

Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome

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Objective: To examine sperm DNA fragmentation in semen used for assisted reproduction procedures to establish this factor's prognostic role in fertilization rate, embryo development, pregnancy rate, and outcome.

Design: Prospective study.

Setting: Department of Medicine and Biology of Reproduction of the Edouard Herriot Hospital in Lyon, France.

Patient(s): 322 couples, divided into 88 cycles of in vitro fertilization (IVF) or 234 cycles of intracytoplasmic sperm injection (ICSI).

Intervention(s): Sperm DNA fragmentation was detected in sperm obtained 2 to 5 months before the ART procedure.

Main Outcome Measure(s): Sperm DNA fragmentation was measured with the terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) technique.

Result(s): There was a negative statistical correlation between the rate of fragmentation and the semen characteristics. A statistically significant negative relationship was found for sperm DNA fragmentation and fertilization when ICSI and IVF were compared. With ICSI, a statistically significant negative relationship was found between fertilization rate and percentage of sperm DNA fragmentation (DNA fragmentation index, or DFI). The risk of nontransfer due to blocked embryo development increased when the DFI exceeded 15% (18.2% for ICSI vs 4.2% for IVF) with an odds ratio of 5.05. The miscarriage risk increased fourfold when the DFI exceeded 15% (37.5% for ICSI vs 8.8% for IVF).

Conclusion(s): Sperm DNA fragmentation measured 2 to 5 months before the assisted reproduction procedure was a prognostic indicator of the fertilization, pregnancy, and miscarriage rates and the pregnancy outcome. (Fertil Steril® 2007;87:93–100. ©2007 by American Society for Reproductive Medicine.)

Key Words: Sperm DNA fragmentation, IVF, ICSI, fertilization, pregnancy rate, pregnancy outcome

The sperm used for assisted reproduction techniques (ART) often shows signs of deterioration; in other cases, the semen characteristics seem positive, yet pregnancy proves impossible to obtain. Ensuring the integrity of spermatozoa DNA is necessary to obtain a pregnancy in good condition through ART. Male factors have been shown to have an effect on embryo development (1). As illustrated by the guidelines of the World Health Organization for standard semen analysis (2), count, motility, and morphology have historically been used as indicators of male fertility potential. However, the values between proven fertile and infertile men have a large overlap; thus, routine semen analysis is not a reliable indicator

of fertility potential or pregnancy outcome. Elevated levels of sperm DNA fragmentation are now a well-recognized factor for negative pregnancy outcome (1, 3), and there is increasing evidence that sperm nucleus integrity also should be routinely examined.

Sperm genome anomaly is often one of the factors involved in failure to obtain an embryo and/or pregnancy (4, 5). Sperm DNA fragmentation may be caused by internal factors, such as apoptosis or the production of free radicals by the spermatozoa (6, 7), or external factors such as leucocytes (8). The impact of the DNA fragmentation on the fertilization rate (9–11) and pregnancy rate have been highlighted (1, 12, 13).

Three major methods are generally used to evaluate sperm DNA integrity: sperm chromatin structure assay (SCSA) (14), the terminal deoxynucleotidyl transferase-mediated

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digoxigenin-dUTP nick-end labeling (TUNEL) technique (10), and the comet assay method (15).

For the SCSA technique, 30% appears to be the consensus pathological threshold (14). For the TUNEL technique, the pathological threshold is currently unclear, oscillating between 12% (16) and 36.5% (17, 18). Sergerie et al. (19) place the threshold value for fertility at 20%, a figure that also had been proposed by Benchaib et al. (12). The Sergerie study (19), which compared fertile men with infertile men, showed that fertile men have a lower percentage of spermatozoa with fragmented DNA than infertile men; however, it did not answer the question what threshold value is relevant for a population of infertile men entering an ART protocol—we expect that the sperm of men who succeed in obtaining a pregnancy with ART has a different rate of fragmented DNA than the sperm of those who fail. There are several possible reasons for the heterogeneity of the threshold value set among the various studies: different treatment populations entering the studies (intrauterine insemination, IVF, and/or ICSI), types of sperm on which the technique is carried out (spermatozoa obtained after a washing alone versus after a selection by gradient), and type of measurement (counting by an operator versus counting with a flow cytometer). The range of the pathological threshold of the percentage sperm DNA fragmentation seems to be between 15% and 20%.

