-0468/04/0400-0119/0 © 2004 Plenum Publishing Corporation

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 normozooapermic 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 μL) 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-μL 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 × 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 μg/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% CO_2 at 37°C for 40 min in darkness. MitoSensor (1 μL) 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 × SSC twice at 42°C, and then with 4 × 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 bandpass 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 ± SD), with 72.4% ± 13.5% of the spermatozoa with motility and 36.2% ± 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 ±

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 ± 2.6[a]	5.0 ± 4.0	74.2 ± 33.0	72.4 ± 13.5	36.2 ± 7.6*
OAT	10	3.2 ± 1.5	4.4 ± 1.8	8.0 ± 7.1	22.5 ± 15.8	2.9 ± 2.8*

[a] mean ± SD.
[b] (% normal).
*X^2 test, $P < 0.05$.

SD), with 22.5% ± 15.8% with motility and 2.9% ± 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 control 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.

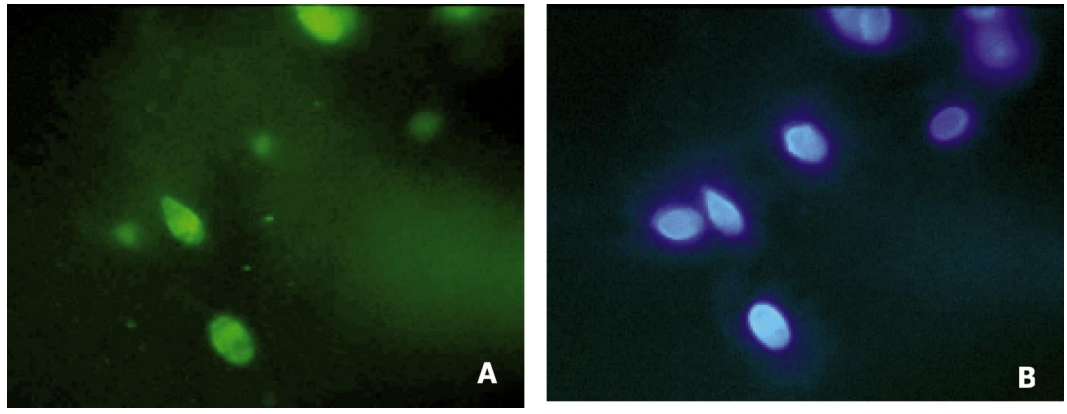

Fig. 1. Results of the TUNEL assay. (A) Spermatozoa with DNA fragmentation showing intense green fluorescence in the nuclear region, in contrast to the negative results without green fluorescence; (B) The spermatozoa (at same field as A) with combined green fluorescence and DAPI, showing the same TUNEL-positive 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]
Normozoo-spermia	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.
*,#X^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 overexposed, 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

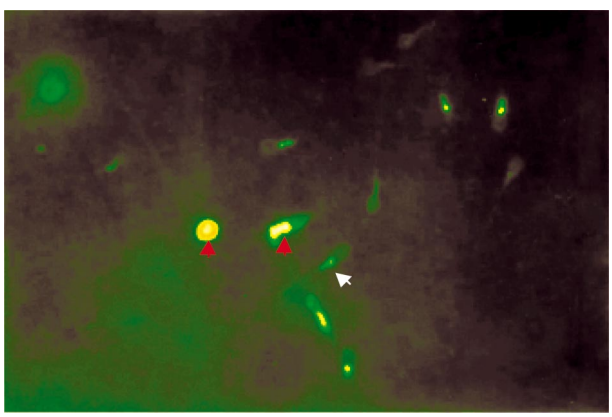

Fig. 2. Results of mitochondrial dysfunction as detected by an Apoalert™ Mitochondrial Membrane Sensor Kit. The white arrow indicates mitochondria damaged in the neck of the spermatozoa (signals in green color). Normal spermatozoa mitochondria are indicated by red arrows (signals in red color).

