Sperm Organelle Morphologic Abnormalities: Contributing Factors and Effects on Intracytoplasmic Sperm Injection Cycles Outcomes

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OBJECTIVE To (1) analyze possible relationships between motile sperm organelle morphology examination (MSOME) and sperm chromatin status, aneuploidy incidence, and patient's age; (2) determine the effects of sperm morphologic abnormalities on intracytoplasmic sperm injection (ICSI) outcomes; and (3) identify the benefits of intracytoplasmic morphologically selected sperm injection (IMSI) in patients with high DNA fragmentation rate.

METHODS The study was performed in 50 patients undergoing ICSI cycles. The MSOME, sperm DNA fragmentation, and sperm aneuploidy incidence were performed in 200 sperm cells of each patient. Regression models were used to assess the relationships among sperm morphology and sperm aneuploidy, sperm DNA fragmentation, patient's age, and ICSI outcomes. In cycles with patients showing a high incidence of DNA fragmentation, oocytes were split into 2 groups according to the sperm selection method: Standard-ICSI (n = 82) and IMSI (n = 79). Fertilization and high-quality embryo rates were compared between the groups.

RESULTS A close relationship between sperm DNA fragmentation and the presence of vacuoles in the MSOME was noted. The patient's age was correlated to the presence of vacuoles. No correlation between sperm aneuploidy and IMSI was observed. Vacuolated cells were negatively correlated with fertilization, pregnancy, and implantation. In patients with a high incidence of sperm DNA fragmentation, fertilization and high-quality embryo rates were similar when comparing IMSI and Standard-ICSI.

CONCLUSIONS Our data demonstrate a correlation between paternal age and the incidence of nuclear vacuoles, as well as an effect of large and small vacuoles on late embryo development. UROLOGY 78: 786–791, 2011. © 2011 Elsevier Inc.

Intracytoplasmic sperm injection (ICSI) is usually performed under an overall optical magnification of 400x that makes it possible to detect, in the living state, most of the sperm anomalies recognized by conventional basic sperm analyses performed on fixed and stained sperm samples. Thus, this system shows severe limitations, because it only enables the observation of major sperm morphologic defects. Minor morphologic defects that seem to be related to the ICSI outcome[1] are often mislaid.

A promising approach that uses a new concept of unstained, real-time, motile sperm organelle morphology examination (MSOME) observation of spermatozoa has been proposed. It is now possible to examine the nuclear morphology of spermatozoa at a magnification of 6600x, using Nomarski differential interference contrast.[2]

Because MSOME is an unstained cytologic technique, its incorporation, together with a micromanipulation system, has allowed the introduction of a modified ICSI procedure called *intracytoplasmic morphologically selected sperm injection* (IMSI).

Positive associations between morphologic sperm normalcy through MSOME and the fertilization rate,[3] percentage of high-quality embryos, implantation rate, pregnancy rate, and miscarriage rate have been previously demonstrated.[4-6]

It is well known that the sperm fertilization potential and embryo development capacity depend on the DNA integrity.[7,8] Moreover, an increased aneuploidy rate has

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been reported in patients with severe testicular damage.[9] However, whether there is a connection between morphologic sperm normalcy (evaluated through high magnification) and sperm DNA integrity, sperm aneuploidy, and the patient's age remains a matter of debate.

The goals for this study were to: (1) Identify the contributing factors associated with sperm morphologic abnormalities, (2) determine the effects of sperm morphologic abnormalities on ICSI outcomes, and (3) identify the benefits of IMSI in patients with a high DNA fragmentation rate.

MATERIAL AND METHODS

Motile sperm organelle morphology, sperm DNA fragmentation, and sperm aneuploidy evaluations were performed in sperm samples from 50 patients undergoing ICSI as a result of male infertility.

Two hundred cells were evaluated in each analysis, and the relationship between those parameters was assessed. All analyses were performed in the same sperm sample.

In cycles with male patients showing a high incidence of DNA fragmentation (≥30%), the oocytes were split into 2 groups corresponding to the method of sperm selection for intracytoplasmic injection: Standard-ICSI (n = 82) and IMSI (n = 79). The fertilization and percentage of high-quality embryos were compared between the groups.

Written, informed consent was obtained, in which patients agreed to share the outcomes of their cycles for research purposes. The study was approved by the local Institutional Review Board.