The majority of studies carried out on sperm DNA fragmentation and ART outcome have examined semen that was used for ART procedures (12, 13, 18). When sperm DNA fragmentation is measured on the same day as the ART procedure, no improvements are possible in cases of a high percentage of sperm DNA fragmentation. Thus, it would be interesting to measure the value of sperm DNA fragmentation before ART, in view of forecasting this factor's role in the procedure and providing the clinician with an opportunity to try to decreasing the percentage of sperm DNA fragmentation before the procedure begins.

The stability of sperm DNA fragmentation values in comparison with standard semen parameters (20, 21) allows for a potential role in predicting ART outcome. The goal of the present study was to confirm that the percentage of sperm DNA fragmentation (measured by the TUNEL technique) could be used as part of the pre-ART male assessment and as a prognostic factor for the success of an ART attempt. In the period between the measurement of sperm DNA fragmentation and the ART procedure's realization, men could be exposed to environmental toxicants, run a high fever, ingest medications that increase sperm DNA fragmentation levels, or encounter a variety of relevant situations, but this would be the case for any other semen parameters measured before an ART procedure as well. Our study examined the relationship of the percentage of sperm DNA fragmentation (DNA fragmentation index, or DFI) and sperm characteristics, fertilization rate, embryonic development, pregnancy rate, and outcome.

MATERIALS AND METHODS

Patients

The present prospective study was carried out between January 2002 and March 2005 at the Department of Medicine and Biology of Reproduction of the Edouard Herriot Hospital in Lyon, France. The study included 322 couples, divided into 88 cycles of IVF and 234 of ICSI. Cycles requiring frozen surgically obtained sperm were excluded; only cycles carried out with fresh, ejaculated sperm were included in the study. Written informed consent was obtained from all participants, and the study was approved by the ethics committee of Edouard Herriot Hospital.

Standard Semen Analysis

Semen specimen were collected by masturbation after a period of 48 to 72 hours of sexual abstinence. After liquefaction, the manual semen analysis was performed according to World Health Organization (2) criteria.

Ovarian Stimulation

After 3 weeks of desensitizing with gonadotropin-releasing hormone (GnRH) analog (Decapeptyl; Ipsen Biotech, Paris, France), ovarian stimulation was obtained by administering recombinant follicle-stimulating hormone (FSH) (Gonal-F, Serono France SA, Boulogne, France; or Puregon, Organon, Oss, The Netherlands), with monitoring by plasma estradiol levels and vaginal ultrasound. When ovarian follicles had reached a diameter of ≥ 18 mm, ovulation was triggered with 10,000 IU of human chorionic gonadotropin (hCG; Organon). Thirty-six hours after their release, oocytes were retrieved under general anesthesia by vaginal ultrasound-guided puncture.

Sperm Preparation for ART

Sperm used for the ART procedure was prepared using a PureSperm discontinuous density gradient (Nidacon, Gothenburg, Sweden). The discontinuous density gradient consisted of three 1-mL layers of PureSperm: 90%, 70%, and 50%. On the 50% layer, 1 mL of semen was deposited. The gradient was then centrifuged at $300 \times g$ for 20 minutes. After centrifugation, the 90% layer was collected and washed with 5 mL of Ferticult Flushing (FertiPro N.V., Beernen, Belgium) at $400 \times g$ for 10 minutes. The sperm pellet was suspended in the IVF medium (Scandinavian IVF, Gothenburg, Sweden) for IVF, or in a HEPES-buffered IVF-medium for ICSI.

Embryos

Sixteen to 18 hours after insemination or microinjection, the oocytes were assessed for the two pronuclei (2PN) stage. Fertilization was calculated as the ratio of the number of resulting embryos to the number of punctured oocytes. Intrauterine embryo replacement was performed on day 2 or day 3 (early replacement) or at the blastocyst stage (late

replacement). In cases of early replacement, supernumerary embryos were frozen when their morphologic state permitted. Embryos that were not frozen were cultivated on a sequential medium until the blastocyst stage was reached; if one or more blastocysts were obtained, these were frozen. In cases of late replacement, embryos were cultivated in a sequential medium: P-1 Medium (Irvine Scientific, Santa Ana, CA) for the first 2 days, and Blastocyst Medium (Irvine Scientific) for the last days of culture. After embryo replacement, the remaining blastocysts were frozen. Clinical pregnancy was assessed by three successive, positive plasma β -hCG concentrations and by ultrasound detection of fetal heartbeat after 6 weeks; miscarriage was defined as a spontaneous abortion after an intrauterine pregnancy had been detected by ultrasound.