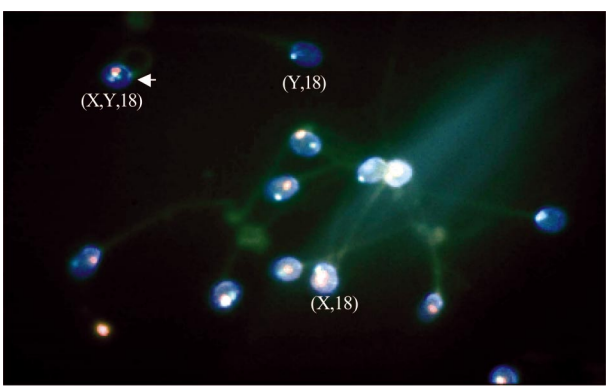

Fig. 3. An example of the result of fluorescence in situ hybridization (FISH). Sperm aneuploidy was detected on chromosomes X, Y, and 18 using triple-color FISH techniques. The probes for X-, Y-, and chromosome 18 were labeled with the fluorescent hapten CEP Spectrum Orange, Aqua, or Green, respectively.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

	Normozoo-spermia (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

*X^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 normozoospemic patients. The results showed increased rates of the three criteria in the OAT patients, as compared with those in the normozoospemic 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 dysfuntion 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

*X^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.

ACKNOWLEDGMENTS

We acknowledge Chia-Yih Chu, Chiu-Ping Chen, and Ming-Chou Hung for their technical assistance. This work was supported by the Chinese Infertility Foundation and partially by an intramural grant to David Pei-Cheng Lin (CSMU-88-OM-B-029).

REFERENCES

1. Ghazzawi IM, Sarraf MG, Taher MR, Khalifa FA: Comparison of the fertilizing capability of spermatozoa from ejaculates, epididymal aspirates and testicular biopsies using intracytoplasmic sperm injection. Hum Reprod 1998;13:348–352
2. Palermo GD, Schlegel PN, Hariprashad JJ, Ergün B, Mielnik A, Zaninovic N, Veeck LL, Rosenwaks Z: Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men. Hum Reprod 1999;14:741–748
3. Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS: Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 1998;59:1037–1046
4. Twigg JP, Irvine DS, Aitken RJ: Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Hum Reprod 1998;13:1864–1871
5. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: A basic biological phenomenon with wide-ranging implication in tissue kinetics. Br J Cancer 1972;26:239–257
6. Nagata S: Apoptosis by death factor. Cell 1997;88:355–365
7. Hikim AP, Wang C, Lue Y, Johnson L, Wang XH, Swerdloff RS: Spontaneous germ cell apoptosis in humans: Evidence for ethnic differences in the susceptibility of germ cells to programmed cell death. J Clin Endocrinol Metab 1998;83:152–156
8. Erkkila K, Hirvonen V, Wuokko E, Parvinen M, Dunkel L: *N*-acetyl-L-cysteine inhibits apoptosis in human male germ cells in vitro. J Clin Endocrinol Metab 1998;83:2523–2531
9. Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U: Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod 1999;4:31–37
10. Sinha Hikim AP, Swerdloff RS: Hormonal and genetic control of germ cell apoptosis in the testis. Rev Reprod 1999;4:38–47
11. Dinsdale D, Zhuang J, Cohen GM: Redistribution of cytochrome *c* precedes the caspase-dependent formation of ultracondensed mitochondria, with a reduced inner membrane potential, in apoptotic monocytes. Am J Pathol 1999;155:607–618
12. Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR: Bax-induced caspase activation and apoptosis via cytochrome *c* release from mitochondria is inhabitable by Bcl-xL. J Biol Chem 1999;274:2225–2233
13. Green DR, Reed JC: Mitochondria and apoptosis. Science 1998;281:1309–1312
14. Jacobson MD, Burne JF, King MP, Miyashrta T, Reed JC, Raff MC: Bcl2 blocks apoptosis in cells lacking mitochondrial DNA. Nature 1993;361:365–369
15. Frade JM, Michaelidis TM: Origin of eukaryotic programmed cell death: A consequence of aerobic metabolism? Bioessays 1997;19:827–832
16. Kroemer G: Mitochondrial implication in apoptosis. Towards an endosymbiont hypothesis of apoptotic evolution. Cell Death Differs 1997;4:443–456
17. Hassold T, Chen N, Funkhouser J, Jooss T, Manuel B, Matsuura J, Matsuyama A, Wilson C, Yamane JA, Jacobs PA: A cytogenetic study of 1000 spontaneous abortions. Ann Hum Genet 1980;44:151–178
18. Bernardini L, Borini A, Preti S, Conte N, Flamigni C, Capitanio GL, Venturini PL: Study of aneuploidy in normal and abnormal germ cells from semen of fertile and infertile men. Hum Reprod 1998;13:3406–3413
19. Bernardini L, Gianaroli L, Fortini D, Conte N, Magli N, Cavani S, Gaggero G, Tindiglia C, Ragni, Venturini PL: Frequency of hyper-, hypohaploidy and diploidy in ejaculate, epididymal, and testicular germ cells of infertile patients. Hum Reprod 2000;15:2165–2172
20. Pang MG, Hoegerman SF, Cuticchia AJ, Moon SY, Doncel GF, Acosta AA, Kearns WG: Detection of aneuploidy for chromosomes, 4,6,7,8,9,10,11,12,13,17,18,21, X and Y by fluorescence in-situ hybridization in spermatozoa from nine patients with oligoasthenoteratozoospermia undergoing intracytoplasmic sperm injection. Hum Reprod 1999;14:1266–1273
21. Pfeffer J, Pang MG, Hoegerman SF, Osgood CJ, Stacey MW, Mayer J, Oehninger S, Kearns WG: Aneuploidy frequencies in semen fractions from ten oligoasthenoteratozoospermic patients donating sperm for intracytoplasmic sperm injection. Fertil Steril 1999;72:472–478