Evaluation of Spermatozoa

Sperm DNA Fragmentation. The sperm DNA fragmentation was evaluated by the terminal deoxyribonucleotidyl transferase-mediated (dUTP) nick-end labeling (TUNEL) assay performed with the in situ Cell Death Detection Kit fluorescein (Roche Diagnostics, Indianapolis, IN). In brief, fluorescein isothiocyanate (FITC)-dUTP was used as a label according to the manufacturer's instructions and was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Two hundred spermatozoa were analyzed and those cells with green fluorescence were considered altered.

Sperm Aneuploidies. The AneuVysion preimplantation genetic testing probe mixture (Vysis, Abbott, Wiesbaden-Delkenheim, Germany), which is designed for use on human blastomere cells, was used for sperm fluorescence in situ hybridization (FISH). This probe mixture consisted of 5 different colored probes for chromosome 13 (red), chromosome 18 (aqua), chromosome 21 (green), chromosome X (blue), and chromosome Y (gold). Thus, a simultaneous evaluation of sperm nuclei in 5 different chromosomes was possible using this probe mixture. In situ hybridization and detection were performed according to the manufacturer's instructions.

Fluorescence Microscopy. Slides were examined using a Nikon E600 fluorescence microscope (Nikon Corp., Tokyo, Japan) with appropriate filter sets (blue/aqua dual bandpass, green/red dual bandpass, and yellow single bandpass).

Using the 5-color FISH technique, the numerical conditions of the sperm chromosomes were evaluated simultaneously. For chromosomes 13, 18, 21, X, and Y, sperm nuclei were considered haploid when only 1 fluorescent signal was seen. Sperm nuclei were scored as nullisomic when they showed no signal from the investigated chromosomes or from the other tested chromosomes. Sperm nuclei were considered disomic for a chromosome when 2 similar fluorescent signals of the same color and size were observed and when the distance between the 2 signals was equal to or greater than the diameter of 1 fluorescent domain. Finally, sperm nuclei were considered diploid when they exhibited 2 signals for each tested chromosome and when the tails were evident.

Motile Sperm Organelle Morphology Examination. The MSOME criteria for the morphologic normalcy of the sperm nucleus were defined according to Cassuto et al.[10] For the present study, the following defects were recorded: (1) Abnormal shape or size of the head on both axes (normal shape and size head determined as an oval head shape with a regular outline, head length 3–5 μm, width 2–3 μm); and (2) presence or absence of small and large vacuoles.

Controlled Ovarian Stimulation

Controlled ovarian stimulation was achieved by pituitary down-regulation using a GnRH antagonist (Cetrotide, Serono, Geneva, Switzerland) followed by ovarian stimulation with recombinant follicle-stimulating hormone (Gonal-F, Serono, Geneva, Switzerland). The follicular dynamic was followed by ultrasound and when adequate follicular growth and serum estradiol levels were observed, recombinant human chorionic gonadotropin (hCG) (Ovidrel, Serono, Geneva, Switzerland) was administered to trigger final follicular maturation. Oocytes were collected 35 hours after hCG administration.

Preparation of Oocytes

After retrieval, oocytes were incubated in culture medium (G-MOPS-V1, Vitrolife, Kungsbacka, Sweden) covered with mineral oil (Ovoil, Vitrolife) at 37°C and 6% CO_2 for 5 hours. Cumulus cells were removed with a 30-second exposure to HEPES-buffered medium containing 80 IU/mL hyaluronidase (Irvine Scientific, Santa Ana, CA), after which coronal cells were manually removed using a finely drawn glass Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, VA).

Denuded oocytes were then assessed for nuclear status. Oocytes that were observed to have released the first polar body were considered mature and were used for ICSI or IMSI. In vitro matured oocytes were excluded from the study.

ICSI and IMSI

In cycles with patients showing a high incidence of DNA fragmentation (≥30%, n = 35), oocytes were split into 2 groups according to the sperm selection method: Standard-ICSI (n = 82) and IMSI (n = 79).

For both groups, oocytes were placed individually in 4-μL droplets of buffered medium (G-Mops-V1, Vitrolife). Sperm were placed in a central 4-μL droplet of polyvinylpyrrolidone solution (Irvine Scientific, Santa Ana) in a 50 × 40-mm glass culture dish covered with warm mineral oil. Sperm injection was carried out on the heated stage (37°C) of an inverted microscope (Eclipse TE 300; Nikon) 40 hours after the hCG trigger.