Sperm Preparation for the Study of DNA Fragmentation

Fragmented DNA was detected in sperm obtained 2 to 5 months before the ART procedure. The spermatozoa were selected by a discontinuous gradient. The TUNEL technique was used, as described by Benchaib et al. (12). Briefly, the cells were spread out over slides. After fixation with acetic acid/methanol mixture (3 volumes/1 volume), cells with fragmented DNA were revealed by TUNEL by using the Apoptag plus kit (Oncor, Illkirch, France). The cells were observed under a microscope (Zeiss, Oberkochen, Germany) equipped with a $\times 100$ magnification lens. Spermatozoa with fragmented DNA had brown-colored nuclei, whereas the other cells were blue-gray (countercoloration with Harris's hematoxylin). On each slide, approximately 500 cells were counted, and the percentage of spermatozoa with fragmented DNA (DFI) was calculated.

Statistical Analysis

The statistical analysis was carried out with SPSS version 12.0 software (SPSS Inc, Chicago, IL). The chi-square test was used to analyze qualitative parameters. For comparison of quantitative parameters, Student's *t*-test and analysis of variance (ANOVA) were used. The Spearman correlation coefficient and odds ratio (OR) were calculated. Logistic regression was used to calculate the OR, taking into account several parameters. $P < .05$ was considered statistically significant.

RESULTS

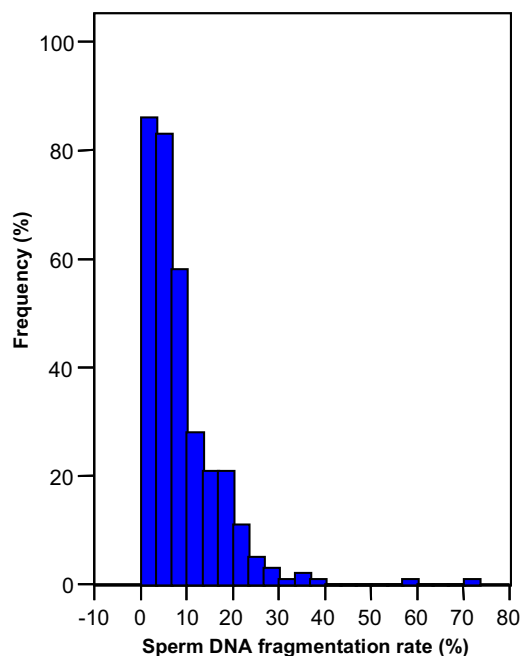
Description of the Population

The study involved 322 couples who underwent IVF (corresponding to 322 cycles, one cycle per couple) between January 2002 and March 2005 for whom sperm fragmentation was studied before the ART procedure.

The average age of the men was of 36.2 ± 5.2 years. Very severe oligospermia (<1 million/mL) was present in 6.6% of the male population, severe oligospermia (<5 million/mL) in 19.2%, and moderate oligospermia (<20 million/mL) in

FIGURE 1

Percentage of sperm DNA fragmentation (DFI) distribution in 322 sperm samples.



Benchaib. Sperm DNA fragmentation and pregnancy outcome. *Fertil Steril* 2007.

34.0%; 40% did not present with a sperm-count anomaly. The average mobility was $28.6\% \pm 14.03\%$. The mean of DFI was $8.6\% \pm 8.0\%$ (Figure 1).

The average age of the women was of 33.5 ± 4.2 years. Tubal deterioration was found in 6.6% of the women, and dysovulation in 27.6%; 2.2% had endometriosis. A uterine anomaly was found in 0.6% of the women. From a total of 3812 oocytes, the average number punctured was 11.8 ± 6.1 . The average number of embryos per couple was 7.5 ± 4.7 (from a total of 2401 embryos).