22. Baschat AA, Kupker W, al Hasani S, Diedrich K, Schwinger EB: Results of cytogenetic analysis in men with severe subfertility prior to intracytoplasmic sperm injection. Hum Reprod 1996;11:330–333
23. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 3rd edn. Cambridge, UK, Cambridge University Press, 1992
24. Kruger TF, Ackerman SB, Simmons KF, Swanson RJ, Brugo SS, Acosta AA: A quick, reliable staining technique for sperm morphology. Arch Androl 1987;18:275–277
25. Williams BJ, Ballenger CA, Malter HE, Bishop F, Tucker M, Zwingman AT, Hassold TJ: Non disjunction in human sperm: Results of fluorescence in situ hybridization studies using two and three probes. Hum Mol Genet 1993;2:1929–1936
26. Hughes CM, Lewis SEM, McKelvey-Martin VJ, Thompson W: A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. Mol Hum Reprod 1996;2:613–619
27. Sun JG, Jurisicova A, Casper RF: Detection of deoxyribonucleic acid fragmentation in human sperm: Correlation with fertilization *in vitro*. Biol Reprod 1997;56:602–607
28. Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF: Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 1998;69:528–532
29. Rotter V, Schwartz D, Almon E, Goldfinger N, Kapon A, Meshorer A, Donehower LA, Levine AJ: Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome. Proc Natl Acad Sci U S A 1993;90:9075–9079
30. Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ: Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 1995;270:96–99
31. Yang JH, Wu MY, Chao KH, Chen SU, Ho HN, Yang YS: Controlled ovarian hyperstimulation and intrauterine insemination in subfertility. How many treatment cycles are sufficient? J Reprod Med 1998;43:903–908
32. Dickey RP, Pyrzak R, Lu PY, Taylor SN, Rye PH: Comparison of the sperm quality necessary for successful intrauterine insemination with World Health Organization threshold values for normal sperm. Fertil Steril 1999;71:684–689
33. In't Veld PA, Broekmans JM, de France HF, Pearson PL, Pieters MH, Kooij RJ: Intracytoplasmic sperm injection (ICSI) RJ and chromosomally abnormal spermatozoa. Hum Reprod 1997;12:752–754
34. Viville S, Mollard R, Bach M-L, Falquet C, Gerlinger P, Warter S: Do morphological anomalies reflect chromosomal aneuploidies? Hum Reprod 2000;15:2563–2566
35. Ushijima C, Kumasako Y, Kihaile PE, Hirotsuru K, and Utsunomiya T: Analysis of chromosomal abnormalities in human spermatozoa using multi-color fluorescence in-situ hybridization. Hum Reprod 2000;15:1107–1111
36. Calogero AE, De Palma A, Grazioso C, Barone N, Burrello N, Palermo I, Gulisano A, Pafumi C, D'Agata R: High sperm aneuploidy rate in unselected infertile patients and its relationship with intracytoplasmic sperm injection outcome. Hum Reprod 2001;16:1433–1439
37. Vegetti W, Van ssche AE, Frias A, Verheyen G, Bianchi M, Bonduelle M, Liebaers I, Steirteghem A: Correlation between semen parameters and sperm aneuploidy rates investigated by fluorescence in-situ hybridization in infertile men. Hum Reprod 2000;15:351–365
38. Mercan R, Lanzendorf SE, Mayer J Jr, Nassar A, Muasher SJ, Oehninger S: The outcome of clinical pregnancies following intracytoplasmic sperm injection is not affected by semen quality. Andrologia 1998;30:91–95