In the ICSI group, sperm morphology selection was assessed using an inverted Nikon microscope with a Hoffmann modu-

lation contrast system under 400x magnification. Sperm selection in the IMSI group was examined during MSOME using a similar inverted microscope equipped with high-power differential interference contrast optics (DIC/Nomarski). The total calculated magnification was 6600x, and sperm cells exhibiting normally shaped and sized nuclei—considered as (1) smooth, (2) symmetric, (3) with an oval configuration, and (4) normal nuclear chromatin content (containing no more than 1 vacuole that occupied <4% of the nuclear area—were selected for injection.

Assessment of Fertilization, Embryo Quality, and Embryo Transfer

Fertilization was assessed 18 hours after ICSI. Normal fertilization was confirmed when 2 clearly distinct pronuclei were present. Embryo quality was evaluated under an inverted microscope on the third day of development. The following parameters were recorded: (1) Number of blastomeres, (2) fragmentation percentage, (3) variation in blastomere symmetry, (4) presence of multinucleation, and (5) defects in the zona pellucida and cytoplasm. High-quality embryos were defined as those having all of the following characteristics: 8–10 cells, <15% fragmentation, symmetric blastomeres, absence of multinucleation, colorless cytoplasm with moderate granulation and no inclusions, absence of perivitelline space granularity, and absence of zona pellucida dysmorphism. Embryos lacking any of the aforementioned characteristics were considered low quality.

Embryo transfer was performed on the third day of development. Embryo selection for transfer was performed blindly in the experimental group (Standard-ICSI or IMSI) and was based on embryo morphology.

STATISTICAL ANALYSIS

Linear regression models were used to assess the relationship among paternal age, motile sperm organelle morphology, and sperm DNA fragmentation as independent variables and normal fertilization rates and implantation rates as dependent variables.

To evaluate the predictive value of motile sperm morphologic abnormalities on the rates of DNA fragmentation and sperm aneuploidy, linear regression models were used. To assess the influence of morphologic abnormalities on the odds of pregnancy, binary logistic models were used.

All regression analysis was adjusted for maternal and paternal age.

Data from the linear regressions are presented as the R^2 value, P value, and slope, indicating the tendency of variation of the equation. The results of the logistic regression are presented as the odds ratio (OR), P value, and 95% confidence interval (CI).

In cycles with male patients demonstrating a high incidence of DNA fragmentation (≥30%), the oocytes were split into 2 groups according to the method of sperm selection for ICSI. The fertilization and percentage of high-quality embryos were compared using the chi-square or Fisher's exact test when the expected frequency was ≤5, and the results are presented as proportions (%).

Results were considered as significant at the 5% critical level (P <.05). Data analysis was carried out using Minitab (version 14, State College, PA), a statistical analysis program.

RESULTS

Couple's General Characteristics

The male and female partner ages were, respectively, 31.4 ± 3.7 and 34.3 ± 3.7 years. The initial sperm concentration, the percentage of motile cells, and the percentage of normal cells according to the Kruger Strict Criteria in the semen sample were 25.7 × 10^6 cells/mL, 43.7%, and 5.5%, respectively.

Contributing Factors to the Incidence of Motile Sperm Organelle Morphology Defects

The presence of vacuoles and abnormal nuclear cell size observed in the MSOME was positively correlated with sperm DNA fragmentation incidence (Table 1).

The sperm DNA fragmentation incidence was also a determinant in the likelihood of fertilization (slope −0.0182, R^2 0.098, P = .007), pregnancy (OR 0.957, CI 0.921–0.996, P = .029), and implantation rates (slope −0.046, R^2 0.448, P = .010).

A close correlation between the patient's age and the percentage of large vacuole or small vacuole cells in the MSOME was noted (Table 1). In addition, a positive correlation was found between age and sperm aneuploidies (slope 0.012, R^2 0.058, P = .013). In addition, a trend toward a positive correlation between patient age and sperm DNA fragmentation was noted (slope 0.569, R^2 0.040, P = .061) (Table 1).

The presence of sperm aneuploidy was not correlated with motile sperm organelle morphology (Table 1).

Effects of the Incidence of Motile Sperm Organelle Morphology Defects on the ICSI Outcomes

The incidence of normal motile sperm organelle cells was positively correlated to the fertilization rate. By contrast, the presence of large and small nuclear vacuoles negatively influenced fertilization (Table 2).

Pregnancy rate was influenced by the percentage of normal motile sperm organelle cells.