Microinjection was carried out in 72.7% of cases (234 cycles), early transfer in 61.5%, and late transfer in 32.3%. Table 1 shows the various characteristics according to the ART protocol, IVF or ICSI.

The semen characteristics in the ICSI population were statistically significantly more faded than in the IVF population ($P < .001$), and the DFI was statistically significantly lower ($6.3\% \pm 9.1\%$ vs $9.4\% \pm 8.0\%$, respectively; $P < .01$). The women undergoing ICSI were statistically significantly younger than those undergoing IVF (33.2 ± 4.3 years vs 34.4 ± 4.3 years, respectively; $P < .01$). The fertilization rates were statistically significantly higher with ICSI than in IVF ($74.4\% \pm 20.7\%$ vs $68.5\% \pm 22.9\%$, respectively; $P < .05$).

TABLE 1**Parameters according to the ART procedure used (IVF versus ICSI).**

	IVF	ICSI	P value
Male age (years)	36.5 ± 5.1	36.1 ± 5.2	NS
Progressive motility (%)	38.0 ± 10.9	25.0 ± 13.4	<.001
Concentration (%)			
≤1 million/mL	0	9.1	<.001
≤5 million/mL	2.3	25.5	<.001
≤20 million/mL	20.7	39.0	<.001
>20 million/mL	77.0	26.4	<.001
Fragmentation rate (%)	6.3 ± 9.1	9.4 ± 8.0	<.01
Female age (years)	34.4 ± 4.0	33.2 ± 4.3	<.05
Oocytes	12.2 ± 5.6	11.7 ± 6.3	NS
Embryos	8.3 ± 4.7	7.1 ± 4.6	NS
Fertilization rate (%)	68.5 ± 22.9	74.4 ± 20.7	<.05
Embryo replacement (%)	95.5	93.2	NS
Embryo replacement state			
Early (%)	56.0	69.3	<.05
Late (%)	44.0	30.7	<.05
Transferred embryos (number)	2.1 ± 0.8	2.1 ± 0.9	NS
Pregnancy rate (%)	33.3 (28/84)	35.8 (78/218)	NS
Early	34.0 (16/47)	35.1 (53/151)	NS
Late	32.4 (12/37)	37.3 (25/67)	NS

Note: Values are presented as mean ± standard deviation. NS: not statistically significant.

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Male Factors and Fragmentation

No statistically significant correlation was found between male age and DFI ($r = -0.4$; not statistically significant [NS]). Negative weak correlations were found between DFI and the standard semen analysis measures. The correlation coefficients between DFI and semen characteristics were as follows: sperm count, $r = -0.2$ ($P < .01$); motility, $r = -0.2$ ($P < .01$); and morphology, $r = -0.2$ ($P < .01$).

Female Factors

No relationship was found between female factors and DFI, or between female factors and pregnancy. So female factors should not be considered as confounding factors in the statistical analysis.

Fragmentation and Embryos

No statistically significant correlation was found between DFI and fertilization rate ($r = 0.04$; NS), regardless of ART protocol: IVF, $r = 0.06$ (NS) and ICSI, $r = -0.02$ (NS). However, when the fertilization rate was transformed into a qualitative variable and considered according to ART protocol, with ICSI it became evident that the fertilization rate decreased as the DFI increased ($P < .05$) (Figure 2) in a linear relationship ($P < .01$). This result was not found with IVF. Late transfer allowed the evaluation of the impact of DFI on