When taking into account each morphologic abnormality, nuclear size and presence of nuclear vacuoles were determinants in the chance of pregnancy (Table 2).

The incidence of motile sperm organelle normal cells was positively correlated with the implantation rate. When the defects were evaluated individually, the presence of large and small vacuoles and the incidence of abnormal nuclear size cells negatively influenced the implantation rate (Table 2).

Fertilization and High-Quality Embryos Rates in Patients with a High Incidence of Sperm DNA Fragmentation

In patients with sperm DNA fragmentation ≥30% (n = 35), 2 groups were formed according to the method of

Table 1. Multivariate regression analysis of factors contributing to motile sperm organelle morphology defects incidence with variables including sperm DNA fragmentation percentage, patient's age, and sperm aneuploidy frequency

Predictor Variables	Response Variable MSOME	Slope	R^2	*P*
Percentage of sperm DNA fragmentation	Normal cells	−0.016	0.030	.145
	Abnormal shape	0.010	0.009	.411
	Abnormal size	0.004	0.189	<.001
	Large vacuoles	0.004	0.067	.029
	Small vacuoles	0.006	0.063	.034
Patient's age	Normal cells	−0.012	0.002	.715
	Abnormal shape	−0.001	0.030	.148
	Abnormal size	0.053	0.156	.135
	Large vacuoles	0.065	0.118	<.001
	Small vacuoles	0.198	0.104	<.001
Sperm aneuploidy	Normal cells	0.00291	0.009	.805
	Abnormal shape	0.00115	0.001	.960
	Abnormal size	0.08637	0.006	.528
	Large vacuoles	0.00291	0.009	.805
	Small vacuoles	0.00115	0.001	.960

Table 2. Multivariate regression analysis of factors contributing to the fertilization and implantation rates and binary logistic regression of factors contributing to pregnancy rate, with variables, including percentage of normal and abnormal cells according to motile sperm organelle morphology

Response Variable	Predictor Variables MSOME	Slope	R^2	*P*
Fertilization Rate	Normal cells	0.015	0.040	.0547
	Abnormal shape	−0.004	0.054	.2183
	Abnormal size	−0.002	0.116	.1231
	Large vacuoles	−0.019	0.012	.044
	Small vacuoles	−0.170	0.056	.005
Implantation rate	Normal cells	0.015	0.168	<.001
	Abnormal shape	−0.006	0.002	.290
	Abnormal size	−0.009	0.147	.004
	Large vacuoles	−0.013	0.139	.002
	Small vacuoles	−0.077	0.340	<.001
	MSOME	OR	CI	*P*
Pregnancy rate	Normal cells	1.158	0.783–1.711	.046
	Abnormal shape	0.860	0.628–1.178	.346
	Abnormal size	0.853	0.735–1.167	.032
	Large vacuoles	0.780	0.630–1.210	.043
	Small vacuoles	1.065	0.953–1.680	.103

sperm selection for ICSI; no significant differences among fertilization (82.6 vs 80.2%, $P = .775$) and high-quality embryo rates (61.6% vs 60.0%, $P = .842$) were noted when comparing IMSI and Standard-ICSI.

COMMENT

With the introduction of a new, meticulous method for sperm morphology examination, it is now possible to select normal spermatozoa in real time for use in an IMSI cycle. In our study, a close relationship between sperm DNA damage and morphology was noted, especially regarding the presence of vacuoles and abnormal nuclear size.

The origin and impact of sperm DNA fragmentation has been the subject of numerous studies. Infertile men with poor sperm motility and morphology have increased DNA fragmentation compared with individuals with normal semen parameters.[11] Nevertheless, men with normal semen analysis may also have a high degree of DNA fragmentation.[12]

Sperm DNA fragmentation may result from defective apoptosis before ejaculation,[13] problems in nuclear remodeling resulting directly from problems during protamine deposition during spermiogenesis,[14] or excessive production of reactive oxygen species in the ejaculate. These abnormalities would lead to populations of abnormal ejaculated spermatozoa with problems at the nuclear level, cytoplasmic level, or both.

Our findings suggest that spermatozoa selected on the basis of normal nuclear morphology have a lower

chance of presenting chromatin damage. Based on this evidence, we hypothesized that IMSI would optimize the sperm injection outcome by favoring the selection of spermatozoa with intact DNA. To test this hypothesis, in cycles with patients demonstrating a high incidence of DNA fragmentation, the oocytes were injected with sperm selected either under 400x or 6600x magnification. However, no significant differences in fertilization or the percentage of high-quality embryos were noted.