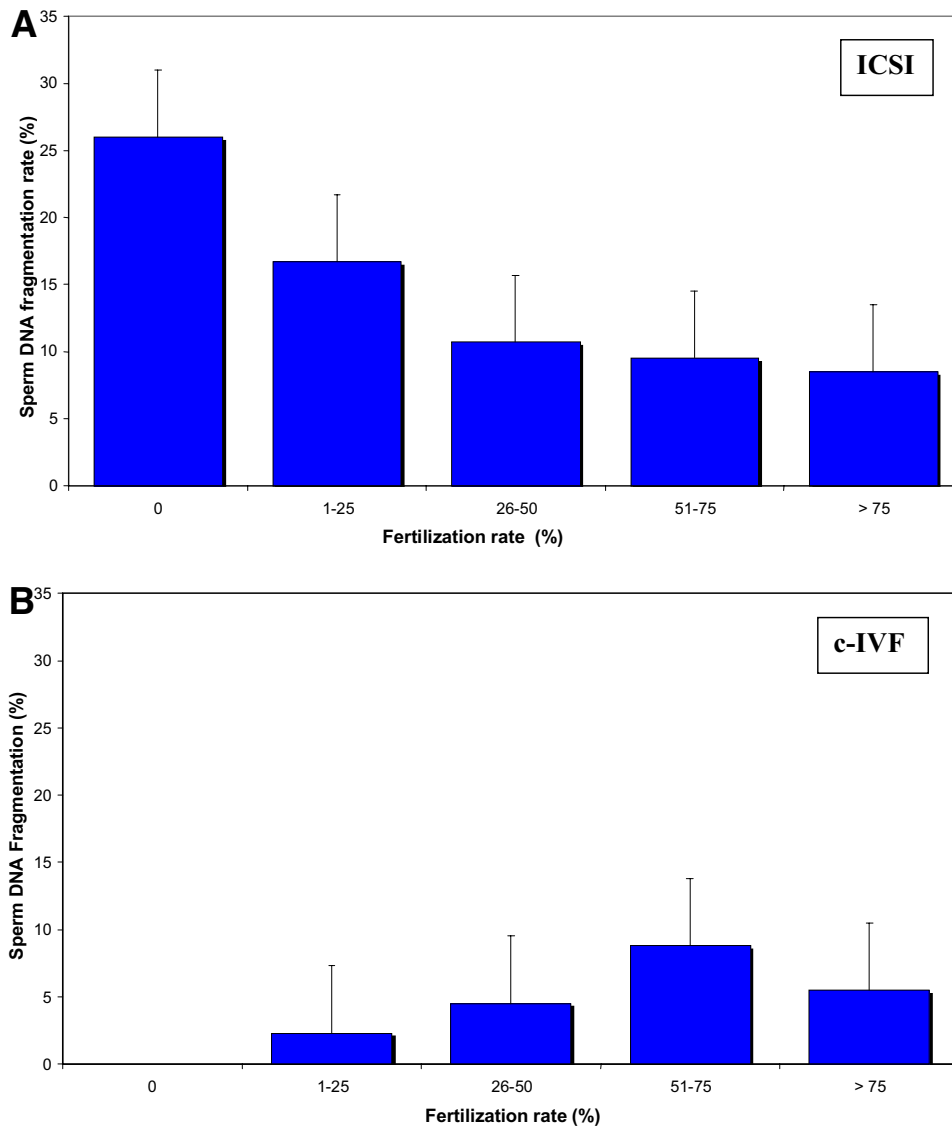
embryo development during the first week. With ICSI, the risk of arrested embryo development increased when the DFI exceeded 15% (18.2% vs 4.2%; $P < .001$). This result, again, was not found with IVF, where the risk was 5% when the DFI was less than 15%, and 0 when it was greater than 15%. Logistic regression for female age, ovarian response, ART procedure, and DFI showed that only DFI had a statistically significant influence on arrested embryo development ($P < .01$), with an OR of 3.8.

Fragmentation and ART Results

Regardless of the ART procedure, no statistically significant difference in pregnancy rate according to DFI was found in cases of embryo replacement. The pregnancy rate was 35.7% when the DFI was less than 15% and 31.8% when it was greater. In IVF, the proportion of patients with a DFI exceeding 15% was too small for statistical testing. In cases of ICSI, the pregnancy rate was 37.4% when the DFI was less than 15%, and 27.8% when it was greater, but this difference was not statistically significant. According to the stage of embryo replacement (early or late), the DNA fragmentation rate did not seem to influence the pregnancy rate. However, when a pregnancy was obtained with a DFI greater than 15%, the risk of miscarriage was increased: 37.5% when the DFI was greater than 15% versus 8.8% when the DFI was less than 15% ($P < .05$, unilateral test) (Table 2).

FIGURE 2

Relationship between sperm DNA fragmentation and fertilization rate by treatment procedure: (A) Intracytoplasmic sperm injection (ICSI). (B) In vitro fertilization (IVF).



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DISCUSSION

The relationship between sperm DNA fragmentation and male factor infertility is beginning to be recognized, but it remains a subject of controversy. The male patients in our study were all young, so it was difficult to highlight the relationship between DFI and age that was found in other studies (16, 22). Alterations in semen characteristics were associated with an increase in DFI, which confirmed the results of other studies (10, 11, 23–26). However, the relationship between semen characteristics and DFI is weak; indeed, DNA deterioration would have only a weak impact on the morphologic and functional characteristics of the

spermatozoa. Spermatozoa having normal characteristics according to World Health Organization criteria could have altered genetic material, fragmentation being only one aspect of the DNA deterioration.

The influence of DFI on the fertilization rate varies from study to study. In our study, in cases of ICSI, the fertilization rate decreased when the DFI was elevated. This result confirmed those of other studies that had used ICSI (11, 27). However, this influence was not found with IVF, again confirming the results of other teams (1, 13, 17, 18, 26, 28, 29). The different behavior of fragmented spermatozoa un-

TABLE 2

Embryo development according sperm DNA fragmentation and procedure.

	IVF			ICSI		
	Sperm DNA fragmentation		P value	Sperm DNA fragmentation		P value
	<15%	>15%		<15%	>15%	
Fertilization rate (%)	68.3 ± 23.6	70.6 ± 16.1	NS	75.4 ± 20.0	70.3 ± 23.4	NS
Arrested development	5.0%	0	NS	4.2%	18.2%	<.05
Pregnancy/transfer	31.6 (24/76)	50.0 (4/8)	NS	37.4 (68/182)	27.8 (10/36)	NS
Pregnancy/transfer/stage						
Early (%)	34.9 (15/28)	25.0 (1/4)	NS	37.6 (47/125)	23.1 (6/26)	NS
Late (%)	27.3 (9/33)	75.0 (3/4)	NC	36.8 (21/57)	40.0 (4/10)	NS
Miscarriage (%)	9.1 (2/22)	50.0 (2/4)	<.05	8.6 (5/58)	30.0 (3/10)	<.05

Note: NS: not statistically significant. NC: not calculated.

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der ICSI or IVF protocols may have been due to the poor quality of sperm used in ICSI, with a greater proportion of spermatozoa with fragmented DNA such that the probability of using an affected spermatozoon for oocyte injection was greater. In this situation, the oocyte's DNA repair capacity would be exceeded (4, 9). Although the ICSI operator tends to choose a normal, mobile spermatozoon for the injection, a spermatozoon can be deemed "normal" while still harboring DNA abnormalities that will result in fertilization failure, as testified by the fall in fertilization rates (11, 26).

To optimize the choice of the spermatozoa injected into the cytoplasm of the oocytes, spermatozoa could be selected under very strong magnification for an intracytoplasmic morphologically selected sperm injection (IMSI) to ensure a better pregnancy rate (30). However, this technique is both recent and time consuming, so more information is needed before it can be proposed systematically in cases of altered spermatozoa. In IVF, the fertilizing spermatozoon may be selected by the pellucid zona, which can reject aneuploid candidates (31).

Controversy remains as to the impact of a high DFI on fertilization, but there is broader agreement about its harmful effect on embryo development (4, 32–34). The present study found a relationship between an elevated DFI (over 15%) and embryo development blocking. When the DFI exceeded 15%, embryo development was blocked in 18% of the cycles; this type of failure occurred in only 4% of cycles when the DFI was lower than 15%. Although this result applied only in case of ICSI, the differential impact on embryo development according to the technique used (ICSI or IVF) had already been reported (1, 12). It has nonetheless been shown that when the DFI is high, the proportion of embryos reaching the blastocyst stage is significantly lower (1, 12, 35). The influence of male inheritance on mouse embryo development was shown by Ahmadi and Ng (4). Spermato-

zoa with DNA damaged by variable amounts of irradiation before insemination still enabled fertilization, but embryonic development to the blastocyst stage was statistically significantly decreased.