Two hypotheses have emerged to explain why the IMSI had no effect on the ooplasmic sperm injection outcomes in patients showing a high DNA fragmentation level.

First, even though a positive correlation between sperm fragmentation and nuclear abnormalities has been noted, the sperm selection under 6600x magnification may not be sensitive enough to select sperm with intact chromatin.

The second theory arises from studies on late paternal effects on embryo development.[14] Four-cell stages are routinely observed before the 50th hour of development, and eight cell stages are usually noted before 72 hours. In the present trial, fertilization and embryo quality in the third day of development (ie, 72 hours after ICSI) were evaluated; however, further development was not analyzed. It is well-recognized that the quality of sperm plays a key role during fertilization and preimplantation embryo development[14]; however, the expression of the embryonic genome, which is a combination of sperm and oocyte contributions, usually starts at the 8-cell stage of human embryo development.[15,16] The eventual disruption of sperm-derived genes is, therefore, unlikely to manifest between fertilization and the 8-cell stage. Therefore, it could be argued that the sperm defects detected under MSOME are associated with a late paternal effect.

In fact, it has been demonstrated that IMSI has no significant effect on embryo quality at day 3 over the conventional ICSI. However, a relationship between defective spermatozoa and higher abortion rates has been shown.[4] Moreover, Hazout et al did not find any difference in embryo quality between conventional and high-magnification ICSI in patients with an increased percentage of DNA fragmentation.[17] It was also suggested that the late paternal effect, but not the early effect, is associated with increased DNA fragmentation.[18]

Although we have not observed any improvement in fertilization rates and embryo quality when the injected sperm was morphologically selected, our evidence has demonstrated that the presence of morphologic abnormalities has a significant impact on pregnancy and implantation rate, especially the incidence of small and large vacuoles and abnormal nuclear size. Such morphologic abnormalities have been shown to be determinants for the impairment of clinical ICSI outcomes. Berkovitz et al conducted a study on the impact of sperm cells with normal nuclear shape but with large vacuoles, identified by high-magnification, on ICSI outcomes. A significant impact of nuclear vacuoles on pregnancy rate was not demonstrated.[19] Even though previous studies have indicated an influence of sperm nuclear vacuoles on embryo development after the onset of paternal DNA content contribution, which starts around day 3 after fertilization, our evidence has demonstrated a close relationship between the incidence of vacuolated cells and the fertilization rate. These data have been supported by other studies indicating a correlation between the classification of the injected sperm and the fertilization rate.[10,20]

In a recently published article, Garolla et al suggested that normal spermatozoa selected by high magnification have a lower incidence of aneuploidy independent of the initial status of the whole sperm sample.[21] We would expect to find an absence of sperm aneuploidies on those cells with normal morphology and no nuclear vacuoles; however, in our study, no correlation between sperm aneuploidies and abnormal nuclear morphology was observed.

For each semen sample, an analysis of aneuploidy was performed in 200 cells by multicolor FISH, and the presence of aneuploidies was very rare for the studied chromosomes. In Garolla's study, a single cell was evaluated under high magnification and analyzed for sperm aneuploidies, which could explain the differences found in the present study.

The current literature has demonstrated a negative effect of advancing paternal age on pregnancy,[22] implantation,[23] miscarriage,[24] failure to conceive,[25] recurrent pregnancy loss,[26] and live birth rates.[27] Moreover, although many studies have demonstrated that sperm quality is severely affected by increasing age,[28,29] to our knowledge, this is the first study on the effects of paternal age on nuclear sperm morphology (MSOME). Indeed, our findings have demonstrated that the higher the paternal age, the higher the incidences of cells exhibiting nuclear vacuoles and of the appearance of sperm DNA fragmentation and aneuploidies. Together with previous studies on the effect of paternal age on later embryo development,[22,27] these findings reinforce the hypothesis of the correlation between nuclear vacuoles and later paternal effects.

In summary, these data suggest that the selection of sperm for ICSI based on the normal nuclear morphology under high magnification may be a useful tool to select spermatozoa without DNA fragmentation. However, whether this technique optimizes the sperm injection outcome by favoring the selection of spermatozoa without DNA fragmentation remains a matter of debate. Moreover, our evidence suggests a correlation between paternal age and the incidence of nuclear vacuoles, as well as the effect of large and small vacuoles on later embryo development.

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