It is generally thought that the first stages of embryo development depend on maternal transcripts, and that the paternal influence only begins at the six to eight cell stage; embryo transfers are generally performed on day 2 or 3 after follicle retrieval, which is before the paternal influence would be felt. However, it has been shown that the paternal genome can play a role very early in embryo development, even at the first cell cycle (36), so that the use of a spermatozoon with a high level of fragmented DNA could cause fertilization failure. These observations could apply in particular situations. For example, it has been shown that the level of fragmented DNA in spermatozoa obtained surgically is lower than found in those punctured from the epididymal (37). Thus, in choosing between testicular or epididymal sperm, especially in the event of obstructive azoospermia, testicular would be preferred to optimize embryo development.

In the present study, high pregnancy rates tended to be obtained with low DFI. The correlation was not statistically significant, but this tendency has been confirmed by other studies that have shown that an elevated DFI is a deleterious factor for obtaining and maintaining pregnancies (1, 4, 12, 17, 26, 27, 29). Considering our total data (IVF and ICSI cycles), in cases of pregnancy where the DFI was greater than 15%, the risk of miscarriage was multiplied fourfold (37.5% versus 8.8%). Sperm DNA fragmentation was a factor in the poor prognosis for pregnancy, and the risk of miscarriage was increased; similar results have been found by other investigators (1, 29).

The exact origin of sperm DNA fragmentation has yet to be established. Several causes have been suggested. A defect

in chromatin replanning and compaction during spermatogenesis has been proposed (38); however, this mechanism has been discarded, because it has been shown that the compaction and the fragmentation of sperm DNA are two independent phenomena (39). Alternatively, the production of reactive oxygen species (ROS) by immature spermatozoa or leukocytes may be at the origin of sperm DNA fragmentation (40). Events taking place in the epididymal section (spermatozoon storage section)—in particular reiterative infections with inflammatory processes—could impact sperm DNA. This concept is based on the fact that ejaculated sperm can exhibit a high DFI while testicular sperm has a low DFI (41), indicating that fragmentation takes place downstream of the testicle.

There is no consensus regarding how to approach a high DFI before an ART procedure or how to “cure” it. However, high DFI has a negative impact on the ART result, so it seems reasonable to bring the DFI under its pathological threshold before undertaking an ART procedure. There are different ways to achieve this. The method of sperm preparation for an ART cycle influences the DNA fragmentation: selecting sperm using a discontinuous density gradient (42) or the swim-up technique (25) can decrease the proportion of spermatozoa with fragmented DNA. In some cases, DFI increases due to oxidative stress.

Selection, however, does not solve all the problems, because some spermatozoa with fragmented DNA can persist and have an impact on the ART result (12). Although it is advisable to treat the cause of DNA fragmentation upstream of the ART attempt, the cause of the sperm DNA fragmentation is not always clear. Sperm DNA fragmentation can be attributed to various pathologic conditions, including cryptorchidism, cancer, varicocele, fever, age, infection, and leukocytospermia. Many environmental conditions can also affect sperm DNA fragmentation, such as chemotherapy, radiation, air pollution, smoking, pesticides, chemicals, heat, and ART preparation protocols. Reactive oxygen species (ROS) activity may be a major factor in DNA strand breakage. In the case of varicocele, curing this pathology could decrease the DFI (43).

When no cause can be found, a treatment based on antioxidants (vitamins C and E) can be given to decrease sperm DNA fragmentation (44). Though antioxidant therapy does not help all patients with elevated sperm DNA fragmentation, improvement has been found in 76% of patients in one study (44). Also, other treatments, such as the commercial dietary supplements ProXeed (Sigma-Tau HealthScience S.p.A. Gaithersburg, MD) or Fertile One (Coast Reproductive, Inc., San Diego, CA), could be considered as therapy. In a case of high DFI in ejaculated sperm, using either testicular sperm where the DFI will be lessened (41) or ejaculated donor sperm (1) has been proposed.

The present results indicate a negative correlation between the DFI and semen characteristics. With ICSI, the risk of nontransfer due to blocked embryo development increases

with a DFI higher than 15%. Moreover, when pregnancy is obtained with a DFI higher than 15%, the risk of miscarriage is increased. Similar results have been found with SCSA (1, 13, 29, 45) and TUNEL (46). Thus, the measurement of sperm DNA fragmentation has a place in pre-ART assessment to optimize the results under certain conditions, such as repeated ART failure, miscarriage, varicocele, inflammatory processes, or infection of the genital tract.

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